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Application of Nanotechnology in Drug Delivery

Edited by Ali Demir Sezer

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Contributors

Mojtaba Salouti, Azam Ahangari, Azam Bolhassani, Fadwa Odeh, Hala Al-Jaber, Dima Khater, Rafik Karaman, Luigi Battaglia, Marina Gallarate, Pier Paolo Panciani, Elena Ugazio, Simona Sapino, Elena Peira, Daniela Chirio, Kubra Elcioglu, Ali Demir Sezer, Seyda Bucak, Haico Te Kulve, Utpal Bhadra, Songul Yasar Yildiz, Ebru Toksoy, Biswajit Mukherjee, Sanghyo Kim, Devasier Bennet, Anthony Attama, Pedro Baptista, Alexandra Fernandes, Joana Silva, Lourdes Rodriguez-Fragoso

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Meet the editor

Dr. Sezer is Ph.D. of Pharmaceutical Biotechnology at the Faculty of Pharmacy, University of Marmara (Turkey). He is a member of many Pharmaceutical Associations and acts as a reviewer of scientific journals and European projects under different research areas such as drug delivery systems, nanotechnology and pharmaceutical biotechnology. Dr. Sezer is the author of many scientific publications in peer-reviewed journals and poster communications. Focus of his research activity are drug delivery, physico-chemical characterization and biological evaluation of biopolymers micro and nanoparticles as modified drug delivery system, and colloidal drug carriers (liposomes, nanoparticles, etc.).

Contents

Preface

Nanotechnology can simply be defined as the technology at the scale of nanosize. It is the design, characterization, synthesis and application of materials, structures, devices and systems by controlling shape and size at nanometer scale. Nanotechnology, being an interdisciplinary field, has three main extensively overlapping areas: nanobiotechnology nanomaterials and nanoelectronics, which find applications in materials, pharmaceuticals and healthcare, biomimetics, environment, energy, electronics, metrology, security, robotics information technology, manufacturing, agriculture, construction, transport, and food processing and storage. Nanotechnology in drug delivery has been manifested into nanoparticles that can have unique properties both and *in vivo* . Nanotechnology is a technique or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. These technologies modify drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance.

Since it was first reported in 1980, site-specific drug delivery nanocarriers have progressed greatly with the development of nanotechnology and biotechnology, especially in the antitumor field. Currently, some of the ligand peptides like RGD have become hot targeting molecules with extensive academic studies and some receptor-medicated nanocarriers are now in clinical trials. On the other hand, new approaches are needed to reduce or to avoid off target toxicities, associated with chemotherapy and their long-term residual effects. Recently, nanotechnology has been employed to enhance cancer therapy, via improving the bioavailability and therapeutic efficacy of anti-cancer agents.

It is critical for the field of drug delivery from a proof of concept to a pharmaceutical product at the beginning of the new millennium. A successful outcome will result in a new clinical modality that represents a revolutionary approach to medicine. One immediate benefit will be to produce a continuous level of therapeutic protein, avoiding the characteristic peak and through behavior of intermittent administrations with drug carrier systems. Novel drug delivery carriers using nanotechnology will have the capability to turn genes on or off on demand, producing a therapy that can treat the disease rather than the symptoms and with minimal side effects.

The aim of this book was to gather all results coming from very fundamental studies. Again, this will allow to gain a more general view of the various drug carrier systems that can prepare using nanotechnology and apply, along with the methodologies necessary to design, develop and characterize them. The reader will be introduced to various aspects of the fundamentals of nanotechnology based drug delivery systems and the application of these sys-

tems for the delivery of small molecules, proteins, peptides, oligonucleotides and genes. How these systems overcome challenges offered by biological barriers to drug absorption and drug targeting will also be described.

Ali Demir Sezer
Department of Pharmaceutical Biotechnology,
Faculty of Pharmacy, Marmara University,
Haydarpaşa, Istanbul, Turkey

Chapter 1

Liposomes as Potential Drug Carrier Systems for Drug Delivery

Melis Çağdaş, Ali Demir Sezer and Seyda Bucak

Additional information is available at the end of the chapter

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1. Introduction

Lipids are amphiphilic molecules, where one part of the molecule is water-loving (hydrophilic) and the other water-hating (hydrophobic). When lipids are placed in contact with water, the unfavorable interactions of the hydrophobic segments of the molecule with the solvent result in the self assembly of lipids, often in the form of liposomes. Liposomes consist of an aqueous core surrounded by a lipid bilayer, much like a membrane, separating the inner aqueous core from the bulk outside. They were first discovered by Bangham and his co-workers in 1961 [1] and described as swollen phospholipid systems [2]. In the following years, a variety of enclosed phospholipid bilayer structures were defined which were initially called bangosomes and then liposomes, which was derived by the combination of two Greek words, "lipos" meaning fat and "soma" meaning body.

Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Drug distribution is then controlled primarily by properties of the carrier and no longer by physico-chemical characteristics of the drug substance only.

Lipids forming liposomes may be natural or synthetic, and liposome constituents are not exclusive of lipids, new generation liposomes can also be formed from polymers (sometimes referred to as polymersomes). Whether composed of natural or synthetic lipids or polymers, liposomes are biocompatible and biodegradable which make them suitable for biomedical research. The unique feature of liposomes is their ability to compartmentalize and solubilize both hydrophilic and hydrophobic materials by nature. This unique feature, coupled with biocompatibility and biodegradability make liposomes very attractive as drug delivery vehicles.

Hydrophobic drugs place themselves inside the bilayer of the liposome and hydrophilic drugs are entrapped within the aqueous core or at the bilayer interface. Liposomal formulations enhance the therapeutic efficiency of drugs in preclinical models and in humans compared to conventional formulations due to the alteration of biodistribution. Liposome binding drugs, into or onto their membranes, are expected to be transported without rapid degradation and minimum side effects to the recipient because generally liposomes are composed of biodegradable, biologically inert and non-immunogenic lipids. Moreover, they produce no pyrogenic or antigenic reactions and possess limited toxicity [3-5]. Consequently, all these properties as well as the ease of surface modification to bear the targetable properties make liposomes more attractive candidates for use as drug-delivery vehicles than other drug carrying systems such as nanoparticles [6, 7] and microemulsions [8, 9]. In the 1970s [1, 10-13], liposomes were introduced as drug delivery vehicles but the initial clinical results were not satisfactory due to their colloidal and biological instability and their inefficient encapsulation of drug molecules.

Subsequent research on their stability and drug interactions resulted in several commercial liposome products in the market in the 1980s and early 1990s [14]. A schematic representation of liposomal drug delivery is given in Figure 1.

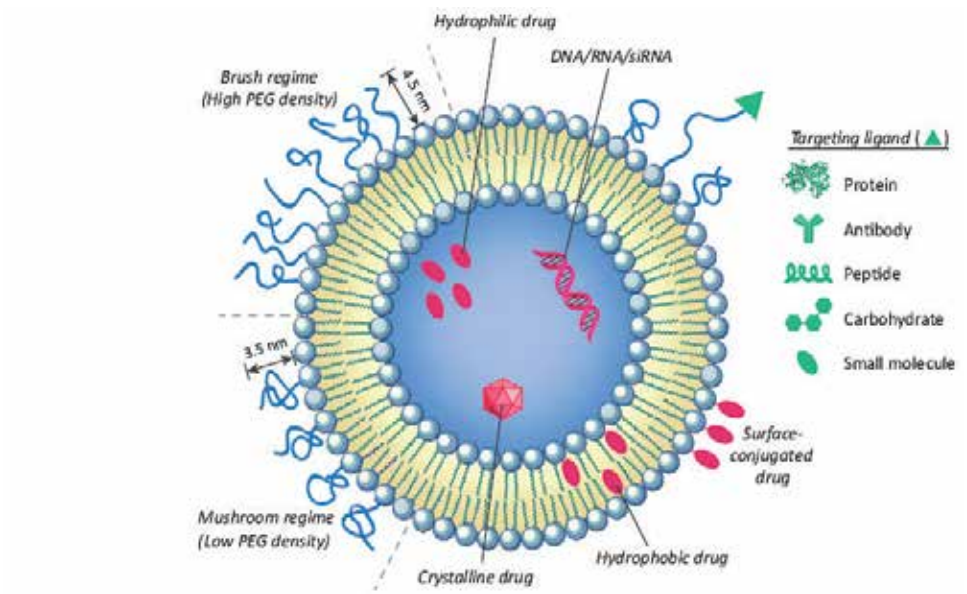

Figure 1. Structural and design considerations for liposomal drug delivery. Liposomes can be surface functionalized to endow stealth through PEGylation and to promote receptor-mediated endocytosis by using targeting ligands such as antibodies, peptides, proteins, carbohydrates, and various other small molecules. PEGylation extends liposomal circulation half-life in vivo by reducing clearance, immune recognition, and the non-specific absorption of serum proteins. Polyethylene glycol (PEG) density determines its structure at the liposome surface, with densities below 9% adopting a mushroom-like globular structure and those above 9% adopting a more rigid, extended, brush-like morphology. Chemotherapeutics or diagnostics can be encapsulated into the aqueous lumen, incorporated into the lipid bilayer, or conjugated to the liposome surface. Abbreviation: siRNA, small interfering RNA [15].

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Liposomes represent versatile and advanced nanodelivery systems for a wide range of biologically active compounds [16]. The final amount of the encapsulated drug is affected by a selection of an appropriate preparation method providing a preparation of liposomes of various size, lamellarity and physicochemical properties [17]. The entrapment of the drugs, both hydrophilic and hydrophobic, into the liposomes is used to bypass the frequent generic toxicity associated with the drug as often seen in cancer drugs [18]. Thus, it represents a very effective route that enhances the drug therapeutic effect. The modification of liposomes permits a passive or active targeting of the tumor site. This effect enables an efficient drug payload into the malignant cell of tumors, while the non-malignant cells become minimally impacted.

In some of the first demonstrations of the improved in vivo activity of liposome-encapsulated drugs in animal models, the anti-cancer drug cytosine arabinoside used which showed a significant increase in the survival times of the mice bearing leukemia [19, 20] which became a popular model for testing the effects of a wide range of liposome characteristics on therapeutic outcomes. Following experiments include liposomal amphotericin B [21] and liposomal doxorubicin [22] that finally led to the first clinical trials of liposomal drugs. Nowadays; the liposomal products (as a suspension, as an aerosol or in a semi-solid form such as a gel, cream, or powder) in the market still include mostly anticancer preparations as well as antifungal and antibacterial preparations and cosmetics. In addition, liposomes are recently used as therapeutic agents to treat a disease because increased gene transfer efficiencies have been obtained via liposomal gene vectors in gene therapy.

The benefits and limitations of liposome drug carriers critically depend and based on physicochemical and colloidal characteristics such as size, composition, loading efficiency and stability, as well as their biological interaction with the cell membranes. There are four major interactions between liposomes and cells [23]. The predominant interaction among them is either simple adsorption or subsequent endocytosis. Adsorption occurs when the attractive forces exceed the repulsive ones and obviously this type of interaction depends on the surface properties of liposomes. In the delivery through endocytosis, liposome and its contents indirectly place themselves in the cytoplasm. Fusion with cell membranes, delivery of the liposomal content directly into the cell through the merge of liposome lipids into the membrane, is much rarer. The last possible interaction is the lipid exchange which is a long-range interaction that involves the exchange of bilayer constituents, such as lipids, cholesterol, and membrane bound molecules with components of cell membranes. Upon entering into the body, the delivered liposomes via one of these interaction types trigger the response of the immune system and the encapsulated material may become inactive. Therefore; substantial researches have been carried out in the development of the biocompatible and nonrecognizable liposomal surfaces.

Various types of liposomes can be prepared by different preparation methods, depending on the required application. In this chapter, these methods are summarized to give a general understanding of the relationship between structure and functionality of liposomes. As one of the advantages of liposomal formulations is the encapsulation ability of both hydrophobic and hydrophilic drugs, incorporation methods are shortly visited. Physical properties of liposomes

such as stability, storage and sterilization are discussed along with the characterization techniques for size, charge, etc. Clinical applications of liposomes are a vast area of research where cancer therapy is the area of highest impact. Different clinical applications of liposomes and most recent advances in cancer therapy are summarized. New generation involving constituents other than conventional ones such as phospholipids prove to be a growing field in nanotechnology. A brief list of different types of new generation liposomes are given with short descriptions at the end of this chapter.

2. Liposome preparation methods

The manufactured liposome features are directly related to the preparation method. Although liposome formation may be spontaneous, often some mechanical agitation is required. In order to have control over the size and structure of the liposomes that are formed, increase the efficiency of entrapment of the desired molecules, and prevent subsequent leakage from the liposomes, different preparation methods have been devised.

There are a few parameters that should be considered during the method selection: 1) the physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients, 2) the nature of the medium in which the liposomes are dispersed, 3) the effective concentration of the encapsulated material and its potential toxicity, 4) additional processes involved during application (delivery of the liposomes), 5) optimum size, polydispersity and shelf-life of the liposomes for the intended application and 6) batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products [24-26].

Liposome size is a crucial parameter in determining the circulation half-life of liposomes in drug delivery. The amount of encapsulated drug is also related with the size and the number of bilayers of the prepared liposome. According to the desired formulation, different liposome preparation methods can be employed. The main difference in these methods is their approach to overcome the low solubility of lipids in water. Accordingly, these methods can be classified as mechanical agitation, solvent evaporation, solvent injection, and detergent solubilization. In all the above mentioned methods, drug loading is passive.

2.1. Mechanical agitation

In this method, lipids are directly solubilized in water upon application of high mechanical agitation, through the use of probe sonication. It is one of the simplest methods of liposome preparation, however, yields small liposomes that are highly unstable in terms of their size and suffers from the drawback that it is impossible to remove completely the risk of lipid degradation by contact with the hot probe, and contamination with titanium from the probe. Its advantage is the exclusion of use of organic solvents as described in the following methods. However, for drug delivery applications, liposomes prepared with mechanical agitation are not suitable due to their size instability and high leakage of encapsulated drugs [27].

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2.2. Solvent evaporation

In general this method consists of four major steps; first is the solubilization of the lipid (and a hydrophobic compound) in an organic solvent; second is solvent evaporation; third is hydration with a buffer (and the hydrophilic compound) and if need the fourth often involves obtaining unilamellar liposomes from the obtained multilamellar ones.

The aqueous volume enclosed within these lipid membranes is very small proportion of total volume used for preparation (5-10%). Consequently, large amount of water soluble drug is wasted during the preparation. On the other hand, lipid soluble drug can be encapsulated with 100% efficiency, providing that they are not present in quantities which overwhelm the structural components of the membrane [28]. The volume of entrapment can be significantly increased by the usage of negatively charged lipids in the membrane which tend to push the bilayers apart from each other. The same effect can also be achieved in the presence of neutral lipids by freezing and thawing repeatedly the obtained liposomes. 30% volume of entrapment can be achieved, which can further be increased at higher lipid concentrations [29]. The freeze-thaw protocol results in a dramatic change in liposome morphology followed by freeze-fracture electron micrographs. Before freeze-thawing, the samples exhibit the tightly packed "onions skin" arrangements of concentric bilayers normally associated with liposomal systems. After a few freeze-thaw steps, however, new structures are observed where the interlamellar spaces are much increased, and where closed lamellar systems can be intercalated between bilayers [30].

2.2.1. Solubilization of the lipid

The starting point of this liposome preparation method is to prepare an organic solution of membrane lipids in order to ensure complete and homogenous mixing of all the components as they are required in the final membrane preparation. Compounds to be incorporated which are lipid soluble will be added to the organic solution, while compounds to be entrapped in the aqueous compartment of liposomes will be dissolved in the aqueous environment. In this method, phospholipids are first dissolved in an organic solvent along with lipid soluble compounds (if any) to be incorporated in the liposome to ensure complete and homogenous mixing.

2.2.2. Solvent evaporation

The next step is the evaporation of the organic solvent. The simplest is to allow the solvent to evaporate in a glass container. A better method is evaporating the solvent using a rotary evaporator connected to a vacuum pump to obtain a thin film of the lipid on the walls of a round bottom flask. In order to increase encapsulation, it is recommended to start with a large volume round bottom flask so that the lipids will be dried down onto a large surface area possible to form a very thin film. The evaporator is detached from vacuum pump and introduced to nitrogen. The container is then removed from the evaporator and fixed to a lyophilizer or exposed to high vacuum overnight to remove the residual solvent.

An alternative method of dispersing the lipids in a finely-divided form before the addition of aqueous media is to freeze-dry the dissolved lipids in an organic solvent [31]. The important concept in this method is the choice of the organic solvent which should have a freezing point above the temperature of the condenser of the freeze-drying and also be inert with regard to rubber seals of commercial lyophilizers. When these restrictions are concerned, the most suitable organic solvent happens to be tertiary buthanol.

2.2.3. Hydration

Evaporation (or freeze-drying) of the solvent is followed by hydration of lipids with the aqueous medium. Often for hydration, a suitable buffer at a temperature above the phase transition temperature of the phospholipid is employed. The solution is swirled manually or mechanically (either with a bath sonicator or vortex mixer) until all the lipids have been incorporated into the solution. The resulting product is a milky suspension of lipids which is allowed to stand for a while for the complete swelling to give MLVs [28]. Further treatment is required for the preparation of ULVs, which will be discussed later in the text.

It is possible to obtain LUVs instead of MLVs during hydration by introducing an aqueous sucrose solution down the side of the flask by inclining the flask to one side and slowly returning the flask to the upright orientation, allowing the fluid to run gently over the lipid layer on the bottom of the flask. The swelling is carried out as usual without any shaking or agitation. The suspension is then centrifuged and the layer of MLVs floating on the surface is removed, leaving LUVs in solution.

2.2.4. Obtaining SUVs from MLVs

After preparation of MLVs by hydration of dried lipid, it is possible to continue processing the liposomes in order to modify their size and other characteristics. For many purposes, MLVs are too large of too heterogeneous population to work with. There are several methods devised to reduce their size. These include techniques such as micro-emulsification, extrusion, and ultrasonication. A second set of methods is designed to increase the entrapment volume of hydrated lipids, and/or reduce the lamellarity of the liposomes formed, and involves procedures such as freeze-drying, freeze-thawing or induction of vesiculation by ions or pH change.

Microemulsification of liposomes is performed with an equipment called micro fluidizer to prepare small vesicles from concentrated lipid suspension. This method can produce liposomes in 50-200 nm size range with the encapsulation efficiency of up to 75% [32].

Sonication [33] disrupts MLV suspensions by using sonic energy to produce SUVs with diameters in the range of 15-50 nm. There are two methods of sonication; bath sonication and probe sonication. The former method is used for large volumes of dilute lipids whereas the latter one is used for suspensions which require high energy, such as high concentration of lipid suspensions. The disadvantage of probe sonication is the contamination of preparation with metal from the tip of the probe which should be removed by centrifugation prior to use. Also, as a result of high energy, probe sonication suffers from overheating the lipid suspension

causing degradation. For these reasons, bath sonicators are the most widely used instrumentation for SUV preparation.

An even gentler method of reducing the size of the liposomes is to pass through a membrane filter of defined pore size [34]. This can be at much more lower pressure and can give populations in which one can choose the upper size limit depending on the exact pore size of the filter used. This membrane extrusion technique can be used to process both LUVs and MLVs in which liposome contents are exchanged with the suspending medium during breaking and resealing of the phospholipid bilayers as they pass through the polycarbonate membrane. In order to achieve as high an entrapment as possible of water-soluble compounds, it is crucial to have these compounds present in the suspending medium during the extrusion. An almost completely unilamellar population can be produced after 5-10 repeated extrusions through two stacked membranes.

In freezing-thawing method, SUVs are rapidly frozen and thawed slowly. The short-lived sonication disperses aggregated materials to LUV. The creation of unilamellar vesicles is as a result of the fusion of SUV throughout the processes of freezing and thawing [35-37].

2.3. Solvent injection

In this type of preparation methods, lipids are first dissolved in an organic solvent and then brought into contact with the aqueous phase containing the materials to be encapsulated within the liposome. The lipids align themselves into a monolayer at the interface between the organic and aqueous phase which is an important step to form the bilayer of the liposome [29]. There are three categories in solvent dispersion method including; (i) a miscible organic solvent with the aqueous phase, (ii) an immiscible organic solvent with the aqueous phase that is used in excess, and (iii) an immiscible organic solvent used in excess with the aqueous phase.

2.3.1. Ethanol injection method

In this method an ethanol solution of lipids is injected rapidly into an excess saline or other aqueous medium by a fine needle [38]. The injection force is usually sufficient to achieve complete mixing, so that ethanol is diluted in water, and lipids are dispersed evenly throughout the medium. This method yields a high proportion of SUVs. This method is extremely simple and it has a very low risk of degradation for sensitive lipids. Its major disadvantages are the limitation of solubility of lipids in ethanol and the volume of ethanol that can be introduced into the medium, which in turn limits the quantity of lipid dispersed, so that the resulting liposome solution is generally dilute. As a result, the percentage encapsulation for hydrophilic materials is very low. One last disadvantage for this method is the difficulty of the removal of ethanol from the lipid membranes.

2.3.2. Ether injection method

This method [39, 40] involves injecting the immiscible organic solution very slowly into an aqueous phase through a narrow needle at a temperature that the organic solvent is removed by vaporization during the process. In this method, large vesicles are formed which might be

due to the slow vaporization of solvent giving rise to an ether: water gradient extending on both sides of the interfacial lipid monolayer, resulting in the eventual formation of a bilayer sheet which folds in on to itself to form a sealed vesicle [29]. Ether injection treats sensitive lipids very gently and runs very little risk of causing oxidative degradation. Since the solvent is removed at the same rate as it is introduced, there is no limit to the final concentration of lipid which can be achieved, since the process can be run continuously for a long period of time, giving rise to a high percentage of the aqueous medium encapsulated within the liposomes. The major drawbacks of this method are the long time taken to produce a batch of liposomes and the need of careful control for the introduction of lipid solution.

2.4. Surfactant (detergent) solubilization method

In this method, the phospholipids are brought into contact with the aqueous phase via the intermediary of surfactants. Phospholipid molecules associate with surfactants and form mixed micelles. The basic feature of this method is the removal of the surfactant from pre-formed mixed micelles containing phospholipids, whereupon unilamellar liposomes form spontaneously. However, removal of surfactants is carried out using techniques such as, dialysis and column chromatography, inevitably remove other small water-soluble molecules, making this method not very efficient in terms of percentage encapsulation values attainable for water soluble compounds. On the other hand, surfactant solubilization method has the ability to vary the size of the liposomes by precise control of the conditions of surfactant removal and to obtain liposomes of very high size homogeneity [29].

The transfer from laboratory to industry was very important for liposomes, as it is for any biotechnological discipline. The first liposomal drug delivery experiments in humans were carried out by freshly prepared liposomes but in order to be a commercial product the liposome-drug formulation must have well-defined stability and a shelf life over a year. Of the several preparation methods described in the literature, only a few of them have the potential to be used in the large scale liposome manufacturing. The crucial problem is the presence of organic solvent residues, pyrogen control, stability, sterility, size and size distribution as well as batch to batch reproducibility.

In the parental administration the liposomes two important conditions involve being sterile and pyrogen free. In the case of animal experiments, the sufficient sterility can be obtained by the passage of the liposome preparations through the 400 nm pore size Millipore filters. In human experiments the sterilization depyrogenation techniques should be taken much more seriously starting from the raw materials, containers and working areas [41].

2.5. Loading of drugs in liposome formulations

2.5.1. Encapsulation of hydrophilic drugs

Once lipids are hydrated in the presence of hydrophilic drugs, a portion of the drug gets entrapped inside the liposome and another portion remains in the bulk, outside the aqueous core of the liposome. As only the entrapped drug is of interest, drug in the bulk should be

removed. This purification is generally done by gel filtration column chromatography (Sephadex G-50, Pharmacia LKB) and dialysis (hollow fiber dialysis cartridge) on the basis of size differences between the liposomes and the non-encapsulated material. In the cases where DNA or proteins are being encapsulated, or where there is concern that non-encapsulated material may form large aggregates, techniques such as centrifugation can be employed due to the differences in the buoyant densities of liposomes and non-encapsulated material [42, 43].

A hydrophilic drug may not be encapsulated with high efficiency because the drug molecules can diffuse in and out of the lipid membrane. Thus, the drug would be difficult to retain inside the liposomes. However, compounds with ionizable groups and those that are both water and lipid soluble can be encapsulated with high efficiency (up to 90%) by the liposomes after the formation of membranes [44] by active loading. In this technique, the pH of the interior part of the liposome is such that the unionized drug which enters the liposome by passive loading is ionized inside the liposome, and ionized drug molecules lose their ability to diffuse through the lipid membrane. Therefore, high concentration of the ionized drug is obtained inside the liposome. For example, doxorubicin and epirubicin can be entrapped in preformed SUV with high efficiency through active loading [45, 46].

The pH difference can be brought about by encapsulating a non-permeating buffer ion such as glutamate inside the liposomes at low pH and replacing the extra-liposomal buffer with one which is iso-osmolar at pH 7.0. Alternatively, charged lipids may be incorporated into the membrane at low pH, followed by adjustment of the suspending medium to neutrality. A similar approach may be adopted by using a potassium gradient, in which the membrane is made selectively permeable to potassium ions entrapped inside the liposome by incorporation of valinomycin into the lipid membrane [47, 48].

2.5.2. Encapsulation of hydrophobic drugs

Hydrophobic drugs are solubilized in the phospholipid bilayer of the liposomes that mainly provide a hydrophobic environment. Once trapped, they remain in the liposome bilayer as they have very low affinity towards the inner or outer aqueous regions of the liposomes. During the preparation of liposomes, hydrophobic drugs are solubilized in the organic solvent along with the phospholipids and during the subsequent hydration phase, they remain entrapped in the hydrophobic bilayer region. For example, the liposomal photosensitizer verteporfin (Visudyne) contains a hydrophobic drug that is rapidly transferred to blood proteins in vivo. Activation of the drug by targeting laser light to blood flowing though the eye causes its site-specific activity in the treatment of wet macular degeneration [49]. Amphotericin B and paclitaxel are the other most commonly investigated hydrophobic drugs in liposome formulations.

3. Stability of liposomes

Liposome stability can be explained by physical, chemical and biological means which are all interrelated. Generally, chemical (degradation of phospholipids structures) and physical

(uniformity of size distribution and encapsulation efficiency) stability determine the shelf-life of liposomes. Once the liposomal formulations have been obtained, maintenance of the physical properties of these preparations can be difficult. Leakage of the encapsulated material due to the permeability of the membrane, change in the size distribution and stability problems due to the hydrolytic and oxidative degradation are the general problems upon storage. Methods are devised to overcome these instability problems, those designed to minimize the degradation processes and those which help liposomes to survive in the face of conditions which encourages these processes.

Two different types of chemical degradation can affect the performance of the phospholipids bilayers; hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and oxidation of the unsaturated acyl chains, if present. The level of oxidation can be kept to a minimum by taking some precautions like starting with freshly purified lipids and freshly distilled solvents, avoiding procedures involving high temperatures, carrying out the manufacturing process in the absence of oxygen, deoxygenating the aqueous solutions by passing nitrogen, storing all liposome suspensions in an inert atmosphere and including an antioxidant, e.g. α-tocopherol [50], a common non-toxic dietary lipid, as a component of the lipids membrane. An alternative solution to the oxidation problem is to reduce the level of oxidizable lipids in the membrane by using saturated lipids instead of the unsaturated ones. Also, the mono-unsaturated ones have much less tendency of oxidation than the polyunsaturated ones. Thus; sphingomyelins, usually having only one double bond, are expected to degrade more slowly than other mammalian origin lipids. Entirely synthetic and saturated phospholipids; DMPC, DPPC and DSPC, can also be considered as a solution for the oxidative degradation of liposomes.

Hydrolysis type of chemical degradation of the ester linkages in the phospholipid structure occurs most slowly at pH values close to neutral. In general, the rate of hydrolysis has a "V-shaped" dependence, with a minimum at pH 6.5 and an increased rate at both higher and lower pH. In the active loading of drugs, as it is mentioned before, low pH levels are required which triggers the hydrolysis. This hydrolysis kind of chemical degradation is also very effective on the aqueous solutions of liposome due to the presence of water. Temperature also triggers the hydrolysis of the lipids which creates the need for refrigeration. In order to keep hydrolysis to a minimum during active loading, attention must be paid for the removal of residual solvent from the dried lipids. To avoid hydrolysis, instead of ester linked lipids, the usage of ether linkage containing lipids (e.g. found in the membrane of halophilic bacteria) would be an absolute solution [51]. Another chemical degradation, oxidation of the lipids in the liposome structures can be prevented by the addition of small amounts of antioxidants during the manufacturing steps.

The problems related to the lipid oxidation and hydrolysis during the shelf-life of the liposomal product can be reduced by the storage of liposomal dispersion in the dry state by freeze-drying (lyophilization), without compromising their physical state or encapsulation capacity [52]. However, freeze-drying of liposome systems without appropriate stabilizers will lead to fusion of vesicles, i.e. physical instability. To promote vesicle stability during the freeze-drying process, cycloprotectants [53-55], including saccharides (e.g. sucrose, trehalose, and lactose)

and their derivatives are employed [56]. Cycloprotectants, especially sucrose because of its high glass transition temperature, are believed to be effective to protect the liposome membranes against possible fracture and rapture that might cause a change in size distribution and a loss of the encapsulated material presumably by forming glasses under the typical freezing conditions used for lyophilization [57]. Lyophilization increases the shelf-life of the finished product by preserving in a relatively more stable dry state. Some liposome products on market or clinical trials are provided as lyophilized powder. For example, AmBisomeTM, a liposomal amphotericin, is the first liposome product to be marketed in several countries is supplied as a lyophilized powder to be reconstituted with sterile water injection. Additionally, paclitaxel-liposome formulations have been developed which show good stability [58, 59]. These formulations once lyophilized can be stored at room temperature for extended time. On the other hand, once the preparation is reconstituted, it is not stable for more than a day in terms of size.

The physical degradation, leakage and fusion of liposomes, can occur as a result of the lattice defects in the membrane introduced during the manufacture, particularly in SUVs that are prepared below the membrane phase transition temperature. Annealing process, incubating the liposomes at a higher temperature than the phase transition temperature, can wipe out these defects by equalizing the differences in packing density between opposite sides of the bilayers. Even in annealed vesicles, aggregation and fusion can occur over a long period of time. In neutral liposomes, aggregation takes place because of the van der Waals interactions and because of the increased surface area it tends to be more pronounced in large liposomes. The simplest solution to overcome this aggregation is to add a small amount of negatively charged phospholipid (e.g. 10% PA or PG) to the liposome composition [29]

SUVs have much more tendency to fusion when compared to large liposomes due to the presence of stress arising from the high curvature of the membrane. Since this can occur specifically at the transition temperature of the membrane, it would be better to store these liposomes at a temperature much lower than the transition temperature of the lipids. For example, SUVs should be stored above their transition temperature for no longer than 24 hours but LUVs can be stored for a longer period of time if the temperature of the solution is kept in a range of 4-8 0C for approximately 1 week before the leakage of the encapsulated material starts due to the hydrolytic degradation on the membrane structure [60]. Also, addition of cholesterol to the phospholipid mixture would be a solution to reduce or eliminate the transition. The presence of cholesterol prevents packing and aggregation by inducing orientation and more rigidity to the phospholipids. Other than cholesterol, peptide incorporation to the lipid membrane also enables the lipid membrane to be more rigid at physiological temperature [61-63].

Permeability of liposome membranes depends highly on the membrane lipid composition, as well as on the encapsulated material. Large polar or ionic molecules will be retained much more efficiently than low molecular weight lipophilic compounds. Generally, for both type of encapsulated material, a rigid, more saturated membrane with a higher ratio of cholesterol forms the most stable lipid membrane concerning the leakage of the encapsulated material.

Many attempts have been made to enhance the physical stability of liposomes. Among these, surface modification of liposomes is an attractive method to improve liposomal stability both in vitro and in vivo. Some improvements in chemical and physical stability of polymer coated liposomes prepared with polysaccharide derivatives, such as mannan or amylopectin, have been demonstrated [64]. Several other substances also have been used for preparation of polymer coated liposomes such as poloxamer, polysorbate 80, carboxymethyl chitosan, and dextran derivatives [65-69]. While the possibility of coating liposomes with these polymers has been reported, few papers have dealt with the systematic evaluation of the physical stability of polymer coated liposomes. Moreover, contravening results have been also reported such as that polymer coated liposomes showed less stability than non-coated ones [65, 70].

In vivo stability of liposomes is also dependent on their charge. In serum, there are several proteins that are both positively and negatively charged. Liposomes with neutral charge are found to be more stable as they have much less electrostatic affinity towards proteins. [71].

Biological liposome stability plays important roles at various stages of drug delivery. However, liposomes are somewhat biologically unstable as a parenteral drug delivery system owing to their rapid uptake and clearance from circulation by cells of the mononuclear phagocytic system (MPS) located mainly in the liver and spleen [72, 73]. Biological stability of liposomes is dependent on the presence of agents such as proteins that interact with liposomes upon application to the subject and the administration route. There have been many strategies to enhance the biological stability of liposomes that improve the liposomal drug delivery in vivo and increase the circulation time in blood stream [74]. The complexation between polymers and liposomes has been studied as a way to increase the long-term stability of liposomes. Grafting hydrophilic polymers onto the head groups of phospholipids, or the addition of water soluble polymers containing several hydrophobic groups has been shown to increase the circulation time in vivo, as well as to inhibit liposome fusion [75-77]. These kinds of liposomes are called stealth liposome [78] or sterically stabilized liposomes [79]. The steric repulsion of these liposomes stabilizes the liposomes against aggregation. One of the most popular and successful methods to obtain long-circulating biologically stable liposomes is to coat the surface of the liposome with poly(ethylene glycol), PEG [80-84]. Although the PEG chemistry is successful in coating the liposome surface, alternative sterically protecting polymers are also under research. The candidate polymers should be biocompatible, soluble, hydrophilic and have highly flexible main chain for drug delivery. Some of these polymers given in the literature are synthetic polymers of vinyl series i.e. poly(vinyl pyrrolidone) (PVP) and poly(acrylamide) (PAA) [85, 86]. PVP has a similar history on pharmaceutical application to PEG [87, 88]. It shows high degree of biocompatibility and also acts as efficient steric protector for liposomes. It was found that the liposomal bilayers containing lipids with covalently attached to polyethylene glycol by which the membrane surface steric inhibits protein and cellular interactions with liposomes drastically prolonging the blood circulation time when injected in animals [89]. Doxil® is the liposomal doxorubicin available in the market which is stable for more than 18 months in the liquid state due to being stabilized by the usage of polyethylene glycol.

4. Sterilization of liposomes

Pharmaceutical industry in general differentiates between two principally different approaches to ensure sterility of a parental product: terminal sterilization of the final product in its container (steam sterilization) and aseptical manufacturing. Terminal sterilization is the commonly used one because of its higher sterility assurance level achieved when compared with the aseptical methods. However, terminal sterilization is not applicable to many liposomal drug carrier formulations.

There are several sterilization methods; such as filtration, gamma irradiation, final steam sterilization, dry heat sterilization, ethylene oxide sterilization, and ultraviolet sterilization. Bearing in mind the susceptibility of liposomes to the previously mentioned physical and chemical degradation mechanisms, the conditions required in conventional sterilization techniques (except filtration) are rather concerning since they involve the usage of heat, radiation and/or chemical sterilizing agents. Therefore, identification of a suitable method for sterilization of liposome formulations is a major challenge.

Sterilization Technique	Advantage(s)	Disadvantage(s)	Convenience
Filtration	Low operation temperature	Applicable to liposomes lower than 200 nm in diameter Operation under aseptic conditions	Low
γ-irradiation	Moderate operation temperature Highest microbial death reliability	Large scale operation Risk of degradation of liposomes	High
Final steam sterilization	Low cost and convenient	Risk of degradation of liposomes	High
Dry heat	Low cost and convenient	Risk of degradation of liposomes	High
Ethylene oxide	Low operation temperature	Possible carcinogenic residues	Low
UV-sterilization	Low cost and convenient	Poor penetration into products Risk of degradation of liposomes	High

Table 1. Summary of the Sterilization Techniques Applied on Liposomal Preparations.

Filtration is the most suitable sterilization technique for the thermolabile liposomes since it does not include any form of heat or condition that can result in the degradation of liposomes or leakage of the encapsulated material. However, filtration has some drawbacks such as; being only applicable to the liposomes that are smaller than 200 nm in diameter and being an expensive method due to the equipment requiring to work under high pressure (25 kg/cm^2 and above). Additionally, this technique must be performed under aseptic conditions [90].

Filtration sterilization is relatively time-consuming and not efficient for the removal of viruses [91]. Studies have shown that polycarbonate membranes are less effective than hydrophobic Fluoropore membrane and cellulose acetate/surfactant-free membrane filtration units [91]. Although the limitations of filtration provoked researches on other sterilization methods, all resulted in the formation of degradation products via the previously mentioned degradation pathways. Filtration and the other methods are summarized according to their applicability on liposomal preparations in Table 1 [92], given above.

5. Characterization of liposomes

After preparation and before application, liposomes have to be characterized in order to ensure their in vitro and in vivo performance. Liposomal properties that are commonly discussed include lamellarity (the number of bilayers present in liposomes), diameter and size distribution, lipid composition and concentration determination, the encapsulant concentration and its encapsulation efficiency.

For the characterization of chemical properties, phospholipids can be quantitatively in terms of concentration either by Bartlett Assay or Stewart Assay. The phospholipid hydrolysis might be followed by HPLC where the column outflow can be monitored continuously by UV absorbance to obtain a quantitative record of the eluted components. Moreover, the phospholipid oxidation can also be followed by a number of techniques i.e., UV absorbance method, TBA method (2-thiobarbutiric acid) (for endoperoxides), iodometric method (for hydroperoxides) and GLC (gas-liquid chromatography) method [93].

The most direct method for determination of liposome size is the electron microscopy due to the possibility of viewing the liposomes individually and obtaining the exact information about the liposome population over the whole range of sizes [94]. As liposomes do not naturally create a contrast to be visible by electron microscopy, either cryo-TEM (Figure 2) should be used or staining of the liposome sample is required. Either way, it is a very time-consuming method and it requires equipments that may not always be immediately accessible. The other method for the determination of liposome size, dynamic light scattering [95, 96], is very simple and rapid to perform but it measures an average size of liposome bulk. More recently, atomic force microscopy is also used to determine the morphology, size and stability of liposomal structures. All these size determination methods are very expensive. If only an approximate size range is required, gel exclusion chromatography might be suitable.

Electrostatic stabilization of liposomes may be a desirable feature to prevent fusion. The surface charge on the liposomes is measured by zeta-potential measurements [98]. These measurements are useful in determination of the in vivo behavior of liposomes. Often zeta potential values <-25 mV or >+25 mV are considered stable [99]. However, as mentioned earlier, charged liposomes have the disadvantage of being unstable in biological conditions.

Residual solvent is very unacceptable for drug delivery applications, therefore residual solvent should be kept at a minimum in the formulations. Quantification of residual solvents as a result

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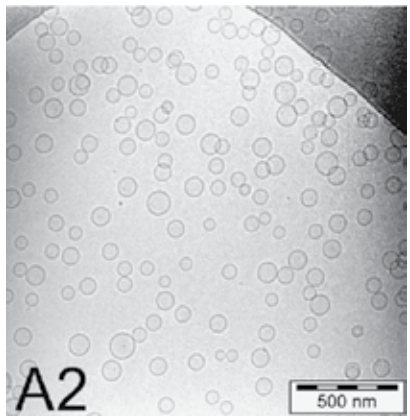

Adopted from Holzer, M., Barnert, S., Momm, J., Schubert, R., 2009. Preparative size exclusion chromatography combined with detergent removal as a versatile tool to prepare unilamellar and spherical liposomes of highly uniform size distribution. J. Chromatogr. A 1216, 5838–5848.

Figure 2. Cryo-TEM pictures of Size Exclusion Chromatography fractions eluted at 90 minutes and prepared from egg-phosphatidylcholine.

of preparation methods is done through gas chromatography (GC) [100-101] This is a very rapid and reliable method and most analytical and organic laboratories are equipped with a GC.

An important feature of liposomes is the existence of a temperature dependant, reversible phase transition, where the hydrocarbon chains of the phospholipid structures undergo a transformation from an ordered gel state to a more disordered fluid, liquid crystalline, state. This transition temperature is important in optimizing the storage conditions (i.e Temperature) to minimize fusion and drug leakage. These changes have been monitored by freeze fracture electron microscopy and much more easily by differential scanning calorimetry (DSC) [102-104, 93].

Entrapped volume is a crucial parameter that governs the morphology of liposomes. This internal volume is defined as the aqueous entrapped volume per unit quantity of lipids. The most promising way to determine the internal volume is to measure the quantity of water by replacing external medium (water) with a spectrophotometrically inert fluid (i.e. deuterium oxide) and then measuring water signal by NMR [93].

It is essential to measure the quantity of the encapsulated material inside liposomal structures before studying the behavior of this encapsulated material physically and biologically since the effects observed experimentally will be dose related. After the removal of the non-encapsulated material by the separation techniques the quantity of material remained can be assumed as 100% encapsulated. Minicolumn centrifugation and protamine aggregation methods are the general separation procedures that are commonly used [93].

Methods for determining the amount of material encapsulated within the liposomes typically rely on the destruction of the lipid bilayer and subsequent quantification of the released material [105]. In these measurements, the signal due to intact liposomes is typically monitored

prior to bilayer disruption. The techniques used for this quantification depend on the nature of the encapsulant and include spectrophotometry [106, 107], fluorescence spectroscopy [108], enzyme-based methods [109] and electrochemical techniques. If a separation technique such as HPLC of field-flow fractionation (FFF) is applied, the percent encapsulation can be expressed as the ratio of the unencapsulated peak area to that of a reference standard of the same initial concentration [110, 111]. This method can be applied if the liposomes do not undergo any purification following preparation. Either technique serves to separate liposome encapsulated materials from those that remain in the extravesicular solution and hence can also be used to monitor the storage stability in terms of leakage or the effect of various disruptive conditions o the retention of encapsulants. Some authors have combined the size distribution and encapsulation efficiency determination in one assay by using FFF-MALS (multi angled light scattering) coupled to a concentration detector suitable for the encapsulant [112].

Since techniques used to separate free materials from liposome-encapsulated contents can potentially cause leakage of contents and, in some cases, ambiguity in the extent of separation, research using methods that do not rely on separation are of interest. Reported methods have included 1H NMR where free markers exhibited pH sensitive resonance shifts in the external medium versus encapsulated markers [113]; diffusion ordered 2D NMR which relied on differences in diffusion coefficients of entrapped and free marker molecules [114]; fluorescence methods where the signal from unencapsulated fluorophores was quenched by substances present in the external solution [115]; electron pin resonance (ESR) methods which rely on the signal broadening of unencapsulated markers by the addition of a membrane-impermeable agent [116, 117].

The drug release from liposomes can be followed by the usage of a well calibrated in vitro diffusion cell in order to predict pharmacokinetics and bioavailability of drug before expensive and time-consuming in vivo studies. For the determination of pharmacokinetic performance of liposomal formulations, dilution-induced drug release in buffer and plasma was employed and for the determination of drug bioavailability, another procedure is followed which involves the liposome degradation in the presence of mouse-liver lysosome lysate [93].

6. Clinical applications of liposomes

New drug delivery systems such as liposomes are developed when the existing formulations are not satisfactory. Among all the nanomedicine platforms, liposomes have demonstrated one of the most established nanoplatforms with several FDA-approved formulations for cancer treatment, and had the greatest impact on oncology to date, because of their size, biocompatibility, biodegradability, hydrophobic and hydrophilic character, low toxicity and immunogenicity [118]. A vast of literature describes the feasibility of encapsulation of a wide range of drugs, including anti-cancer and antimicrobial agents, peptide hormones, enzymes, other proteins, vaccines and genetic materials, in the aqueous or lipid phases of liposomes which showed enhanced therapeutic activity and/or reduced toxicity in preclinical models and in humans when compared to their non-liposomal formulations.

Liposome applications in drug delivery depend, and are based on, physicochemical and colloidal characteristics such as composition, size, loading efficiency and the stability of the

carrier, as well as their biological interactions between liposomes and cells. Based on these liposome properties, several modes of drug delivery can be listed: the major ones are enhanced drug solubilization (e.g. amphotericin B, minoxidil), protection of sensitive drug molecules (e.g. cytosine arabinose, DNA, RNA, antisense olgionucleotides, ribozymes), enhanced intracellular uptake (all agents, including antineoplastic agents, antibiotics and antivirals) and altered pharmacokinetics and biodistribution of the encapsulated drug.

Although lipid based formulations have advantages as drug carriers, drug-delivery systems based on unmodified liposomes are limited by their short blood circulation time, instability in vivo and lack of target selectivity [119, 120]. To increase accumulation of liposomal formulations in the desired cells and tissues, the use of targeted liposomes including surface-attached ligands such as; antibodies, folates, peptides and transferrin that are capable of recognizing and binding to the desired cells. Despite of some improvements in targeting efficiency by these immunoliposomes, the majority of these modified liposomes were still eliminated rapidly by the reticulo endothelial system, primarily in the liver [120]. Better target accumulations are expected if liposomes can be made to remain in the circulation long enough.

Schematic drawing of cytosolic delivery and organelle-specific targeting of drug loaded nanoparticles (i.e. most frequently liposomes) via receptor-mediated endocytosis is shown in Figure 3.

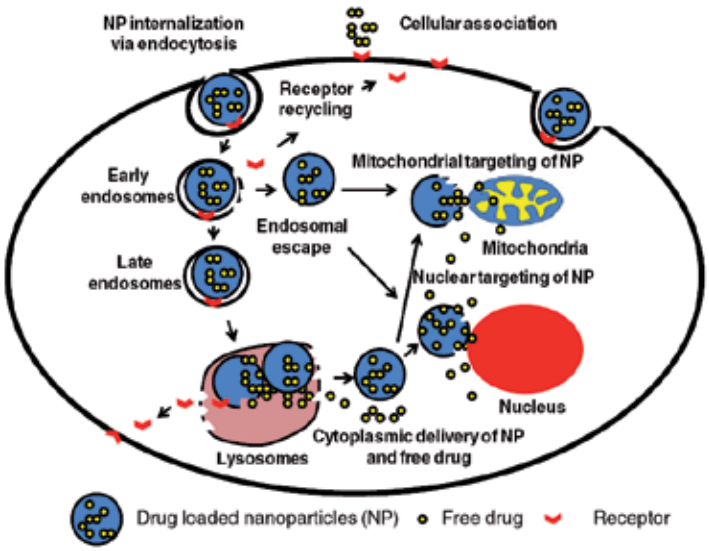

Figure 3. Schematic drawing of the cytosolic delivery and organelle-specific targeting of drug loaded nanoparticles via receptor-mediated endocytosis. After receptor mediated cell association with nanoparticles, the nanoparticles are engulfed in a vesicle known as an early endosome. Nanoparticles formulated with an endosome disrupting property disrupt the endosomes followed by cytoplasmic delivery. On the other hand, if nanoparticles are captured in early endosomes, theymaymake theirway to lysosomes as late endosomes where their degradation takes place. Only fraction of non-degraded drug released in the cytoplasm interacts with cellular organelles in a random fashion. However, cytosolic delivery of a fraction of organelle-targeted nanoparticles via endosomal escape or from lysosomes travel to the targeting organelles to deliver their therapeutic cargo [121].

Different methods have been suggested to achieve liposomes with high stability and long circulation times in vivo, including the surface coating of the liposomes with inert, biocompatible polymers such as PEG (stealth liposomes), which forms a protective layer over the liposome surface and slow down liposome recognition by opsonins and therefore subsequent clearance of liposomes [80, 84]. Long circulating liposomes are now being investigated in detail and are widely used in vitro and in vivo studies due their flexibility and also they found their place in the clinical applications. The flexibility allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface [122, 123]. Long-circulating liposomes demonstrate dose-dependent, non-saturable, log-linear kinetics and increased bioavailability [124].

The studies that attempt to combine the properties of long-circulating liposomes and immunoliposomes in one preparation place themselves in the literature as the further development in the liposomal formulations as drug carriers [125, 126]. In the early experiments, simple co-immobilization of an antibody and PEG on the surface of the same liposome has been performed despite the possibility of PEG creating steric hindrance for target recognition with the targeting moiety [125]. To achieve better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand via a PEG spacer arm, so that the ligand is extended outside the dense PEG brush which reduces steric hindrance of binding to the target [127]. The use of PEG-conjugated immunoliposomes for increasing drug carrying capacity of monoclonal antibody has been demonstrated [128]. In addition to costly monoclonal antibodies, common molecules such as folic acid, trensferrin and RGD peptides have also been studied for tumor targeting with enhanced selective uptakes [120].

Encouraging results of liposomal drugs in the treatment or prevention of a wide spectrum of diseases in experimental animals and in human, indicate that more liposome-based products for clinical and veterinary applications may be forthcoming. These could include treatment of eye and skin diseases in therapeutic applications, antimicrobial and anticancer therapy in clinical applications, metal chelation, enzyme and hormone replacement therapy, vaccine and diagnostic imaging, etc. Some of the liposome applications in terms of drug delivery are discussed below.

6.1. Ocular applications

The eye is protected by three highly efficient mechanisms (a) an epithelial layer which is the barrier to penetration (b) tear flow (c) the blinking reflex. All these mechanisms are responsible for the poor drug penetration into the deeper layers of the cornea and the aqueous humor and for the rapid wash out of drugs from the corneal surface. Initially, in 1981 the enhanced efficiency of liposomes encapsulated idoxuridine in herpes simplex infected corneal lesions in rabbits was reported [129]. In 1985, it was concluded that ocular delivery of drugs can be either promoted or impeded by the use of liposome carriers, depending on the physicochemical properties of the drugs and the lipid mixture employed [130]. The use of mucoadhesive polymers, carbopol 934P and carbopol 1342 to retain liposomes at the cornea was proposed [131]. While precorneal retention times were indeed significantly enhanced under appropriate

conditions, liposomes even in the presence of the mucoadhesive had migrated toward the conjuctival sac with very little activity remaining at the corneal surface.

6.2. Pulmonary applications

Lung is a natural target for the delivery of therapeutic and prophylactic agents such as peptides and proteins. The past 15 years have been marked by intensive research efforts on pulmonary drug delivery not only for local therapy but also for systemic therapy as well as diagnostic purposes, primarily due to the several advantages the pulmonary route offers over other routes of drug administration. Drugs that undergo gastrointestinal degradation (such as proteins and peptides) are ideal candidates for pulmonary delivery.

Targeted drug delivery to the lungs has evolved to be one of the most widely investigated systemic or local drug delivery approaches. The use of drug delivery systems for the treatment of pulmonary diseases is increasing because of their potential for localized topical therapy in the lungs. This route also makes it possible to deposit drugs more site-specific at high concentrations within the diseased lung thereby reducing the overall amount of drug activity while reducing systemic side effects. To further exploit the other advantages presented by the lungs, as well as to overcome some challenges, scientists developed interests in particulate drug delivery systems for pulmonary administration, such as liposomes, micelles, nano-and microparticles based on polymers.

The use of liposomes as drug carriers for pulmonary delivery has been reported for different kinds of therapeutics such as anti-microbial agents, cytotoxic drugs, antioxidants, anti-asthma compounds and recombinant genes for gene therapy in the treatment of cystic fibrosis.

Liposomes as carrier systems for pulmonary delivery offer several advantages over aerosol delivery of the corresponding non-encapsulated drug. Liposomes might be used to solubilize poorly soluble drugs, provide a pulmonary sustained release reservoir prolonging local and systemic therapeutic drug levels, facilitate intracellular delivery of drugs especially to alveolar macrophages, tumor cells or epithelial cells, prevent local irritation of lung tissue and reduce the drug's toxicity, target specific cell populations using surface bound ligands or antibodies and be absorbed across the epithelium to reach the systemic circulation intact [132].

Local delivery of medication to the lungs is highly desirable, especially in patients with specific pulmonary diseases such as cystic fibrosis, asthma, chronic pulmonary infections or lung cancer. The principal advantages include reduction of systemic side effects and application of higher doses of the medication at the site of drug action. Although simple inhalation devices and aerosols containing various drugs have been used since the early 19th century for the treatment of respiratory disorders, the past 15 years have been marked by intensive research efforts on pulmonary drug delivery not only for local therapy but also for systemic therapy as well as diagnostic purposes due to the several advantages the pulmonary route offers over other routes of drug administration. Lung is a natural target for the delivery of therapeutic and prophylactic agents such as peptides and proteins due to the large surface area available for absorption, the very thin absorption membrane and the elevated blood flow which rapidly distributes molecules throughout the body. Moreover, the lungs exhibit relatively low local

metabolic activity, and unlike the oral route of drug administration, pulmonary inhalation is not subject to first pass metabolism [133].

Inhaled drug delivery devices can be divided into three principal categories: nebulizers, pressurized metered-dose inhalers and dry powder inhalers; each class presents unique strengths and weaknesses. A good delivery device has to generate an aerosol of suitable size and provide reproducible drug dosing. It must also protect the physical and chemical stability of the drug formulation.

For controlled delivery of drug to the lung, liposomes are one of the most extensively investigated systems in recent studies given that they can be prepared with phospholipids such as egg phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC) and dipalmitoylphosphatidylcholine (DPPC) endogenous to the lung.

A significant disadvantage of many existing inhaled drugs is the relatively short duration of resultant clinical effects, which requires most medications to be inhaled at least twice daily. This often leads to poor patient compliance. A reduction in the frequency of dosing would be convenient, particularly for chronic diseases such as asthma. The advantages of such an approach include reduced dosing, increased effectiveness of rapidly cleared medicine and enhanced residence time at the target site for the treatment of infection. Many challenges exist in developing controlled release inhalation medicine, which is reflected in the fact that no commercial product exists. Cytotoxic agents, bronchodilators, anti-asthma drugs, antimicrobial and antiviral agents and drugs for systemic action, such as insulin and proteins are being investigated.

6.3. Cancer therapy

The numerous anti-cancer agents that have a high cytotoxic effect on the tumor cells in vitro exhibit a remarkable decrease of the selective ant-tumor effect for in vivo procedures applicable in the clinical treatment. One of the significant limitations of the anti-cancer drugs is their low therapeutic index meaning that the dose required to produce an anti-tumor effect is toxic to normal tissues. The low therapeutic index of these drugs results from the inability to achieve therapeutic concentrations at the specific target sites, tumors. Further, it results from the non-specific toxicity to normal tissues such as bone marrow, renal, gastrointestinal tract, and cardiac tissue and also from the problems associated with a preparation of a suitable formulation of the drugs [134].

Many different liposome formulations of various anticancer agents were shown to be less toxic than the free drug so that most of the medical applications of liposomes that have reached the preclinical stage are in cancer treatment [135-137]. Entrapment of these drugs into liposomes resulted in increased circulation lifetime, enhanced deposition in the infected tissues, and protection from the drug metabolic degradation, altered tissue distribution of the drug, with its enhanced uptake in organs rich in mononuclear phagocytic cells (liver, spleen and bone marrow) and decreased uptake in the kidney, myocardium and brain. To target tumors, liposomes must be capable of leaving the blood and accessing the tumor. However, because of their size liposomes cannot normally undergo transcapillary passage. In spite of this, various

studies have demonstrated the accumulation of liposomes in certain tumors in a higher concentration than found in normal tissues [138, 139]. Anthracyclines are drugs which stop the growth of dividing cells by intercalating into the DNA and therefore kill predominantly quickly dividing cells. These cells are not only in tumors but are also in hair, gastrointestinal mucosa, and blood cells; therefore, this class of drugs is very toxic. Many research efforts have been directed towards improving the safety profile of the anthracyclines cytotoxics, doxorubicin and daunorubicin, along with vincristine. Encapsulation of these drugs into the liposomes showed reduced cardiotoxicity, dermal toxicity and better survival of the experimental animals compared to the controls receiving free drugs [138]. Such beneficial effects of liposomal anthracyclines have been observed with a variety of liposome formulations regardless of their lipid composition and provided that lipids used high cholesterol concentration of phospholipids with high phase transition temperature are conducive to drug retention by the vesicles in the systemic circulation [45].

Active targeting of cancer drugs to the tumors is shown schematically in Figure 4.

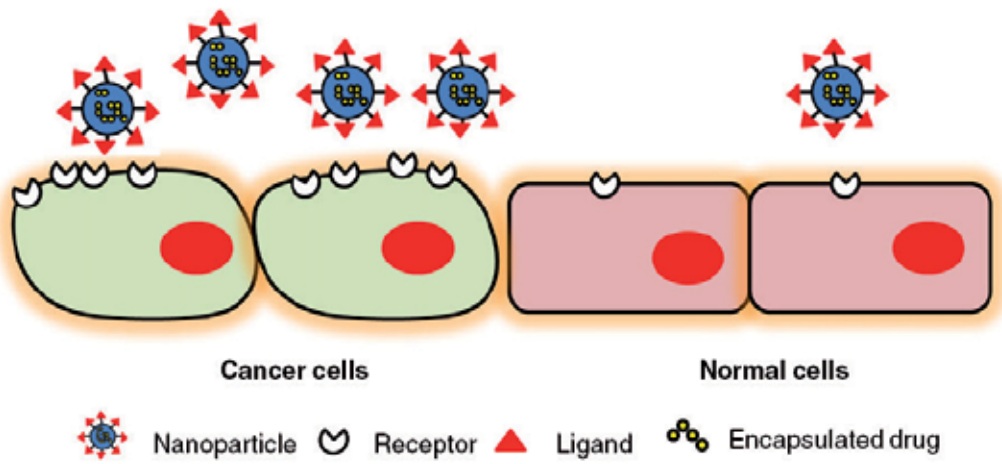

Figure 4. Representation of active targeting via receptors [121].

Currently several liposomal formulations are in the clinical practice containing different chemotherapeutics such as doxorubicin (Doxil1/Caelyx1), doxorubicin (Myocet1), daunorubicin (DaunoXome1) and cytarabine (DepoCyte1) for treating the ovarian cancer, AIDS related Kaposi's sarcoma, multiple myeloma, lymphomas, leukemia with meningeal spread. Several other liposomal chemotherapeutic drugs containing doxorubicin, annamycin, mitoxantrone, cisplatin, oxaliplatin, camptothecine, 9-nitro-20 (S)-camptothecin, irinotecan, lurtotecan, topotecan, paclitaxel, vincristine, vinorelbine and floxuridine are at the various stages of clinical trials [140].

Two liposomal formulations have been approved by the US Food and Drug Administration (FDA) and are commercially available for the treatment of AIDS-related Kaposi's sarcoma. Doxil, first liposomal drug approved by FDA and has been on the market since 1995, is a

formulation of doxorubicin precipitated in sterically stabilized liposomes and has been on the market since 1995 [141], while DaunoXome, approved six months later than Doxil, is daunorubicin encapsulated in small liposomes with very strong and cohesive bilayers, which can be referred as mechanical stabilization [142].

DaunoXome is composed of small unilamellar vesicles containing distearoylphosphatidylcholine-cholesterol (2:1) with daunorubicin loaded by a pH gradient [137]. These liposomes are selectively stable in the circulation because they are small and their membrane is electrically neutral and mechanically very strong [142]. This reduces the charge-induced and hydrophobic binding of plasma components but does not protect against van der Waals adsorption. Also, uncharged liposomes are colloidally less stable than charged ones.

Doxil is a liquid suspension of 80-100 nm liposomes (2000PEG-distearoylphosphatidylethanolamine-hydrogenated-soya-bean phosphatidylcholine-cholesterol, 20 mM) loaded with doxorubicin HCl by ammonium sulfate gradient technique and additionally precipitation with encapsulated sulfate anions. These liposomes circulate in patients for several days, which increase their chances of extravasating at sites with a leaky vascular system. Their stability is due to their surface PEG coating as well as to their mechanically very stable bilayers [141, 142].

Cytarabine (Ara-C) is an effective hydrophilic chemotherapeutic agent used widely for the treatment of acute myelogenous leukaemia and lymphocytic leukeamia [143]. It has often been utilized in the combination chemotherapy, against solid tumors and leukaemias. Cytarabine is a cell cycle-dependent drug; hence, prolonged exposure of cells to cytotoxic concentrations is critical to achieve maximum cytotoxic activity. The toxicity of cytarabine is reduced if it is able to maintain an effective therapeutic level for a long period of time and, thus, it is a suitable candidate for administration in a controlled-release dosage form. Liposome encapsulated liposomes (DepoCytTM) are now commercially available.

Etoposide (VP-16-213) is another successful chemotherapeutic agents used for the treatment of human cancers. The drug is currently in its third decade of clinical use and is a front line therapy for a variety of malignancies, including leukaemias, lymphomas and several solid tumors [144]. It has a short biological half-life (3.6 h) with a terminal half-life of 1.5 h intravenously and a variable oral bioavailability ranging from 24% to 74%. Although intraperitoneal injection would result in initial high local tumor concentrations, prolonged exposure of tumor cells may not be possible [145].

The harmful and even destructive effect of cytotoxic drugs on healthy body cells makes it necessary to search for new delivery methods for drugs like cytarabine and etoposide. There are many articles describing the results of investigations of incorporation of cytarabine [146] and etoposide [147] into liposome. However, there is no information about their simultaneous incorporation, in spite of the fact that these two drugs have been used for more than 30 years.

Taxanes are complexes of diterpenoid natural products and semisynthetic analogs. Presently, these drugs belong to prominent anticancer agents used for combined chemotherapy [148]. Paclitaxel (PTX), the prototype of this class, emerges from a natural source [149]. This drug have been used for various cancers including ovarian, breast, head and neck, and non-small cell lung cancers [150].

The commercial PTX preparation (Taxol®) is formulated in the vehicle composed of Cremophor EL® (polyethoxylated castor oil used as a solubilizing surfactant) and dehydrated ethanol, which provides a homogenous preparation. However, some drawbacks have been reported for its clinical applications of this formulation such as severe hypersensitivity reactions, neurotoxicity and neutropenia [151, 152]. It was reported that these adverse effects associated with this formlation would be due to Cremophor EL rather than PTX itself [153]. PTX solubilized in Cremophor EL shows also an incompatibility with the polyvinyl chloride of the administration sets [152]. Furthermore, the short-term stability of PTX upon dilution with aqueous media can result in possible drug precipitation [154].

Special requirements regarding a proper filter device as well as appropriate containers and infusion bags for the storage and administration of the drug have to be fulfilled in order to overcome the problems of incompatibility and instability during the clinical application of Taxol®. Hence, the development of an improved delivery system for PTX is of high importance. Current approaches are focused mainly on the development of formulations that are devoid of Cremophor EL, investigation of the possibility of a large-scale preparation and a request for a longer-term stability. There are some promising possibilities to replace Taxol® by a less irritable preparation such as micelle formulations, water-soluble prodrug preparations, enzyme-activatable prodrug preparations conjugated with antibodies or albumin, parenteral emulsions, microspheres, cyclodextrins, and nanocrytals [155-162].

The preparation of an optimal PTX formulation requires important considerations such as the optimization of the liposomal composition, the balance of the PTX amount encapsulated in the liposomes and the stability of the prepared PTX liposomes during storage in aqueous media [163]. The main characteristics of PTX molecule are asymmetry, bulkiness, hydrophobicity, low solubility and tendency to crystallization in aqueous media. All these factors affect the final design and preparation of a suitable drug formulation.

Liposomes provide suitable environment enhancing the solubility of the hydrophobic nature PTX by associating the molecule within the membrane bilayers. Commonly prepared formulations of PTX with liposomes were able to encapsulate the highest achievable content of PTX, 3-4 mol% with stability for weeks to months whereas 4-5 mol% paclitaxel was stable in the time range of just several hours to a day, and 8% paclitaxel loading only resulted in 15 minutes of liposome stability. Generally, increasing the encapsulated amount of PTX causes a reduction in the stability of the liposomal-PTX formulation due to the crystallization of the drug molecule. Thereby, to achieve a high drug/lipid ratio while retaining the long-term physical-chemical stability, a freeze-drying method is employed to obtain a dry drug-lipid powder, which is rehydrated in an aqueous solution immediately before use [58]. The encapsulation of PTX into liposomes enhances the drug therapeutic efficacy, thus, the same therapeutic effect could be reached by a decreased PTX-dose. On the other hand, the maximum tolerated dose (MTD) of liposome-encapsulated PTX increased compared with the Taxol® [Straubinger, R.M. and S.V. Balasubramanian, Preparation and Characterization of Taxane-containing Liposomes, Methods Enzymol. 391 (2005) 97-117.]. [163].

Taxane liposomes have shown slower elimination, higher antitumor activity against various murine and human tumors and lower systemic toxic effect compared to Taxol® [58]. They

have also shown antitumor effect in Taxol-resistant tumor models [164]. Abraxane®, the only nonliposomal preparation of PTX, (albumin nanoparticle-based PTX preparation) and Lipusu® (liposomal PTX approved by State FDA of China) have entered the field of clinical applications. LEP-ETU (NeoPharm) and EndoTAG®-1 (Medigene) have reached the phase II of the clinical trials. Generally, liposomes and protein nanoparticles represent a promising approach to the optimization of PTX delivery. Their commercialization is at the doorstep of modern drug delivery market.

7. New generation liposomes

Liposomes made up of commonly used ester phospholipids such as phosphatidylcholine are referred as conventional liposomes. These structures are very attractive for encapsulation and drug delivery applications to entrap both hydrophilic and hydrophobic materials due to the presence of aqueous core part as well as the lipid bilayer. Up to this date, there are many formulations in the market and also in the clinical trials. However, none of them truly overcome their chemical and physical instability problems especially during the transfer to the site of action [120]. Various attempts like modification of the liposome surface with i.e. hydrophilic polyethylene glycol polymers, using cryoprotectants or incorporation of high amount of cholesterol into the bilayer have led to only limited success. Other than instability problems, liposomal drug vehicles show extensive leakage of water-soluble drugs during the passage through the gastrointestinal tract and they are heterogeneous in terms of size distribution. Therefore, scientists have been looking for new drug delivery formulations that could address these issues about liposomes, which lead to the so-called new generation of liposomes which will be summarized in this section.

Archaeosomes are liposomal formulations that are prepared with one or more lipids, mainly containing diether and/or tetraether linkages, found in archaeobacterial membrane [165]. These archaeobacterial lipids present unique features and higher stabilities to several conditions (high or low temperatures, high salinity, acidic media, anaerobic atmosphere, high pressure) over conventional liposomes [166]. The definition of archaeosomes also includes the use of synthetically derived lipids that have the properties of archaeobacterial ether lipids, that is, regularly branched phytanyl chains attached via ether bonds at sn-2,3 glycerol carbons [167]. The surprising stability of archaeosomes can be attributed to some properties brought by the archaeobacterial lipids' structure: (i) the ether linkages that are more stable than esters over a wide range of pH, and the branching methyl groups help both to reduce crystallization and permeability; (2) the stability towards oxidative degradation of these lipid membranes are provided by the fully saturated alkyl chains in the archaeobacterial lipids; (3) the unusual stereochemistry of the glycerol backbone ensures the resistance of the membrane to enzymatic attack; (4) the bipolar lipids span the membranes and enhance their stability properties [167, 168].

Archaeosomes can be prepared by using conventional procedures (hydration of a thin film followed by sonication or extrusion, detergent dialysis) at any temperature in the physiolog-

ical range or lower, thus making it possible to encapsulate thermally labile compounds. Additionally, they can be prepared and stored in the presence oxygen without any degradation. According to the clinical experiments, in vivo and in vitro, these new drug delivery vehicles are not toxic. Thus, the biocompatibility and better stability of archaeosomes in numerous conditions offer advantages over conventional liposomes for their usage in biotechnology including vaccine and drug/gene delivery [167]. Consequently, they can be considered as better carriers than conventional liposomes, especially for protein and peptide delivery due to their high stability. Li et al. showed the superiority of archaeosomes over conventional liposomes in their study in which they used insulin as a model peptide for its oral delivery [169].

Another development aiming to enhance tissue targeting is virosomes in which the liposome surface is modified with fusogenic viral envelope proteins [170]. Virosomes have been used for the intracellular delivery of drugs and DNA [171, 172] as well as the basis of the newly developed vaccines which are very effective in the delivery of protein antigens to the immune system [173]. As a result, a whole set of virosomes-based vaccines have been developed for human and animal use. Special attention has been paid to the delivery of influenza vaccine using virosomes containing the spike proteins of influenza virus. Virosome-based vaccines were found to be highly immunogenic and well tolerated in children. A similar approach was used to prepare virosomal hepatitis A vaccine that elicited high antibody titres after primary and booster vaccination of infants and young children which was also confirmed for the healthy adults and elderly patients [174-176]. In general, virosomes can provide an excellent opportunity for the efficient delivery of both various antigens and many drugs, including nucleic acids, cytotoxic drugs and toxoids [177, 178], although they might present certain problems associated with their stability, leakiness and immunogenicity.

Niosomes, exhibiting a similar behavior to liposomes, are the vesicles that are made up of nonionic surfactants (e.g. alkyl ethers and alkyl esters) and cholesterol. These structures are stable on their own and they increase the stability of the encapsulated drugs. No special conditions are needed for handling and storage of these surfactants. Niosomes improve the oral bioavailability of poorly absorbed drugs, and enhance skin penetration of drug. When compared with liposomes, their oral absorption is better due to the replacement of phospholipids with nonionic surfactants which are less susceptible to the action of bile salts, parenteral, as well as topical routes. These delivery systems are biodegradable, biocompatible and non-immunogenic. Niosomes improve the therapeutic performance of drug molecules by delaying the clearance from the circulation and protecting the drug from biological environment [179].

The transdermal delivery is one of the most important routes of drug administration. The main factor which limits the application of transdermal route for drug delivery is the permeation of drugs through the skin. Human skin has selective permeability for drugs. Lipophilic drugs can pass through the skin but the drugs which are hydrophilic in nature can not pass through. Water soluble drugs either show less or no permeation. To improve the permeation of drugs through the skin various mechanisms have been investigated, including use of chemical or physical enhancers, such as iontophoresis, sonophoresis, etc. Liposomes and niosomes are not

suitable for transdermal delivery due to poor skin permeability, breaking of the system, aggregation, drug leakage, and fusion of vesicles [180].

A new type of carrier system, suitable for transdermal delivery, called transfersome has been proposed for the delivery of proteins and peptides like insulin, bovin, serum albumin, vaccines, etc. These systems are soft and malleable carriers that offer noninvasive delivery of drug into or across the deeper skin layers and/or the systemic circulation [181]. Transfersomes improve the site specificity while providing the safety of the drug. Transfersomes are the lipid supramolecular aggregates which make them very flexible. This flexibility as well as their good penetration ability causes them to be used in the effective delivery of non-steroidal anti-inflammatory agents like ibuprofen and diclofenac [182].

Alternatively, unlike classic liposomes [183, 184], that are known mainly to deliver drugs to the outer layers of skin, ethosomes can enhance permeation through the stratum corneum barrier [185-187]. Ethosomes, developed by Touitou in 1997, are the slight modification of well established drug carrier liposome, containing phospholipids, alcohol (ethanol or isopropyl alcohol) in relatively high concentration and water [188]. The size of these soft vesicles can vary from nanometers to microns [189-193]. The high concentration of ethanol makes the ethosomes unique. The ethanol in ethosomes causes disturbance in the skin lipid bilayer organization, hence when incorporated into a vesicle membrane, it enhances the vesicle's ability to penetrate the stratum corneum. Also, because of the high concentration of ethanol the lipid membrane is packed less tightly than conventional vesicles but has equivalent stability, allowing a more malleable structure and improves drug distribution ability in stratum corneum lipids. Ethosomes can be used for many purposes in drug delivery for the treatment of many diseases such as Minoxidil for baldness, testosterone as steroidal hormone, Trihexyphenidyl hydrochloride for Parkinson's disease, Zidovudine and Lamivudine as anti-HIV, Bacitracin as antibacterial, Erythromycin as antimicrobial, DNA for genetic disorders, Cannabidol in the treatment of rheumatoid arthiritis and many others [190, 192, 194-201].

Novasomes are the modified forms of liposomes [202] or a type of niosomes prepared from the mixture of monoester of polyoxyethylene fatty acids, cholesterol and free fatty acids with the diameter of 0.1-1.0 microns. They consist of two to seven bilayer shells that surround an unstructured space occupied by a large amorphous core of hydrophilic or hydrophobic materials [203]. The inner amorphous core can be loaded up to 80-85% with a medical drug and the surfaces of novasomes can be positive, negative or neutral.

Novasomes offer several advantages to the owners of the product such as: Both hydrophilic and hydrophobic products can be incorporated in the same formulation, drugs showing interactions can be incorporated in between bilayers to prevent incompatibility, they can be made site specific due to their surface charge characteristics, they can deliver a large volume of active ingredient, thus also reducing the frequency of application, and they have the ability of adhering skin or hair shafts which makes novasomes applicable in the cosmetic formulations [204].

Novasomes have extensive utilization in fields of foods, cosmetics, personal care, chemical, agrochemical and pharmaceuticals. The technology enhances absorption rate via topical

delivery of pharmaceuticals and cosmeceuticals by utilizing non-phospholipid structures. Various FDA-regulated products such as human pharmaceuticals and vaccines can be developed by this technology [205, 206]. These nonionic vesicles composed of glyceryl dilaurate with cholesterol and polyoxyethylene-10-stearyl ether have been known to deliver greater amounts of cyclosporine into and through hairless mouse skin than phosphatidyl choline or ceramide based vesicles [206]. Among various liposomal formulations, novasomes appeared more effective when delivered under non-occluded conditions from a finite dose [206]. Various vaccines based on novasomes have been licensed for the immunization of fowl against Newcastle disease virus and avian rheovirus [135]. Some of the novasome-based vaccines against bacterial and viral infections have been developed such as small pox vaccine while still many are under development [207]. Novasomes inactivate viruses such as orthomyxoviruses, paramyxoviruses, coronaviruses and retroviruses, etc., by fusing with enveloped virus and that the nucleic acid of the virus denatures shortly after the fusion [208].

Although liposomes are like biomembranes, they are still foreign objects of the body. Therefore, liposomes are known by the mononuclear phagocytic system (MPS) after contact with plasma proteins. Accordingly, liposomes are cleared from the blood stream. For more than two decades, various PEG derivatives have been used to stabilize for increasing efficiency in drug or gene delivery. Most 'stabilized' liposomes, the so-called stealth liposomes [78], or cryptosomes [84], contain a certain percentage of PEG-derivatized phospholipids, which reduce the uptake by MPS, thereof prolonging the circulation times and making available abundant time for these liposomes to leak from the circulation through the leaky endothelium. Unlike, conventional liposomes, PEG-liposomes do not show dose dependent blood clearance kinetics [209]. Vesicles containing PEG-conjugated lipids at various concentrations, molecular weights, or various sizes of PEG-containing vesicles were reported to have different circulation times [81, 84, 210-212]. These kind of liposomal systems are generally used in the ligand-mediated drug targeting [213]. This stealth principle has been used to develop the successful doxorubicin-loaded liposome product that is presently available in the market as Doxil (Janssen Biotech, Inc., Horsham, USA) or Caelyx (Schering-Plough Corporation, Kenilworth, USA) for the treatment of solid tumors.

Cryptosomes is a liposomal composition for targeted delivery of drugs. The composition comprises poloxamer molecules and liposomes encapsulating one or more delivery agents. Poloxamers are polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide triblock co-polymers of different molecular weights. The hydrophobic PPO group in the middle links the two hydrophilic PEO groups. The hydrophilic PEO groups of a poloxamer, on either side of the central PPO unit, can provide steric protection to a bilayer surface. The amphiphilic nature of the poloxamers makes them extremely useful in various applications as emulsifiers and stabilizers. It is considered that the central PPO unit, being hydrophobic, would tend to push into the bilayer interior serving as an anchor. Dislodging the poloxamer molecule from the bilayer is achieved by reducing its hydrophobicity which is achieved by decreasing the temperature. In an aqueous medium, poloxamers stay as individual molecules at temperatures below their critical micelle temperature (CMT), but at temperatures above the CMT, they form micelles due to their amphiphilic nature. In the presence of lipid bilayers, some poloxamer

molecules would partition into the bilayers as well as forming micelles with other poloxamer units. If the temperature again goes below the CMT, the poloxamer molecules lose their amphiphilic nature and disassociate from the lipid bilayer or micelle [179].

Emulsome, having the characteristics of both liposomes and emulsions, is a novel lipoidal vesicular system with an internal solid fat core surrounded by phospholipid bilayer. Emulsomes comprise a hydrophobic core (composed of solid fates instead of oils) as in standard oil-in-water emulsions, but the core is surrounded and stabilized by one or more envelopes of phospholipid bilayers as in liposomes allowing water insoluble drugs in the solution form without requiring any surface active agent or co-solvent. Emulsomes differ from liposomes since their internal core is a lipid, whereas the internal core in liposomes is an aqueous compartment. The drug loading is generally followed by sonication to produce emulsomes of smaller size [214]. These systems are often prepared by melt expression or emulsion solvent diffusive extraction. The lipid assembly of emulsomes, stabilized by cholesterol and soya lecithin (5-10% by weight), has features that are intermediate between liposomes and oil-in-water emulsions droplets. Emulsomes provide the advantages of improved hydrophobic drug loading in the internal solid lipid core and the ability of encapsulating water-soluble medicaments in the aqueous compartments of surrounding phospholipid layers.

Beside the other vesicular formulations, emulsomes are much stabilized and nano range vesicles. It is a new emerging delivery system and therefore could play a fundamental function in the effective treatment of life-threatening viral infections and fungal infections such as hepatitis, HIV, Epstein-Barr virus, leishmaniasis, etc. appear promising for the treatment of visceral leishmaniasis specifically and hepato-splenic candidiasis [214-216]. Emulsomes could be utilized in order to improve oral controlled delivery of drug, vaccine, and biomacromolecules. It is due to the fact that they are nano sized in range and could be utilized for the intravenous route. The common application areas of emulsomes are drug targeting, anti-neoplastic treatment, leishmaniasis (a disease in which a parasite of the genus Leishmania invades the cells of the liver and spleen) treatment, and biotechnology. Moreover, emulsomes could represent a more economical alternative to current commercial lipid formulations for the treatment of viral infections and fungal infections. Emulsomes provide a controlled and sustain release of drug. In comparison to the liposomes, emulsomes provide a prolong release of drug up to 24 hours, whereas liposomes have shown release up to 6 hours [217-219]. Emulsomes are nano size range in comparison to other vesicular delivery system such as niosomes and ethosomes. Due to the reduced size (10-250 nm) they can be used to enhance bioavailability to drug and as the best carrier for the intravenous drug delivery as well as oral drug delivery.

The lipid core of emulsomes may contain one or more anti-oxidants which are generally α-tocopherol or its derivatives that are the members of Vitamin-E family. The presence of anti-oxidants reduces the formation of oxidative degradation products of unsaturated lipids such as peroxides. The need of anti-oxidant can be prevented by the usage of saturated fatty acids during the preparation of the lipid core [220]. In the formation of emulsomes, like in the case of liposomes, cholesterol is essential component for the system that influences the stability of emulsomal systems and plays an important role in the drug encapsulation [221-224].

The most important advantage of emulsomes is their ability to protect the encapsulated drug from harsh gastric environment of stomach before oral administration because the drug is inside the triglyceride lipid core which can be supported that the gastric pH and the gastric enzymes are unable to hydrolyze triglycerides. Also, they resist development of multi drug resistance, often associated with over expression of a cell membrane glycoprotein, which cause efflux of the drug from the cytoplasm and results in an ineffective drug concentration inside the cellular compartment [225].

The development of emulsomes, however, is still largely empirical, and in vitro models that are predictive of oral bioavailability enhancement are lacking. There is a need for in vitro methods for predicting the dynamic changes involving the drug in the gut in order to monitor the solubilization state of the drug in vivo. Attention also needs to be paid to the interactions between lipid systems and the pharmacologically active substance. The characteristics of various lipid formulations also need to be understood, so that guidelines can be established that allow identification of suitable candidate formulations at an early stage. Future research should involve human bioavailability studies as well as more basic studies on the mechanisms of action of this fascinating and diverse group of formulations.

Unilamellar vesicles or liposomes are commonly used as simple cell models and as drug delivery vehicles to follow the release kinetics of lipophilic drugs that require compartmental models in its therapeutics and triggers. The localization of the drug at the site of action, rate of achieving the therapeutic index and circulation lifetime are the key parameters for a liposome. Lately, their arises a need for a multi-compartment structure consisting of drug-loaded liposomes encapsulated within another bilayer, is a promising drug carrier with better retention and stability due to prevention enzymes or proteins reaching the interior bilayers. A vesosome is a more or less heterogeneous, aggregated, large lipid bilayer enclosing multiple, smaller liposomes that offer a second barrier of protection for interior compartments and can also serve as the anchor for active targeting components [226, 76]. The multi-compartment structure of vesosome can also allow for independent optimization of the interior compartments and exterior bilayer; however, just the bilayer-within-a-bilayer structure of the vesosome is sufficient to increase drug retention from minutes to hours [227, 228].

In nature, eukaryotes increased their ability to optimize their response to their surroundings by developing multiple compartments, each of which has a distinct bilayer membrane, usually of quite varied composition and physical structure. Mimicking this natural progression to nested bilayer compartments led to the development of the vesosome, or vesicles deliberately trapped within another vesicle. The vesosome has distinct inner compartments separated from the external membrane; each compartment can encapsulate different materials and have different bilayer compositions. In addition, while it has proven difficult to encapsulate anything larger than molecular solutions within lipid bilayers by conventional vesicle self-assembly, the vesosome construction process lends itself to trapping colloidal particles and biological macromolecules relatively efficiently [229, 230]. The nested bilayer compartments of the vesosome provide a degree of freedom for optimization not possible with a single membrane enclosed compartment and a more realistic approximation of higher order biological organization.

The vesosome structure could be used to deliver a cocktail of antibiotics or antimicrobials to sites at a fixed ratio; such mixtures have been shown to act synergistically when delivered in a single liposome [231]. Such multi-drug formulations may be useful to avoid inducing pathogen resistance to a single drug.

As vesosomes are simply liposomes within liposomes, it should be possible to directly translate the extensive body of research on liposome drug delivery to the vesosome with only minor changes, and perhaps significant major improvements. The vesosome is created by simply self-assembly steps very similar to those used in making conventional unilamellar liposomes [229]. An important question is whether such additional effort in developing new structures will provide a therapeutic benefit over direct injection of the free drug or drug delivery by conventional unilamellar liposomes. The most obvious potential application for the vesosome is for drugs that have already shown increased efficacy by delivery with conventional liposomes. As an example, ciprofloxacin (cipro), a synthetic bactericidal fluoroquinolone antibiotic with broad spectrum efficacy, is released much more quickly from unilamellar liposomes in serum relative to saline [232, 233]. Conventional pH-loaded liposomes can retain essentially all encapsulated ciprofloxacin when stored in buffer for 12 weeks at 21 0C and 8 weeks at 37 0C [234, 235]. Although liposomal cipro has shown increased efficacy due to prolonged residence of cipro in the blood (free cipro is cleared in minutes), the half-life of release from the liposomes was only 1 hour, yet the liposomes themselves circulated for more than 24 hours [232, 235]. A second example is vincristine, a naturally occurring dimeric catharanthus alkaloid that has been used extensively as an antitumor agent since 1960's. The therapeutic activity of vincristine is dictated by the duration of therapeutic concentrations at the tumor site [236, 238, 239]. However, conventional liposomes, while offering improved bioavailability, also cannot encapsulate vincristine for sufficient time to give optimal results [234, 236, 237]. Future work will determine if multiple compartment structures like vesosome give sufficient enhancement of small drug entrapment to lead to new therapeutics.

Genetics play an increasingly important role in medicine and is used routinely to diagnose diseases and to understand malfunctions at the molecular level. The active approach of trying to amend genetic defects or insufficiencies is a logical next step. Major elements in the successful advance of gene therapy are identification of the disease and target cells, tissues and organs as well as construction of appropriate gene vectors, effective gene transfer and expression in the targeted cells. Many inherited diseases follow the Mendelian inheritance pattern in which the cause is due to a single genetic defect. Because the existing therapeutic treatments of such diseases are in most cases very limited, it is hoped that by transfecting appropriate cells with the correct gene or by adding a missing one, the disease could be alleviated. Examples of such potential treatments are for cystic fibrosis, hemophilia, sickle cell anemia or hypercholesterimia and mutant tumor suppressor genes.

The aim of gene therapy is to deliver DNA, RNA or antisense sequences to appropriate cells in order to alleviate symptoms or prevent the occurrence of a particular disease, i.e. repair the defect and also its cause. The major approaches to gene therapy include gene replacement, addition of genes for production of natural toxins, stimulation of the immune system or over

expression of highly immunogenenic genes for immune self-attack and sensitization of cells to other treatments.

Recently, the studies on gene delivery into eukaryotic cells by the use of non-viral-lipid-based macromolecular delivery systems have been experiencing a growing interest owing to the appearance of clinical protocols for gene therapy. Although the efficiency and specificity of such non-viral delivery systems are not yet very high, some of the problems concerning transfection methods are being successfully solved. To date, the transfection mediators that ensure effective and directed gene delivery into various cells have been created. Transfection of plasmid DNA is closely connected to the problem of condensation of its molecule since the plasmid is too large (13-15 kb) to effectively overcome the cellular membrane barrier. Besides, free DNA has to be protected from destruction by endogenous nucleases. Lastly, it is necessary to neutralize the negative charge on DNA.

Genosomes are the artificial functional complexes for functional gene or DNA delivery to cell [238]. For the production of genosomes, cationic phospholipids were found to be more suitable because they possess high biodegradability and stability in the blood stream. Gene delivery is a vast area of research and a detailed summary of work in that field is beyond the scope of this chapter.

New generation liposomes and their features are summarized in Table 2.

Type	Main constituent	Advantage
Liposomes	Phospholipids	
Archaesomes	One or more lipids containing diether linkages	High stability at several conditions
Niosomes	Non-ionic surfactant and cholesterol	Less prone to action of bile salts
Novasomes	Monoester of polyoxyethylene fatty acids, cholesterol and free fatty acids. Two to seven bilayer shells	High loading of drugs
Transfersomes	Lipid supramolecular aggregates	More flexible hence better transdermal delivery
Ethosomes	Phospholipids and alcohol in relatively high concentration	More distruptive in the skin lipid bilayer organization hence better transdermal delivery
Virosomes	Lipids surface modified with fusogenic viral envelope proteins	Intracellular delivery of antigens, drugs and DNA
Cryptosomes	Phospholipids and polaxamers or PEG	More stable
Emulsomes	Internal solid fat core surrounded by phospholipid bilayer	Better for encapsulation of hydrophobic drugs
Vesesomes	Multilamellar liposomes	Multidrug formulations are possible
Genosomes	Complex of cationic phospholipids and a functional gene or DNA	Suitable for gene delivery

Table 2. New generation liposomes and their features.

Extensively motivated by the need to increase the stability and bioavailability of drugs, and to reduce their side effects by targeting to the site of action, research in new drug delivery vehicles has taken giant steps. Liposomes and their derivatives, so called new generation liposomes, present a vast area in this field where several advances have already been achieved as summarized in this chapter. However, still further research is required to overcome the limitations faced today in terms of prolonged stability, drug loading and active targeting.

8. Conclusion

In the last decade from the concept of clinical utility of liposomes to their recognized position in mainstream of drug delivery systems, the path has been long and winding. The liposome systems have been explored in the clinic for applications as diverse as sites of infection and imaging, for vaccine, gene delivery and small molecular drugs, for treatment of infections and for cancer treatment, for lung disease and for skin conditions etc. Several liposomal formulations are already on the market, while quite a few are still in the pipeline for treatment of diseases. Conventional techniques for liposome preparation and size reduction remain popular as these are simple to implement and do not require sophisticated equipment. However, not all laboratory scale techniques are easy to scale-up for industrial liposome production. Many conventional methods, for preparing small and large unilamellar vesicles, involve use of either water miscible/immiscible organic solvents or detergent molecules. The need for improvements in the design and stability of liposomal diagnostic and therapeutic systems will continue to motivate innovative and efficient routes to their production.

Author details

Melis Çağdaş[1], Ali Demir Sezer[2] and Seyda Bucak[3*]

*Address all correspondence to: seyda@yeditepe.edu.tr

1 Department of Chemistry, Boğaziçi University, Bebek, Istanbul, Turkey

2 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Haydarpaşa, Istanbul, Turkey

3 Department of Chemical Engineering, Yeditepe University, Kayışdağı, Istanbul, Turkey

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Chapter 2

Techniques for the Preparation of Solid Lipid Nano and Microparticles

Luigi Battaglia, Marina Gallarate,
Pier Paolo Panciani, Elena Ugazio, Simona Sapino,
Elena Peira and Daniela Chirio

Additional information is available at the end of the chapter

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1. Introduction

SLN are nanoparticles made of solid lipids with a photon correlation spectroscopy (PCS) mean diameter approximately between 50 and 1000 nm [1].

General ingredients include solid lipid(s), surfactant(s) and water. The term lipid is used here in a broad sense and includes triglycerides (e.g. tristearin), partial glycerides, fatty acids (e.g. stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). All classes of surfactants (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion [2].

SLN were developed in the early 1990s and up today they have been considered to be promising drug carrier systems, especially with the aim to give a sustained release profile to the incorporated active substances. In fact, compared to liquid lipid formulations, such as nanoemulsions, drug mobility is really lower in solid lipids than in liquid oils. Compared to polymeric nanoparticles, they show a better biocompatibility since they're made of lipids similar to physiological one, so the toxicity is reduced. Moreover SLN are physico-chemically stable and can be produced easily on a large industrial scale, and raw material and production costs are relatively low [1].

SLM have equivalent composition to SLN, but higher particle size (>1000 nm), meaning that their application domains and administration routes can be different [3].

There are different techniques for the preparation of solid lipid nano-and microparticles: generally, the preparation of nano-and microparticles requires a dispersed system as precursor or template, otherwise particles are produced through the use of a particular instrumentation.

The most important precursor are emulsions (hot homogenisation technique, melt dispersion technique, PIT method, solvent evaporation-diffusion from emulsions), microemulsions (microemulsion dilution and microemulsion cooling techniques), micellar solutions (coacervation technique). Some preparation techniques are based on supercritical fluids. The most important techniques which involve the use of particular instrumentation are: membrane contactor technique, spray-drying, spray-congealing and electrospray.

In Table 1 a scheme of the main preparation techniques of solid lipid nano-and microparticles is reported.

Precursor system	Technique	Nanoparticle type
Emulsion	Hot homogenisation (high pressure homogenisation, high shear homogenisation, ultrasound homogenisation)	SLN
Emulsion	Melt dispersion	SLM
Emulsion	PIT method	SLN
Microemulsion	Microemulsion dilution	SLN
Microemulsion	Microemulsion cooling	SLN
Micellar solution	Coacervation	SLN
Organic solvent solution	Solvent injection	SLN
Organic solvent emulsion	Solvent evaporation from emulsions	SLN
Organic solvent emulsion	Solvent diffusion from emulsions	SLN
Gas-saturated solution/suspension	Particles from gas-saturated solutions/ suspensions	SLN/SLM
Supercritical fluid emulsion	Supercritical fluid extraction of emulsions	SLN
	Membrane contactor technique	SLN
	Cryogenic micronisation	SLM
	Spray-drying	SLM
	Electrospray	SLN/SLM
	Spray-congealing	SLM

Table 1. Preparation techniques of solid lipid nano-and microparticles

2. Emulsion precursors

The most important precursor used for the preparation of solid lipid particles is the emulsion.

Emulsion consists of a mixture of two immiscible liquids (eg. water – W-and oil-O), where one is dispersed in the other in the form of droplets, thanks to the use of emulsifying agent.

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According to the character of the continuous and dispersed phase, emulsions can be direct (O/W), reversed (W/O) and multiple (W/O/W and O/W/O).

Emulsions can be used as precursors for solid lipid particles preparation since lipids, that are solid at room temperature, can be heated 5-10 °C above their melting point to obtain a liquid lipid that can be emulsified with water at the same temperature: the obtained hot emulsion is then cooled to room temperature and the droplets solidify in the form of solid lipid particles.

Owing to the size of the hot emulsion droplets, nano-or microparticles can be obtained.

SLN can be obtained from hot O/W nanoemulsion (<1 μm droplet size), prepared through the so called hot homogenisation technique. Different methods can be used to achieve hot homogenisation: high pressure homogenisation, high shear homogenisation, ultrasound homogenisation.

SLM, instead, can be obtained from hot emulsions (>1 μm droplet size) by the so called melt dispersion technique: O/W or multiple W/O/W precursors can be used.

2.1. Hot homogenisation

2.1.1. High pressure homogenisation

High pressure homogenisation (HPH) was applied for the first time in the nineties by Müller and Lucks [4] for SLN production and represents the main method established for them.

In the HPH technique the drug is dissolved in the lipid being melted at approximately 5-10 °C above its melting point. The drug-containing melt is dispersed under stirring in a hot aqueous surfactant solution of identical temperature, in order to obtain pre-emulsion, which is then homogenised using a piston-gap homogeniser; the produced hot O/W nanoemulsion is cooled down to room temperature, the lipid re-crystallises and leads to SLN [1].

Practically, SLN can be derived from the emulsions for parenteral nutrition just by replacing the liquid lipid (oil) of the emulsion droplets by a solid lipid. In contrast to emulsions for parenteral nutrition which are normally stabilised only by lecithin, the SLN are stabilised also by other surfactants or polymers which can act as a suspending agent in order to avoid particle aggregation.

This is a technique well established on the large scale for parenteral emulsions since the fifties and it is already available in the pharmaceutical industry. The production lines for parenteral emulsions are in most cases equipped with temperature control units because an increased temperature facilitates emulsion production, this means that existing production lines can be used for producing SLN by the hot homogenisation technique [1]. This technique allows the production of colloidal suspensions of SLN without solvents, which are generally used for polymeric nanoparticles, and this is a further advantage of this method from a toxicological point of view.

An important problem of SLN prepared through hot homogenisation is drug expulsion from nanoparticles, that can happen during lipid re-crystallisation on cooling of the hot nanoemul-

sion. Consequently, in order to improve drug loading within nanoparticles and to prevent drug leakage during storage, a new generation of lipid nanoparticles has been prepared [5,6]. NLC (nanostructured lipid carriers) are characterised by a solid lipid core consisting of a mixture of solid and liquid lipids: the resulting matrix of the lipid particles shows a melting point depression compared to the original solid lipid, but the matrix is still solid at body temperature. Depending on the method of production and on the lipid blend composition, different types of NLC are obtained: imperfect, amorphous and multiple type. In the imperfect type, lipid crystallisation is altered by small amounts of oils. In the amorphous type, the lipid matrix is solid but not crystalline (amorphous state): this can be achieved by mixing special lipids, e.g. hydroxyoctacosanyl hydroxystearate with isopropyl myristate. In the multiple type the solid lipid matrix contains tiny oil compartments: they are obtained by mixing a solid lipid with a higher amount of oil. The basic idea is that by giving a certain nanostructure to the lipid matrix, the payload for active compounds is increased and expulsion of the compound during storage is avoided [5,6].

SLN prepared through hot homogenisation are not suitable for the encapsulation of hydrophilic drugs, since they would partition preferentially to the external aqueous phase of the hot nanoemulsion: to overcome this limitation lipid drug conjugate (LDC) nanoparticles were developed. In a typical process, an insoluble drug-lipid conjugate bulk is first prepared either by salt formation (e.g. with a fatty acid) or by covalent linking (e.g. esters or ethers). The obtained LDC is then processed as the lipid for nanoparticles preparation [7,8].

However, besides all its advantages and its versatility, HPH involves some critical process parameters like high temperatures, high pressures (cavitation forces), that may cause significant thermodynamic and mechanical stress for the resulting product: in particular this method is not suitable for thermo-sensitive drugs. Moreover, melted lipids are not good solvents for many drugs and this can cause a low drug loading in the SLN. For these reasons suitable alternative and easy handling production methods for lipid nanoparticles preparation have been widely investigated in last years [9].

2.1.2. High shear homogenisation and ultrasound homogenisation

High shear homogenisation and ultrasound homogenisation [10,11] are dispersing techniques which were initially used for the production of solid lipid nanodispersions. Both methods are widespread and easy to handle. However, dispersion quality is often compromised by the presence of microparticles. Furthermore metal contamination has to be considered if ultrasounds are used [2]. In the case of high shear homogenisation, SLN can be obtained owing to the use of proper emulsifying agent, which allow the formation of the hot nanoemulsion under simple high shear mixing [10].

2.2. Melt dispersion technique

In the melt dispersion technique, SLM can be obtained either from O/W or multiple W/O/W emulsions, according respectively to the lipophilic or hydrophilic character of the drug.

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In the former case, lipophilic drug is dissolved into the melted lipids, which are emulsified with a hot surfactant solution through a high shear mixer: the obtained O/W emulsion is then cooled to room temperature to obtain solidification of lipid microparticles [12].

In the latter case, a heated hydrophilic drug solution is emulsified with a melted lipid in order to form a primary W/O emulsion, which is then put in contact with an external aqueous phase at the same temperature; the resulting W/O/W multiple emulsion is then cooled to room temperature to form microparticles [13].

2.3. PIT method

The PIT (phase inversion temperature) method is commonly used for the preparation of nanoemulsions. The PIT concept uses the specific ability of some polyethoxylated surfactants to modify their affinities for water and oil as a function of the temperature. In the PIT nanoemulsion preparation method, the use of such surfactant type leads to an emulsion inversion from O/W macroemulsion to a W/O emulsion when temperature is increased above the PIT, and to the formation of a O/W nanoemulsion when the temperature is next lowered below the PIT [14].

The PIT method has been used also for the preparation of LNC (lipid nanocapsules), nanoparticles organised in an internal liquid or semiliquid oil core and an external lipid layer solid at room temperature [15].

Recently it has been adapted for the preparation of SLN. In this case two main components are used: an oil phase, constituted by solid lipids and non-ionic surfactants and an aqueous phase containing NaCl. The aqueous phase and the oil phase are separately heated at ~90° C (above the PIT); then the aqueous phase is added dropwise, at constant temperature and under agitation, to the oil phase, in order to obtain a W/O emulsion. The mixture is then cooled to room temperature under slow and continuous stirring. At the PIT, the turbid mixture becomes clear, then below the PIT an O/W nanoemulsion is formed, which turns in SLN below the lipid melting point [16].

3. Microemulsion templates

Another precursor which can be used for SLN preparation is the microemulsion.

Microemulsions are clear, thermodynamically stable, isotropic liquid mixtures of oil, water and surfactant, frequently in combination with a cosurfactant. The aqueous phase may contain salt(s) and/or other ingredients, and the "oil" may actually be a complex mixture of different hydrocarbons and olefins.

The concept of microemulsion was first introduced by Hoar and Schulman in 1943 [17]; they prepared the first microemulsion by dispersing oil in an aqueous surfactant solution and adding an alcohol as a cosurfactant, leading to a transparent, stable formulation.

The existence of this theoretical structure was later confirmed by the use of various technologies, and today we can adopt the definition given by Attwood [18]: “a microemulsion is a

system of water, oil, and amphiphilic compounds (surfactant and cosurfactant) which is a transparent, single optically isotropic, and thermodynamically stable liquid".

The main difference between emulsions and microemulsions lies in the size — and shape — of the particles dispersed in the continuous phase: these are at least an order of magnitude smaller in the case of microemulsions (10 – 200 nm) than those of conventional emulsions (1 – 20 μm). Also, whereas emulsions consist of roughly spherical droplets of one phase dispersed into the other, microemulsions are dynamic systems, constantly evolving between various structures ranging from droplet-like swollen micelles to bicontinuous structures.

Microemulsions are formed when and only when the interfacial tension at the oil/water interface is brought to a very low level and when the interfacial layer is kept highly flexible and fluid.

These two conditions are usually met by a careful and precise choice of the components and of their respective proportions, and by the use of a "cosurfactant" which brings flexibility to the oil/water interface [17].

This situation leads to a thermodynamically optimised structure, which is stable — as opposed to conventional emulsions — and does not require high input of energy (i.e. through agitation) to be formed. In contrast to ordinary emulsions, microemulsions form upon simple mixing of the components and do not require the high shear conditions generally used in the formation of ordinary emulsions.

Because the size of the particles is much smaller than the wavelength of visible light, microemulsions are transparent and their structure cannot be observed through an optical microscope.

The three basic types of microemulsions are direct (O/W), reversed (W/O) and multiple (W/O/W and O/W/O).

Hot microemulsion can be used as template for SLN production: in this case the oil phase is made up of the solid lipid, liquefied above its melting point; SLN can be obtained through dilution of the hot microemulsion in cold water (microemulsion dilution technique) or by simple cooling of the hot microemulsion itself (microemulsion cooling technique).

Because of their unique solubilisation properties, microemulsions have attracted increasing attention as potential drug delivery systems for poorly water soluble active pharmaceutical ingredients (API) [19]; also the good solubilisation properties of hot microemulsion precursor allows an advantageous drug loading within SLN for many drugs, especially for poorly water soluble drugs.

3.1. Microemulsion dilution technique

Gasco [20] was the first researcher to use a microemulsion template for the preparation of SLN. Lipids are heated above their melting point and an aqueous phase containing surfactants and cosurfactants is added at the same temperature in order to form a clear O/W microemulsion under stirring; lipophilic drug can be dissolved in the hot microemulsion. Multiple W/O/W

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microemulsion can be prepared, too, in order to encapsulate hydrophilic drugs within SLN [21,22]. SLN with reduced mean particle size and narrow size distribution can be obtained after dilution in cool (2-10° C) water of the hot microemulsion.

The lipids employed can be triglycerides, fatty acids, fatty alcohols. Surfactants can be chosen among bile salts, phospholipids, polysorbates and cosurfactants among short chain alcohols and glycols (butanol, hexanol, hexanediol, propylene glycol), short chain fatty acids (butyric acid, hexanoic acid), phosphoric acid alkyl esters and benzyl alcohol [20].

Dilution of the microemulsion can be performed with a volume of water 10 to 200 compared to microemulsion volume.

3.2. Microemulsion cooling technique

Recently, Mumper and Jay [23,24] patented a microemulsion based method to produce SLN. The technique is simple, reproducible, and easy to scale-up. In particular for the production of SLN the authors start from an O/W microemulsions: an emulsifying wax is melted (37-55 °C) and water is then added at the same temperature under minimal stirring to form an homogeneous milky slurry. Upon the addition of defined amounts of a suitable pharmaceutically-acceptable polymeric surfactant in water, a clear and stable liquid matrix O/W microemulsion is formed. SLN are precipitated from this O/W microemulsion by cooling of the undiluted O/W microemulsion to room temperature or to 4 °C.

The matrix material may be selected from a variety of excipients including for examples waxes, such as emulsifying wax (Polysorbate 60, PEG-150 Stearate and Steareth-20), the polymeric surfactants can be chosen among the Brij®-type, polyethylene glycol derivatives of fatty acids such as PEG-400 monostearate, and phospholipids such as phosphatidylcholine (lecithin).

An advantage of this invention is that the described nanoparticle system can be formulated at mild operating temperatures, rapidly, reproducibly and cost-effectively from the microemulsion precursor in an one-step process and contained in a manufacturing vessel, vial or container.

Moreover all ingredients are potentially biocompatible, well-defined and uniform solid nanoparticles (50 to 300 nm) may be reproducibly made, no organic solvents were used during the preparation, very high entrapment efficiencies are achievable [25], especially for water-insoluble drugs, and cell-specific ligands can easily be incorporated on the surface of the nanoparticles [26].

4. Coacervation method

Recently, a new method was developed to prepare-in a controlled way-SLN by coacervation. This method allows the incorporation of drugs, without using very complex equipment or dangerous solvents, and is therefore inexpensive for laboratory and industrial application. It is based on the interaction between a micellar solution of a fatty acid alkaline salt (soap) and

an acid solution (coacervating solution) in the presence of different amphiphilic polymeric stabilizing agents: fatty acid nanoparticles precipitate as proton exchange occurs between the coacervating solution and the soap solution [27,28].

In this method the precursor for SLN preparation is a soap micellar solution, obtained at a temperature above its Krafft point (that is the solubilisation temperature of the soap in water): drug can be dissolved directly in the micellar solution, or pre-dissolved in a small amount of ethanol, in order to enhance micellisation. As for microemulsion templates, the good solubilising properties of micellar solutions allow an advantageous drug loading within SLN for many drugs, especially for poorly water soluble drugs [29, 30].

The salt of fatty acid is chosen from the group consisting from sodium stearate, sodium palmitate, sodium myristate, sodium arachidate and sodium behenate in a concentration preferably between 1 and 5% w/w. The stabilizing agent is selected from the group of surface active non ionic polymers: polyvynilacetate/polyvynilalcohol and polyoxyethylene/polyoxypropylene copolymers, dextrans, hydroxypropylmethylcellulose. Normally the acidification takes place at a temperature between 40 and 50 °C, above the Krafft point of the fatty acid sodium salt; sodium arachidate and behenate need higher temperatures. Then the obtained suspension is rapidly cooled to 15 °C [27,28].

A very important feature of this method is the possibility to control the size of SLN changing the reaction conditions. In order to obtain a homogeneous and stable nanoparticle suspension, a key role is played by the right coupling between the fatty acid alkaline salt and the proper coacervating solution. Particle size is highly influenced by the lipid concentration: increasing the micellar solution concentration the SLN size increases. Also the type and the grade of the polymer used as stabiliser influence the SLN mean particle size [28].

5. Solvent based methods

Solvent based methods have been proposed in order to encapsulate molecules with stability and bioavailability problems, despite toxicological issues of the solvent are a limiting aspect. One of the main advantages of solvent based methods is the mild operating temperature, which can be useful for the encapsulation of thermosensitive drugs. According to the different precursor/method used, solvent based methods can be divided in:

- solvent injection;
- solvent evaporation from emulsions;
- solvent diffusion from emulsions.

5.1. Solvent injection method

In solvent injection (or solvent displacement) method the lipid and the drug are dissolved in a water-miscible organic solvent (ethanol, acetone, isopropanol) and this solution is injected through a syringe needle in water under stirring: lipid precipitates as nanoparticles while

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contacting water, encapsulating the drug. Particle size can be influenced by lipid type, surfactant and solvent used, and from the viscosity of the outer phase [31,32].

5.2. Solvent evaporation – Diffusion from emulsions

SLN can also be prepared starting from emulsion precursor, whose organic phase is constituted by a solvent, which can be either volatile or partially water miscible.

O/W or W/O/W emulsions can be prepared: O/W emulsions are used for lipophilic drugs, that are dissolved in the inner organic phase of the system [33,34], together with the lipid. W/O/W emulsions are suitable for hydrophilic drugs, that are dissolved in the inner aqueous phase, while the lipid is dissolved in the intermediate organic phase of the multiple system [35,36].

Nanoparticles are formed when the solvent is removed either by evaporation (solvent evaporation technique for volatile solvents) [33, 35] or by water dilution (solvent diffusion technique for partially water miscible solvents) [34, 36]: owing to solvent removal lipid precipitates as nanoparticles encapsulating the drug.

Solvent evaporation technique is quite outdated and shows many drawbacks, due to the toxicity of chlorinated solvents used; solvent diffusion is more innovative and most of the solvent employed show a better safety profile compared to volatile solvents.

6. Supercritical fluid based methods

Supercritical fluid (SCF) technology has gained increasing interest in the last years for nanoparticle production. SCF is obtained above its critical pressure and temperature: above this fluid's critical point, the solubility of a substance in the fluid can be modulated by a relatively small change in pressure. Due to its low critical point at 31 °C and 74 bar, and its low cost and non toxicity, carbon dioxide (CO_2) is the most widely used SCF [37].

The four main SCF processes used to produce nano-or microparticles are:

1. Rapid Expansion of Supercritical Solutions (RESS);
2. Gas Anti-Solvent (GAS) process;
3. Particles from Gas-saturated Solutions/Suspensions (PGSS);
4. Supercritical Fluid Extraction of Emulsions (SFEE).

6.1. RESS

RESS, which is also called supercritical fluid nucleation (SFN), is based on a simple principle: the matrix is dissolved in SCF, which is then expanded through a nozzle, in order to form the particles [38]. This major limitation of RESS lies in the too low solubility of compounds in SCF, that precludes production at acceptable costs. In fact its applications to lipid particles is very limited.

A modified RESS process has been used for lipid coating of bovine serum albumin (BSA) microcrystals [39]. The lipid coated microparticles are prepared as follows: coating material and BSA crystals are placed in an autoclave, equipped with a rotating impeller, heated and pressurised with CO_2 (temperature typically ranging between 35 and 45 °C and pressure about 200 bar). The system is allowed to equilibrate at these conditions, so as to solubilise the coating material. Then, cooling the autoclave induced a pressure decrease and a phase change from SCF to liquid state, therefore insolubilising the coating material that precipitated upon the insoluble BSA crystals dispersed in the medium. Afterwards, the autoclave is vented to ambient conditions and the coated particles are collected from the bottom of the autoclave.

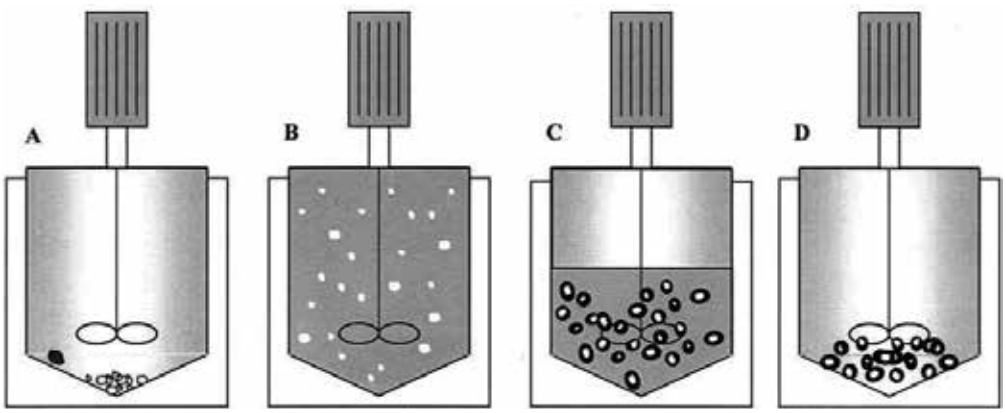

Figure 1. Schematic representation of the coating process. (A) Filling step: BSA crystals represented in white, coating material in black. (B) Solubilisation of the coating material in the SCF CO_2. Insoluble BSA crystals are dispersed in the medium. (C) Decompression phase: insolubilisation of the coating material. (D) Coated particles are harvested after autoclave has been vented.

6.2. GAS

GAS process has been developed in order to achieve nanosizing of the hydrophobic materials that can not be processed by RESS technique owing to their poor solubility in SCF. The origin of GAS process is based on the fact that when a solution is expanded sufficiently by a gas, the liquid phase is no longer a good solvent for the solute and nucleation occurs [38]. Up to now GAS technique has very limited applications for lipid particles.

6.3. PGSS

PGSS involves melting of the material to be processed, which then dissolves the SCF under pressure. The saturated solution is then expanded across a nozzle where the SCF, which is more volatile, escapes, leaving dry fine particles. As the solubilities of compressed gases in liquids and solids are usually high, and much higher than the solubilities of such liquids and solids in the compressed gas phase, the process consists in solubilizing CO_2 in melted or liquid-suspended substance(s), leading to a so-called gas-saturated solution/suspension that is

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further expanded through a nozzle with the formation of solid particles or droplets. The advantage of this process is that the substances need not be soluble in CO_2 [38].

In literature some examples of PGSS applied to lipid nano-and microparticles can be recovered. The most important is the so called Gas Assisted Melting Atomisation (GAMA) [40,41,42].

Lipids are placed in a thermostated mixing chamber (CM), where they are melted and kept in contact with supercritical CO_2 at selected temperature and pressure conditions (Figure 2). Then, the lipid saturated fluid is forced through the nozzle by opening the valve at the bottom of the CM, in order to produce microparticles. A CO_2 reservoir is allowed to keep constant the pressure in the CM, and the flow rate through the nozzle as well. Particles are gathered by a collection system and dispersed in water by vortexing and sonicating, in order to obtain suspensions. Polyethylenglycol (PEG) can be added to the formulation in order to increase the rate of dispersion in water.

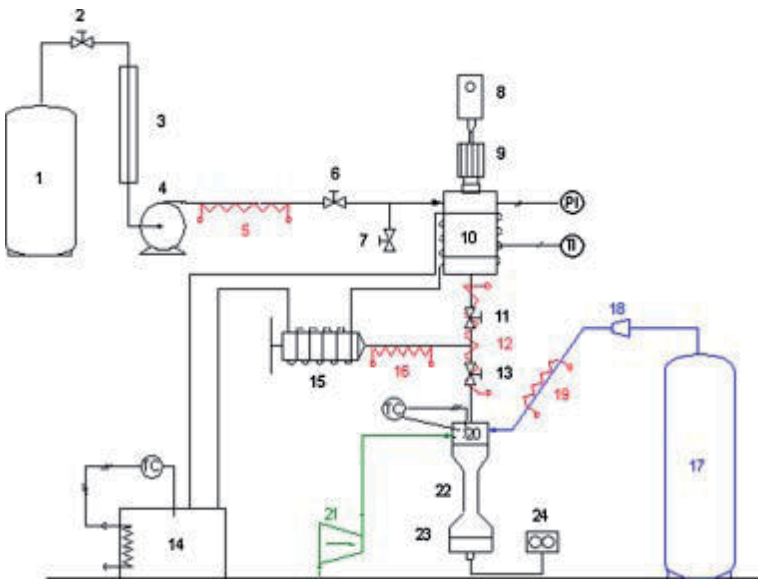

Figure 2. GAMA apparatus for micro and nanoparticles production: supercritical CO_2 supplier (1), on–off valves (2,6,7,11,13), heater exchanger (3), pump (4), electrical resistances (5,12,16,19), pressure indicator (PI), temperature indicator (TI), temperature controller (TC), mixing device (8,9), melting chamber (10), heating system (14), spraying pump (15), air supplier (17), pressure reducer (18), 100 mm diameter nozzle (20), compressor for tangential air flux (21), precipitation vessel (22), filter (23), flowmeter (24).

6.4. SFEE

SFEE is based on a simple principle, whereby the lipid nanosuspensions are produced by supercritical fluid extraction of the organic solvent from O/W emulsions. O/W emulsion is prepared by dissolving lipid and drug in a volatile solvent (i.e. chloroform), dispersing this solution into an aqueous phase of a surfactant and passing the mixture through a high pressure

homogeniser in order to form fine emulsions with a mean droplet size ranging between 30–100 nm.

SLN suspensions are obtained using a continuous extraction method. The O/W emulsions are introduced into an extraction column from the top; simultaneously, supercritical CO_2 (at constant pressure of 80 bar and temperature 35 °C) is introduced counter-currently from the bottom [43,44] (Figure 3).

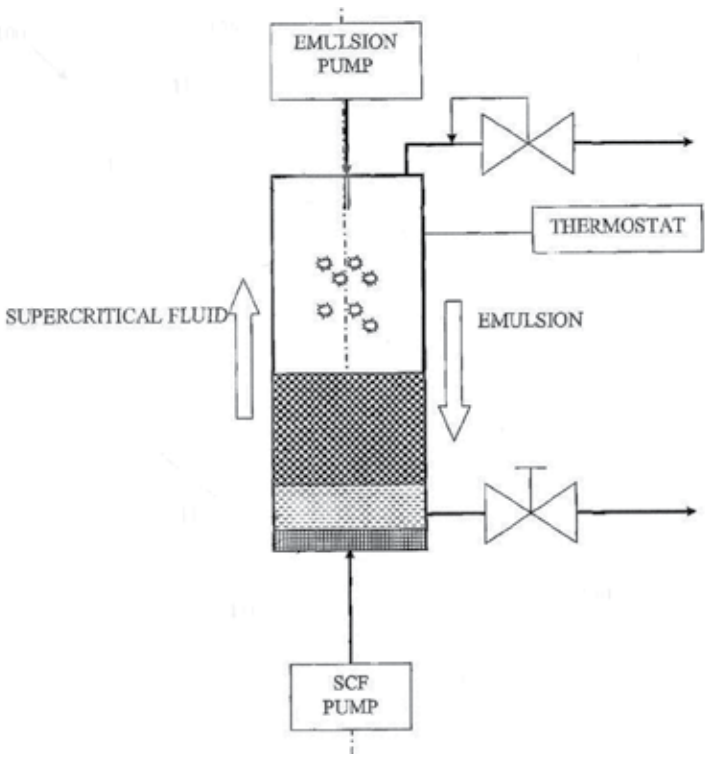

Figure 3. Extraction column for emulsion with SCF in counter-current

The operating pressure and temperature conditions have to be selected to minimise losses of product due to lipid and drug dissolution into the CO_2 phase at maximum extraction efficiency. The residence time required for producing pure aqueous suspensions of SLN is approximately two minutes, with the product continuously removed from the bottom of the extraction column.

When the O/W emulsion containing the lipid and the drug is introduced into the supercritical CO_2 phase, parallel processes of solvent extraction into the supercritical CO_2 phase, and the inverse flux of CO_2 into the emulsion droplets occurs, leading to expansion of the organic phase of the emulsion. This in turn leads to precipitation of lipid-drug material dissolved in the organic phase as composite particles [44].

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One of the advantages of this technique is that the solvent extraction efficiency using supercritical CO_2 is much higher than for the conventional methods, such as solvent evaporation/diffusion from emulsions. It therefore provides for a fast and complete removal of the solvent and more uniform particle size distribution. Supercritical CO_2 also tends to extract other low-molecular weight impurities, purifying the lipids. The size of SLN obtained in the SFEE process is directly related to the emulsion droplet size and it is therefore dependent upon the method of formulation; a mean particle diameter between 20–90 nm can be obtained [44].

7. Membrane contactor technique

SLN can be produced by using a membrane contactor [45]: a proper module has been realised (Figure 4), including a Kerasep ceramic membrane (0.1, 0.2, 0.45 μm pore size), which separates the water phase, allowed to circulate tangentially to the membrane surface, and the lipid phase; the lipid phase is heated in a pressurised vessel above its melting point, conveyed through a tube to the module (Figure 5) and pressed through the membrane pores, allowing the formation of small droplets, which are detached from the membrane pores by tangential water flow. SLN are formed after cooling of the obtained water dispersion [46].

SLN particle size depends on many process parameters: larger sizes are obtained with higher lipid phase content, reduced lipid phase pressure and aqueous cross-flow velocity. If the temperature of the aqueous phase is below the melting point of the lipid, smaller SLN are obtained: this is due to the fact that the lipid phase solidifies suddenly in the aqueous phase. Instead the SLN size decreases when the lipid temperature increases. Particle size is also highly influenced by type and concentration of surfactants added to the formulation [47].

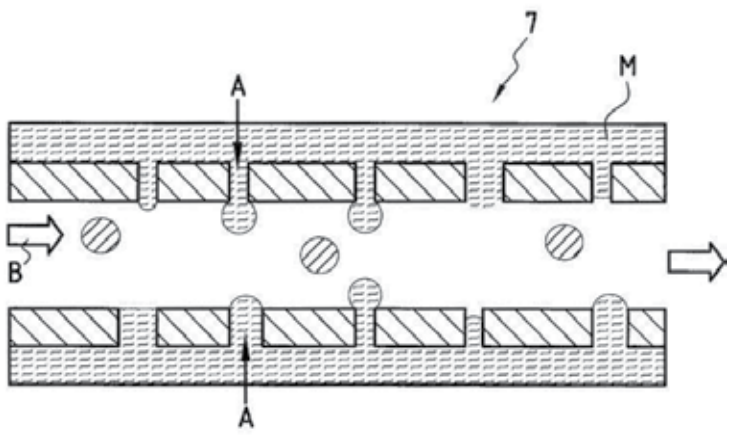

Figure 4. Module for membrane contactor. A (lipid phase), B (water phase), M (porous membrane), 7 (tangential flow filtration unit).

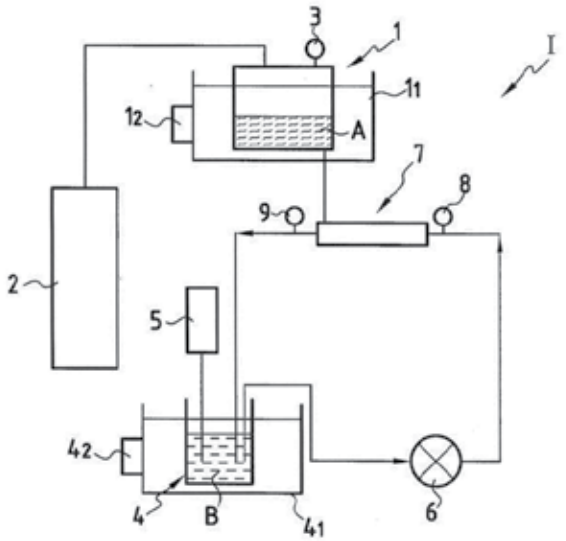

Figure 5. Scheme of the apparatus for producing SLN with a membrane contactor. A (lipid phase), B (water phase), 1 (pressurised vessel containing the lipid phase), 1_1 (thermostated bath), 1_2 (thermostat), 2 (nitrogen bottle), 3 (manometer), 4 (vessel containing the water phase), 4_1 (thermostated bath), 4_2 (thermostat), 5 (stirrer), 6 (pump), 7 (tangential flow filtration unit), 8-9 (manometer).

8. Cryogenic micronisation

In the cryogenic micronisation process, lipid matrices are obtained either by melt dispersion (the drug is mixed in a molten lipid) or solvent stripping (the drug and lipid are co-dissolved into a solvent mixture under stirring, e.g. benzyl alcohol, ethanol). In the cryogenic micronisation step, cooling is performed by insufflating liquid nitrogen nearly at-80 °C before the drug loaded lipid matrix being micronised by grinding.

Finally, the obtained powders are sieved in an automatic sieving apparatus. Regarding to the sieving step, this operation is depending on particle size requirements and on the type of mill used, the particle size of the product obtained by micronisation can already be suitable for some applications and therefore sieving is not necessary.

This technique can be used for the production of SLM of 1 to 500 μm in diameter according to the chosen sieves [48,49].

9. Spray-drying

Spray-drying is an one-step process which converts a liquid feed to a dried particulate form. The feed generally is a solution, but it can also be a coarse or fine suspension or a colloidal

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dispersion (e.g., emulsions, liposomes, etc.); this feed is first atomised through various techniques (centrifugal, pneumatic, ultrasonic and electrostatic atomisation) to a spray form, that is put immediately into thermal contact with a hot gas, resulting in the rapid evaporation of the solvent to form dried solid particles [50]. The dried particles are then separated from the gas by means of a cyclone, an electrostatic precipitator or a bag filter (Figure 6).

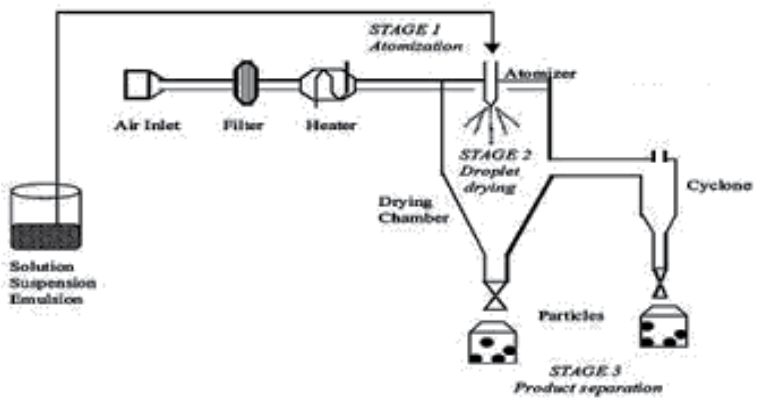

Figure 6. Spray-drying apparatus

The design may be "open cycle", when the drying gas (usually air) is not recirculated and is vented into the atmosphere. If an organic solvent is employed, a "closed cycle" layout is more suitable than an open one, since the risk of inflammability and explosion is higher in the latter when organic feeds are heated in the presence of oxygen [51].

The main advantage of the spray-drying technique is the ability to manipulate and control a variety of parameters such as solvent composition, solute concentration, solution and gas flow rate, temperature and relative humidity, droplet size, etc. So the optimisation of particle characteristics can be performed in terms of size, size distribution, shape, morphology and density, in addition to macroscopic powder properties like bulk density, flowability and dispersibility [51].

However, the spray-drying process may induce degradation of some macromolecular drugs as a result of a number of factors such as thermal stress during droplet drying, high shear stress in the nozzle and peptide/protein adsorption at the greatly expanded liquid/air interface of the spray solution.

Sebti *et al.* have developed a spray-drying technique to produce SLM. These SLM are composed of biocompatible phospholipids and cholesterol, and can be used as a carrier or filler to deliver drugs directly to the lungs via a dry powder inhaler [52,53]. SLM are obtained by starting from an ethanolic solution of lipids and drug, which undergo to spray-drying process. Compared to other SLM production methods, spray-drying produces particles which are characterised by smaller and more homogeneous particle size distribution. However, the produced particles are not always spherical and may have convoluted surfaces, asperities and cavities. The shape

is influenced by the drying rate, the surface tension and the viscosity of the liquid which constitutes the liquid feed.

10. Electrospray

In a general process, a solution of the matrix, which constitutes the particles, is contained in a syringe, with a metal capillary connected to a high-voltage power supply, working as an electrode. A metal foil collector is placed opposite the capillary as a counter electrode (Figure 7). Depending on the properties of the liquid, the flow rate and the voltage applied can be modulated.

The cone-jet mode is the method used to prepare nanoparticles, in which liquid emerging at the nozzle forms hemispherical drops because of the surface tension. By increasing the electrical field, the hemispherical drops can be changed to a conical shape, which breaks up into highly charged droplets. By selecting suitable conditions, droplets can be produced with a close size distribution and nano-or micrometer size range. Particles can be formed by evaporating the solvent from the droplets produced travelling through the electrical field.

The electrospray method has been employed to prepare monodisperse lipid-based nano-and microparticles: lipid has been dissolved in aliphatic alcohols and this solution has been used for particles production. Particle shape and size depend on the solvent and excipients used and on the applied voltage [54,55].

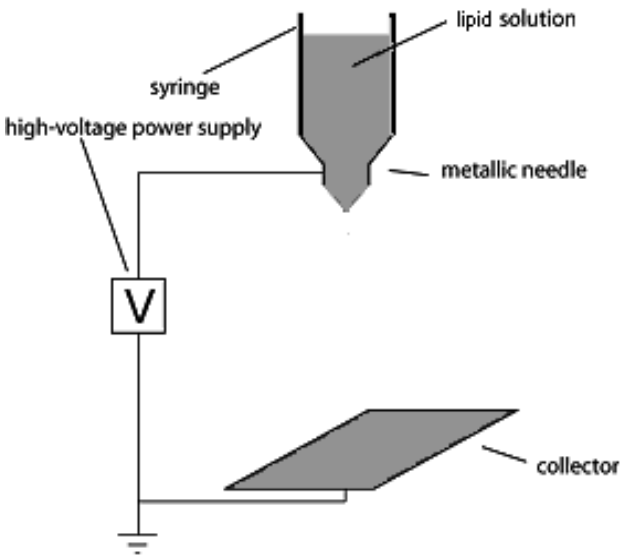

Figure 7. Scheme of electrospray apparatus

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11. Spray congealing

In the spray congealing technique, lipids are heated to a temperature above their melting point and the drug is dissolved or suspended into the melted lipid. The hot mixture is then atomised through a pneumatic nozzle into a vessel, where the atomised droplets can solidify in the form of microparticles [56]. In this technique some variations can be performed, especially in the atomisation device.

The Wide Pneumatic Nozzle (WPN) (Figure 8A) is an innovative external mixing atomiser: the molten fluid and the atomisation air get in contact outside the nozzle; the former is delivered to the orifice by the Venturi effect, while the latter is delivered in radial direction with respect to the molten fluid. The atomisation occurs where the air input converges with the molten fluid [57].

The Air Pressure Nozzle (APN) (Figure 8B) is an internal mixing device: the molten fluid and the atomisation air get in contact in the mixing chamber inside the nozzle. The swirling fluid impinges on the plate and the interaction between the fluid and the air creates extreme turbulence in the chamber, and then flows through an orifice, where the droplets are exposed to shear forces, before coming in contact with a circular deflector ring and leaving the nozzle as a finely atomised spray cone [57].

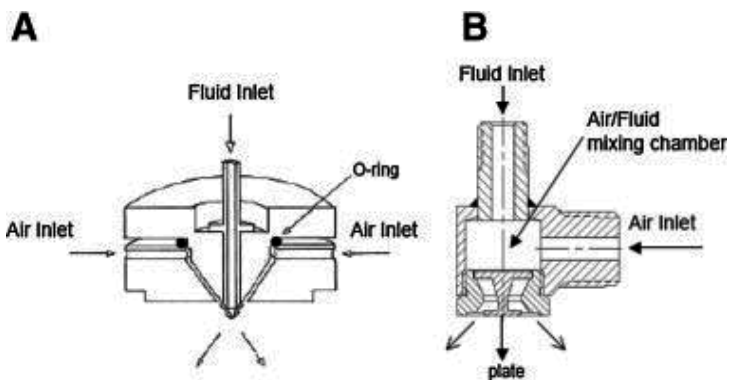

Figure 8. A) Diagram of the external-mixing WPN. B) Diagram of the internal mixing APN

Another particular type of atomiser is based on ultrasounds [58]. The ultrasound-atomiser basically consists of three parts (Figure 9):

1. the ultrasound piezoelectric generator;
2. an interchangeable booster allowing for the modification of the amplitude of the ultrasound wave;
3. a titanium vibrating surface (sonotrode) on which the atomisation of the liquid occurs.

The atomiser is also provided of an inductive coil to keep the sonotrode at suitable temperature.

The melted lipid mixture, fed to the sonotrode by a thermostated reservoir through a funnel, is atomised by ultrasound energy into small droplets that fall freely and solidify by cooling at room temperature in a collector.

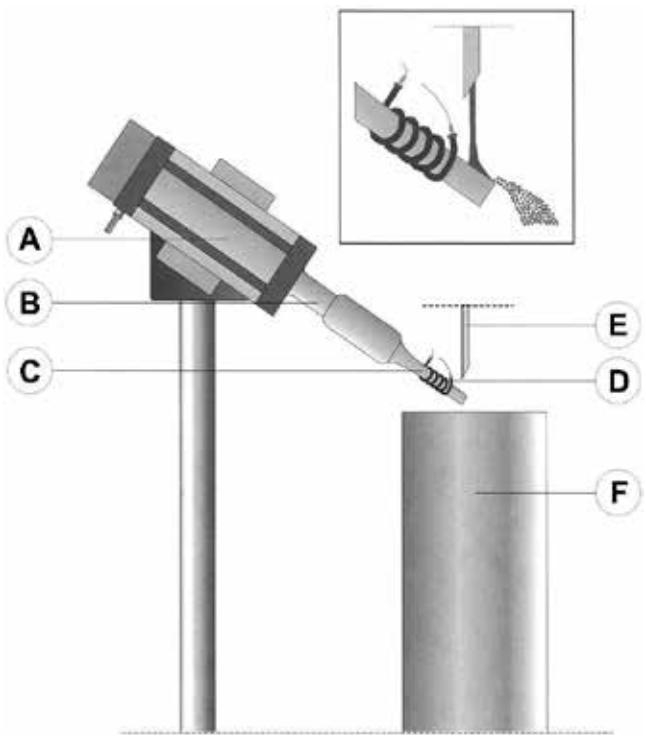

Figure 9. Scheme of the ultrasonic atomiser (not in scale): (A) ultrasound generator; (B) booster; (C) sonotrode; (D) inductive coil; (E) supply funnel; (F) cylindrical chamber (collector).

Another variant of the spray congealing method is to use a rotating disc [59]. With this technique, the melted mixture is dropped onto a high-speed rotating disc. The rotation induces the molten mixture to spread between the disc periphery and the cooled surface on which microparticles are collected.

Owing to this technique SLM with particle size ranging from 50 to 2000 μm can be produced.

12. Conclusion

Different techniques have been developed for the production of solid lipid particles with the possibility to obtain different size and shape. The size can influence the pharmacological properties of the particles, but it is not the unique parameter considered to compare the various techniques.

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Toxicological issues are also very important: the materials used must be biocompatible and biodegradable, while the use of solvents can be a relevant drawback, since they can remain in traces in the final product.

From a technological point of view, the possibility to scale up the process is very important, but also the feasibility of the method is relevant: in fact the use of expensive and complex machine can hamper the production on lab scale.

Finally the drug entrapment is very important: nowadays more and more complex molecules are entrapped within solid lipid particles. These molecules have different physico-chemical properties (solubility, hydrophobicity, etc.) and stability issues (temperature, pH, etc.). The chosen preparation technique should be the most suitable to enhance drug loading and encapsulation efficiency within the nanoparticles, without hampering the chemical stability of the molecule itself: the working temperatures and operating conditions used to prepare the particles can affect the physico-chemical stability of the drug.

In Table 2 some important parameters, like particle size, solvents used, instrumentation needed, working temperatures and operating conditions, of the various techniques are shown for comparative purposes.

Technique	Particle size	Solvent used	Instrumentation needed	Working temperature	Operating conditions
High pressure homogenisation	50-1000 nm		High pressure homogeniser	5-10°C upon lipid mp	cavitation forces
High shear homogenisation	50-1000 nm		High shear homogeniser	5-10°C upon lipid mp	
Ultrasound homogenisation	50-1000 nm		Ultrasound apparatus	5-10°C upon lipid mp	Ultrasound treatment
Melt dispersion	1-250 µm		High shear homogeniser	5-10°C upon lipid mp	
PIT	30-100 nm			90°C	
Microemulsion dilution	50-800 nm			5-10°C upon lipid mp	
Microemulsion cooling	50-300 nm			37-55°C	
Coacervation	200-1000 nm			40-75°C, according to the lipid matrix	pH shifts
Solvent injection	100-500 nm	Ethanol, acetone, isopropanol		25°C	

Technique	Particle size	Solvent used	Instrumentation needed	Working temperature	Operating conditions
Solvent evaporation from emulsions	30-500 nm	Chlorinated solvents	High shear/pressure homogeniser	25°C	
Solvent diffusion from emulsions	100-2000 nm	Partially water miscible solvents	High shear/pressure homogeniser	40-50°C	
PGSS	0.2-20 μm		GAMA apparatus	5-10°C upon lipid mp	Pressure>74 bar
SFEE	20-90 nm	Chlorinated solvents	High pressure homogeniser SFEE apparatus	>31°C	Pressure>74 bar
Cryogenic micronisation	1-500 μm			-80°C	
Membrane contactor method	100-200 nm		Membrane contactor	5-10°C upon lipid mp	
Spray-drying	0.3-10 μm	Ethanol	Spray-drier	70°C	
Electrospray	Nearly 1 μm	Aliphatic alcohols	Electropray apparatus	25°C	High voltage
Spray-congealing	50-2000 μm		Spray-congealing apparatus	5-10°C upon lipid mp	

Table 2. Main parameters for the various preparation techniques of solid lipid nano-and microparticles

As it can be noticed, each technique leads to the formulation of particles with different size and shows some limitations and drawbacks: the choice of a preparation technique should be done basing on the desired particle size, on the technology available, on the toxicological issues and on the characteristics of the drug encapsulated within the particles.

Author details

Luigi Battaglia[1*], Marina Gallarate[1], Pier Paolo Panciani[2], Elena Ugazio[1], Simona Sapino[1], Elena Peira[1] and Daniela Chirio[1]

*Address all correspondence to: luigi.battaglia@unito.it

1 University of Turin, Department of Drug Science and Technology, Turin, Italy

2 Department of Neurosciences, University of Brescia, Brescia, Italy

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Chapter 3

Nanoemulsions — Advances in Formulation, Characterization and Applications in Drug Delivery

S.A. Chime, F.C. Kenechukwu and A.A. Attama

Additional information is available at the end of the chapter

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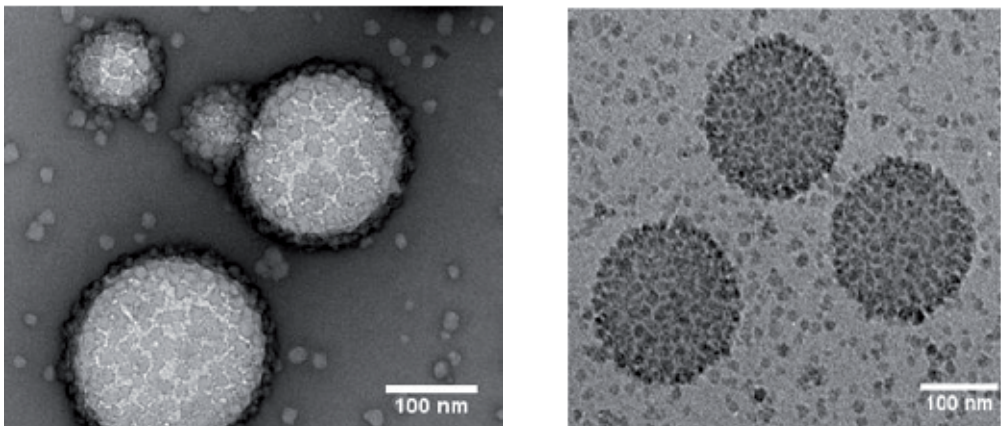
1. Introduction

An ideal drug delivery system fulfils the objective of maximizing therapeutic effect while minimizing toxicity. With the progress in time and advances in science and technology, dosage forms have evolved from simple mixtures and pills, to highly sophisticated systems, which are known as novel drug delivery systems. Nanoemulsions are novel drug delivery systems consisting of emulsified oil and water systems with mean droplet diameters ranging from 50 to 1000 nm. Usually, the average droplet size is between 100 and 500 nm and can exist as oil-in-water (o/w) or water-in-oil (w/o) form, where the core of the particle is either oil or water, respectively. Nanoemulsions (Figs. 1 and 2) are made from pharmaceutical surfactants that are generally regarded as safe (GRAS). The surfactant type and concentration in the aqueous phase are chosen to provide good stability against coalescence. Several types of oils-natural semi-synthetic and synthetic are used in the formulation of nanoemulsions. The capacity of nanoemulsions to dissolve large quantities of low soluble drugs along with their mutual compatibility and ability to protect the drugs from hydrolysis and enzymatic degradation make them ideal drug delivery vectors [1]. The major advantages of nanoemulsions as drug delivery carriers include increased drug loading, enhanced drug solubility and bioavailability, reduced patient variability, controlled drug release, and protection from enzymatic degradation [2].

A lot of techniques are available for enhancing absorption of poorly water-soluble drugs, like use of lipid-based systems. Thus enhancement of aqueous solubility in such case is a valuable goal to successfully formulate them into bioavailable dosage forms. A range of novel strategies are currently being developed for efficient delivery of poorly water-soluble drugs, such as the formulation of amorphous solid form, nanoparticles, microemulsions, solid dispersions, melt extrusion, salt formation and formation of water-soluble complexes. Among all, the most



Nanoemulsions courtesy of the Chemical, Materials and Surfaces unit at SP Technical Research Institute of Sweden (former YKI, Institute for Surface Chemistry). Obtained via: http://www.vironova.com/nanoemulsions-casebody. Accessed on April 27, 2014.

Figure 1. A silicate particle-stabilized oil in water nanoemulsion imaged using negative stain TEM (left panel) and cryo TEM (right panel).

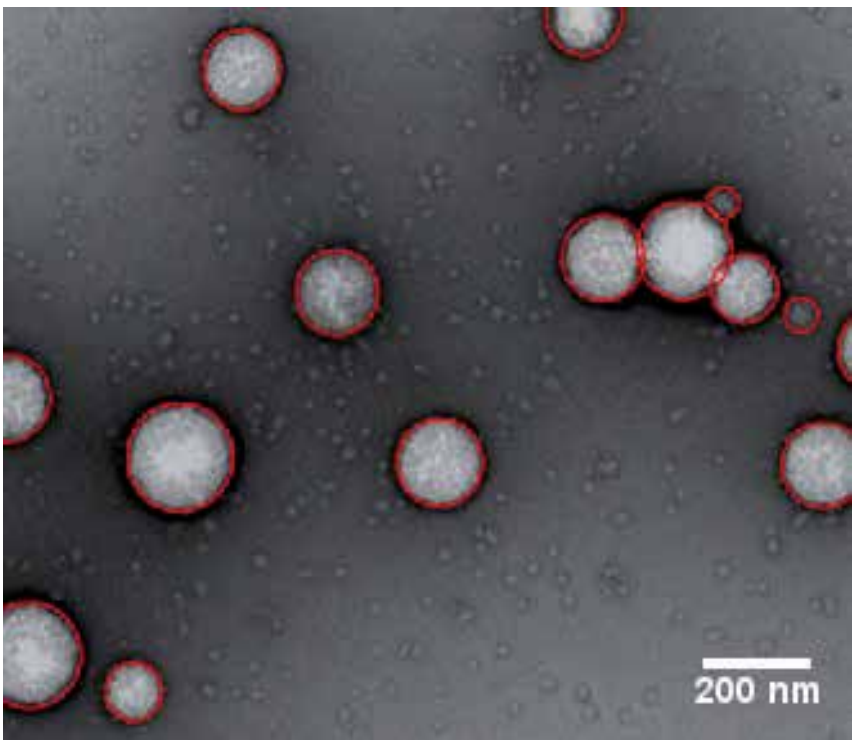


Figure 2. Negative stain TEM image of a silicate particle-stabilized oil in water nanoemulsion. Obtained via: http://www.vironova.com/nanoemulsions-casebody. Accessed on April 27, 2014.

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accepted approach is the lipid-based formulation approach [3,4]. Lipid-based formulations enhance the absorption by enhancing solubilization, prolonging gastric residence time, stimulating the intestinal lymphatic transport pathway, altering intestinal permeability, reduced activity of efflux transporters and reduced metabolism. Lipid-based formulations present a large range of optional systems such as solutions, suspensions, self-emulsifying systems and nanoemulsions.

Nanoemulsions can be prepared by high- and low-energy methods. Both high-energy and low-energy methods can produce stable nanoemulsions. High-pressure homogenizer or ultrasound generator can be used for the preparation of nanoemulsion by high-energy emulsification method. Self emulsification and phase inversion methods-phase inversion temperature and phase inversion composition are low-energy methods for the preparation of nanoemulsions. Low-energy emulsification methods depend on the phase behaviour and properties of the constituents, and they utilize the stored energy of the system to form nano droplets. The emulsification can be brought about by changing the parameters such as temperature and composition, which would affect the hydrophilic lipophilic balance of the system.

This chapter focused on recent advances in the formulation, characterization and application of nanoemulsions in drug delivery. Nanoemulsion can be formulated for delivery of drugs through various routes. Nanoemulsions are well tolerated orally and on the skin and mucous membranes when used to deliver topically active drugs. As a result they are used as vehicles for drugs active against herpes labialis, fungal infections, bacterial infections, vaginitis, etc. Nanoemulsion globules can fuse with membranes of lipid-containing organisms facilitating penetration and transfer. Less amount of surfactant is required in nanoemulsions compared to other emulsion systems. This can increase the bioavailability of poorly soluble drugs since small particles easily cross the absorption membrane. Furthermore, very small size provides large surface area which eases the solubilization and penetration through the skin or epithelial layer [5].

2. Nanoemulsion based delivery system: types and properties

2.1. Self emulsifying formulations (SEFs)

Self-emulsifying formulations (SEFs) are mixtures of oil, surfactant, co-surfactant, and co-solvents (absence of external phase water) and forms a transparent isotropic solution, which emulsify under gentle agitation similar to those which would be encountered in gastro intestinal tract (GIT). It has been recognized that this formulation when administered orally undergo spontaneous emulsification in aqueous GI fluids [6]. This emulsified oil (triglycerides) stimulates bile secretion and drug containing oil droplets are further emulsified by bile salts. Lipid droplets are then metabolized by lipases and co lipases, secreted from the salivary gland, gastric mucosa and pancreas, which also hydrolyze the triglycerides into di-and monoglycerides and free fatty acids. Further, solubilization of these molecules occurs during the passage through the GI tract and eventually forms a range of emulsion droplets, vesicular structures

and mixed micelles containing bile salts, phospholipids and cholesterol [6]. Upon mixing with water the system SEFs have an ability to form fine colloidal droplets with very high surface area. In many cases, this accelerates the digestion of the lipid formulation, improves absorption, and reduces food effect and inter-subject variability [7]. Self emulsifying formulations distribute readily in the GI tract, the digestive motility of the stomach and the intestines provides sufficient agitation enough for the spontaneous formation of emulsions [8,9].

SEFs prepared using surfactants of HLB < 12 possess high stability and improved dissolution (for poorly soluble drugs) due to enhancement in surface area on dispersion [6]. Therefore, their absorption is independent of bile secretion and ensures a rapid transport of poorly soluble drugs into the blood [6].

According to Reiss [10], self emulsification occurs when the entropy changes that favor dispersion is greater than the energy required to increase the surface area of the dispersion [10]. The free energy of the conventional emulsion is a direct function of the energy required to create a new surface between the oil and water phase and can be described by the equation:

$$\Delta G = \sum N_i 4\pi r_i^2 \sigma \tag{1}$$

where, ΔG is the free energy associated with the process, r_i is the radius of droplets, N_i is the number of droplets, σ is the interfacial energy [11]. The two phases of the emulsion tend to separate with time to reduce the interfacial area and thus, minimize the free energy of the system(s). The conventional emulsifying agents stabilize emulsions resulting from aqueous dilution by forming a monolayer around the emulsion droplets, reducing the interfacial energy and forming a barrier to coalescence. On the other hand, emulsification occurs spontaneously with SEDDS, as the free energy required to form the emulsion is low, whether positive or negative [12]. For emulsification to take place, it is vital for the interfacial structure to offer negligible or no resistance against surface shearing [13]. The ease of emulsification has been suggested to be related to the ease of water penetration into various liquid crystals or gel phases formed on the surface of the droplet [14-16]. The interface between the oil and aqueous continuous phases is formed upon addition of a binary mixture (oil/non-ionic surfactant) to water [14]. This is followed by solubilization within the oil phase, as a result of aqueous penetration through the interface. Invariably, this tends to occur until the solubilization limit is attained close to the interphase. Further, aqueous penetration will lead to the formation of the dispersed liquid crystal phase. Ultimately, everything that is in close proximity with the interface will be liquid crystal, the actual amount of which depends upon the emulsifier concentration in the binary mixture. Hence, following gentle agitation of the self-emulsifying system, water rapidly penetrates into the aqueous cores leading to interface disruption and droplet formation [16].

When compared with emulsions, which are sensitive and metastable dispersed forms, SEFs are physically stable formulations that are easy to manufacture. Thus, for lipophilic drugs that exhibit dissolution rate-limited absorption, these systems may offer an improvement in the rate and extent of absorption and result in more reproducible blood time profiles [17]. SEFs

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have been transformed into solid dosage forms using techniques such as melt granulation, where the lipid excipient acts as a binder and solid granules are produced on cooling. Solvents or supercritical fluids can be used with semisolid excipients, which are solubilized and then the solvent evaporated to produce a waxy powder. Spraying techniques can be used to produce powder formulations. These techniques enable the production of granules or powders that can then be compressed into a tablet form or filled into capsules. In all cases, the lipid excipients used must be semi-solid at room temperature [18].

2.1.1. Self emulsifying drug delivery systems (SEDDS)

Self-emulsifying drug delivery system (SEDDS) is a strategy that has drawn wide research interest, basically due to its distinct capacity to solubilize and improve the bioavailability of hydrophobic drugs. This it does by ensuring aqueous solubility of the lipophilic drug [19]. The presence of oil makes SEDDS unique and distinguishes them from ordinary surfactant dispersions of drugs [20]. SEDDS are isotropic combination of drug, lipid/oil, cosolvents and surfactants [19]. On dilution by an aqueous phase they form fine stable oil-in-water (o/w) emulsions or fine lipid droplets which is the characteristic feature of these systems. When such a formulation is released into the lumen of the GIT, it disperses to form a fine emulsion generally o/w emulsion. SEDDS are generally formulated with triglyceride oils and ethoxylated nonionic surfactants. In general, the concentration of surfactant is greater than 25% in the formulation. The size of droplets ranges approximately less than 100 nm [19].

SEDDS are believed to be superior compared with lipid solutions due to the presence of surfactants in the formulations leading to a more uniform and reproducible bioavailability [7]. Advantages of SEDDS include more consistent drug absorption, selective targeting of drug(s) toward specific absorption window in GIT, protection of drug(s) from the gut environment, control of delivery profiles, reduced variability including food effects, enhanced oral bioavailability enabling reduction in dose and high drug loading efficiency [21]. Self emulsifying formulations spread readily in the gastrointestinal tract (GIT), and the digestive motility of the stomach and intestine provide the agitation necessary for self emulsification. These systems advantageously present the drug in dissolved form and the small droplet size provides a large interfacial area for the drug absorption. SEDDSs typically produce emulsions with turbid appearance, and droplet size between 200 nm to 5 μm while self micro emulsifying drug delivery systems (SMEDDSs) form translucent micro-emulsions with droplet size of less than 200 nm. However, self nano emulsifying drug delivery systems (SNEDDS) produces clear or transparent emulsion with droplets size less than 100 nm [22,23]. When compared with emulsions, which are sensitive and metastable dispersed forms, SEFs are physically stable formulations that are easy to manufacture. Thus, for lipophilic drug compounds that exhibit dissolution rate-limited absorption, these systems may offer an improvement in the rate and extent of absorption and result in more reproducible blood time profiles [17]. SEDDS are prepared in two forms: Liquid and solid SEDDS (S-SEDDS). S-SEDDS are prepared by solidification of liquid self-emulsifying components into powder. This powder is then used to produce various solid dosage forms, for example self-emulsifying pellets, self-emulsifying tablets etc [19]. S-SEDDS do not suffer with the problems like liquid SEDDS (L-SEDDS). It has

the advantages like low manufacturing cost, more stability and is more patient compliance, because they are available as solid dosage form in tablets or pellet form. In many studies it have been reported that SEDDS are used for delivering and targeting hydrophobic drugs such as coenzyme Q10, halofantrine, vitamin E and cyclosporine-A [19]. The solid SEDDS focus on the incorporation of liquid/semisolid ingredients into powders employing diverse solidification techniques like spray drying, melt granulation, moulding, melt extrusion, and nanoparticle technology. The powders can then be formulated as solid dosage forms like self-emulsifying tablets and self-emulsifying pellets [16]. Alternative approaches for the development of solid SEDDS comprise adsorption by solid carriers like microcrystalline cellulose, colloidal silica and various viscosity grades of HPMC, and use of high melting point solid excipients like Lutrol® and Gelucire® [16]. The idea of blending the potential SEDDS with that of the pellets through the inclusion of a self-emulsifying mixture into microcrystalline cellulose, and the production of pellets using extrusion-spheronization was first introduced by Newton *et al* [24].

High levels of surfactant typically present in SEDDS formulations can invariably lead to severe GI side-effects. Hence, a new class of SEDDS formulations, i.e., supersaturable SEDDS (S-SEDDS) has been designed to reduce the amount of surfactant by incorporating a water soluble polymeric precipitation inhibitor (PPI) [25] Such formulations have been developed specifically to reduce the surfactant side-effects and achieve rapid absorption of poorly soluble drugs [16]. The system is intended to generate and maintain a metastable supersaturated state *in vivo* by preventing or minimizing the precipitation of the drug through the use of a suitable PPI. Supersaturation is intended to increase the thermodynamic activity of the drug beyond its solubility limit, and therefore, to result in an increased driving force for transit into and across the biological barrier [26]. The S-SEDDS formulations have been demonstrated to improve both the rate and extent of the oral absorption of poorly water-soluble drugs quite effectively [25, 27, 28]. The inclusion of cellulosic polymers in the S-SEDDS formulation tends to effectively suppress the precipitation of drugs [29]. Various viscosity grades of hydroxypropyl methylcellulose (HPMC) are well-recognized for their ability to inhibit crystallization and, thereby, generate and maintain their supersaturated state for extended time periods [16]. *In vitro* dilution of the S-SEDDS formulation results in the formation of a microemulsion, followed by slow crystallization of the drug on standing indicating that the supersaturated state of the system is prolonged by HPMC in the formulations. In the absence of HPMC, the SEDDS formulation undergoes rapid precipitation, yielding a lower drug concentration [25]. The significantly reduced amount of surfactant used in the S-SEDDS formulation approach significantly reduces toxicity and improves safety profile over the conventional SEDDS formulations [16].

Positively charged SEDDS have also been produced; many physiological studies have proved that the apical potential of absorptive cells, as well as that of all other cells in the body, is negatively charged with respect to the mucosal solution in the lumen [16]. The drug exposure of the positively charged SEDDS has been found to be higher as well as the conventional formulations especially for bioavailability enhancement. The binding of the cationic SEDDS has been found to be much higher compared with the anionically charg-

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ed formulation, suggesting increased adhesion of the droplets to the cell surface due to electrostatic attraction [16].

Different dosage forms of S-SEDDS include the dry emulsions, self-emulsifying capsules, self-emulsifying sustained/controlled-release tablets, self-emulsifying sustained/controlled-release pellets, self-emulsifying solid dispersions, self-emulsifying beads, self-emulsifying sustained-release microspheres, self-emulsifying nanoparticles, self-emulsifying suppositories and self-emulsifying implant [29 – 34].

2.1.1.1. Advantages of SEDDS

SEDDS possess the following advantages among others [11]:

- Improvement and reduction in the variability of GI absorption of poorly water soluble, lipophilic drugs.
- Possible reduction in, or elimination of, a number of development and processing steps (e.g., salt selection or identification of a stable crystalline form of the drug, coating, taste masking, and reduced need for containment and clean-up requirements during manufacture of highly-potent or cytotoxic drug products).
- Food does not interfere with the absorption of drug by use of such systems.
- Relative ease of manufacture using readily available equipment.
- The dose ranging from less than 25 mg to greater than 2000 mg can be administered by using these systems.
- These systems enhance oral bioavailability due to bypass of hepatic metabolism and delivers drug directly into systemic circulation.
- Inhibition of p-glycoprotein mediated drug efflux and pre-absorptive metabolism by gut membrane bound cytochrome enzyme.
- Protection of sensitive drug substances
- High drug payloads.
- Liquid or solid dosage forms.
- Reduced energy requirement for emulsion formation.
- Control of delivery profile
- Promotion of lymphatic drug transport.
- They enhance absorption of lipophilic drugs by stimulating pancreatic and biliary secretions and by prolongation of gastric residence time.

2.1.1.2. Disadvantages of SEDDS

Disadvantages of SEDDS include [11, 35]:

- Lack of good predicative *in vitro* models for assessment of the formulations.
- Traditional dissolution methods do not work, because formulations are independent on digestion prior to release of the drug.
- *In vitro* model needs further development and validation.
- Different prototype lipid based formulations needs to be developed and tested *in vivo*.
- Chemical instabilities of drugs and high surfactant concentrations in formulations (approximately 30-60%) may irritate GIT.
- Volatile co solvents may migrate into the shells of soft or hard gelatin capsules, resulting in the precipitation of the lipophilic drugs.
- The precipitation tendency of the drug on dilution may be higher due to the dilution effect of the hydrophilic solvent.
- Formulations containing several components become more challenging to validation.

2.1.2. Self nano emulsifying drug delivery systems (SNEDDSs)

Self-nano emulsifying drug delivery systems (SNEDDS) are isotropic mixtures of oil, surfactant, co-surfactant and drug that form fine oil-in-water nanoemulsion when introduced into aqueous phases under gentle agitation. SNEDDS spread readily in the gastrointestinal tract, and the digestive motility of the stomach and the intestine provide the agitation necessary for self-emulsification [36]. SEDDSs typically produce emulsions with turbid appearance, and droplet size between 200 nm to 5 μm, while self micro emulsifying drug delivery systems (SMEDDSs) form translucent micro-emulsions with droplet size of less than 200 nm. However, self nano-emulsifying drug delivery systems (SNEDDS) produce clear or transparent emulsion with droplets size less than 100 nm [22, 23].

Successful formulation of SNEDDS depends on the thorough understanding of the spontaneous nano-emulsification process and also on the physicochemical and biological properties of the components used for the fabrication of SNEDDS. The factors influencing the phenomenon of self nano-emulsification are:

- The physicochemical nature and concentration of oily phase, surfactant and co-emulsifier or co surfactant or solubilizer (if included)
- The ratio of the components, especially oil-surfactant ratio
- The temperature and pH of the aqueous phase where nano-emulsification would occur
- Physicochemical properties of the drug, such as hydrophilicity/lipophilicity, pKa and polarity.

These factors should receive attention while formulating SNEDDS. In addition, the acceptability of the SNEDDS components for the desired route of administration is also very important while formulating SNEDDS [36].

SNEDDS offer a reduction in bioavailability and can offer reproducibility in plasma profiles of drugs. The ability of the SNEDDS in improving C_{max} and oral bioavailability or therapeutic effect has been established for various hydrophobic drugs. The improvement in bioavailability can be translated into reduction in the drug dose and dose-related side effects of many hydrophobic drugs, such as antihypertensive and antidiabetic drugs [37]. Transretinol acetate SNEDDS emulsion, anti hyperlipidemic, probucol, estrogen receptor antagonist, tamoxifen citrate, calcium channel blocker, felodipine,and beta blocker, carvedilol have been formulated as SNEDDS [36]. SNEDDS are used for enhancing the solubility of anti-inflammatory drugs such as indomethacin [38]. Fibrinolytic drugs such as simvastatin, atorvastatin, valsartan, gemfibrozil were also formulated as SNEDDS for improved bioavailability. Super-SNEDDS of simvastatin show increased bioavailability compared to the conventional SNEDDS due to the increased drug loading [39]. Solid SNEDDS of valsartan enhanced the bioavailabilty potential due to the presence of porous carriers and also showed stability for about six months which is an important factor [40]. Hormones such as ondasteron hydrochloride and insulin were also delivered orally by using SNEDDS. Solid SNEDDS of ondasteron showed increased bioavailbility than the pure drug [41]. Insulin was formulated into SNEDDS by first forming insulin-phospholipid complex (IPC) and this was used as oil phase in the formulation. This showed good hypoglycemic effect in diabetic Wistar rats for oral administration. Hence IPC can be used for the oral delivery of insulin [42].

Anti-cancer drugs were also formulated as SNEDDS. They include raloxifene hydrochloride, cyclosporine A, paclitaxel, flutamide. In raloxifene, the uptake of the drug by endocrine organs was assessed by administering the SNEDDS in alkalinized and non-alkalinized form to Wistar rats. Non-alkalinized form showed good uptake by the endocrine organs than the alkalinized form [43]. SNEDDS pellets of cyclosporine A were formulated by fluid bed coating technique and this improved the *in vivo* performance of the drug [44]. The drug release profile of paclitaxel was improved by SNEDDS [45] and the dissolution rate was also faster compared to that of the pure drug in flutamide [37, 46].

SNEDDS are given in the form of soft or hard gelatin capsules. They reach the gastro intestinal tract and the GI motility of the stomach provides the agitation for self-emulsification. Because of this self-emulsification the drug is given as small droplets with size less than 5μm for improved solubility. After administering orally, lingual and pancreatic lipases act on the oily phase of the SNEDDS that result in the formation of emulsified mono-glycerides, di-glycerides and fatty acids. This in the presence of bile acids leads to the formation of intestinal mixed micelles. When these mixed micelles pass through the enterocytes, it leads to the formation of chylomicrons. These drain the drug into the lymphatic vessels and not in the blood vessels thus bypassing the first pass effect. Thus the oral bioavailability gets increased [37].

2.1.2.1. Advantages of SNEDDS

Advantages of SNEDDS include [36]:

- Protection of sensitive drug substances.
- Selective targeting of drug(s) toward specific absorption window in GIT.

- Enhanced oral bioavailability enabling reduction in dose.
- High drug payloads.
- It can be easily stored since it belongs to a thermodynamics stable system.
- Fine oil droplets would pass rapidly and promote wide distribution of the drug throughout the GIT, thereby minimizing the irritation frequently encountered during extended contact between bulk drug substance and the gut wall.
- As compared with oily solutions, they provide a large interfacial area for partitioning of the drug between oil and water [36].

2.1.2.2. Disadvantages of SNEDDS

Disadvantages of SNEDDS include [36]:

- Lack of good predicative *in vitro* models for assessment of the formulations because traditional dissolution methods do not work, because these formulations potentially are dependent on digestion prior to release of the drug. To mimic this, an *in vitro* model simulating the digestive processes of the duodenum has been developed.
- Need of different prototype lipid based formulations to be developed and tested *in vivo* in a suitable animal model

2.1.2.3. Factors affecting SNEDDS

There are many factors that affect SNEDDS viz:

- Drugs which are administered at very high dose are not suitable for SNEDDS, unless they exhibit extremely good solubility in at least one of the components of SNEDDS, preferably lipophillic phase. The drugs exhibit limited solubility in water and lipids are most difficult to deliver by SNEDDS.
- The ability of SNEDDS to maintain the drug in solubilized form is greatly influenced by the solubility of the drug in oily phase. If the surfactant or co-surfactant is contributing to a greater extent for drug solubilization, then there could be a risk of precipitation, as dilution of SNEDDS will lead to lowering of solvent capacity of surfactant or co-surfactant [36].

2.1.3. Solid self-nanoemulsifying drug delivery systems (SSNEDDSs)

Solid SNEDDS was developed in order to eliminate the disadvantages associated with liquid SNEDDS handling, manufacturing and stability. Solid SNEDDS in the form of dry, solid powders would help in overcoming the limitations associated with liquid SNEDDS. Solid dosage forms are most stable and are convenient for handling; therefore, attempts are made to convert the liquid systems into solid SNEDDS. Various techniques, such as spray drying, freeze drying and adsorption on carriers, can be employed to convert liquid SNEDDS into solid SNEDDS compressed into tablets. The selection of a particular process for preparation of solid SNEDDS would depend on the content of oily excipient of the formulation,

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properties of active pharmaceutical ingredients, such as solubility, heat stability and compatibility with other ingredients [47].

The simplest technique to convert liquid SNEDDS to solid SNEDDS is by adsorption onto the surface of carriers or by granulation using liquid SNEDDS as a binder. This technique is uncomplicated, cost effective, easily optimized and industrially scalable. It can be used for heat-and moisture-sensitive molecules, thus providing an advantage over other techniques, such as spray drying and freeze drying. Various excipients utilized for the preparation of solid oral dosage forms can be employed for adsorption. The excipients should possess large surface areas to adsorb sticky and sometimes viscous oily SNEDDS formulation [47].

The ability of different excipients, such as dibasic calcium phosphate, lactose, microcrystalline cellulose, colloidal silicon dioxide and Neusilin, to adsorb cefpodoxime proxetil SNEDDS have been studied [47]. Solidification techniques for converting liquid/semisolid SEDDS/SNEDDS to solids include:

- **Capsule filling with liquid and semisolid self-emulsifying formulations:** Capsule filling is the simplest and the most common technology for the encapsulation of liquid or semi solid SE formulations for the oral route. The advantages of capsule filling are simplicity of manufacturing, suitability for low-dose highly potent drugs and high drug loading up to 50% (w/w) potential [48].

- **Spray drying:** This technique involves the preparation of a formulation by mixing lipids, surfactants, drug, solid carriers, and solubilization of the mixture before spray drying. The solubilized liquid formulation is then atomized into a spray of droplets. The droplets are introduced into a drying chamber, where the volatile phase (e.g. the water contained in an emulsion) evaporates, forming dry particles under controlled temperature and airflow conditions. Such particles can be further prepared into tablets or capsules [48].

- **Spray cooling:** Spray cooling also referred to as spray congealing is a process whereby the molten formula is sprayed into a cooling chamber. Upon contact with the cooling air, the molten droplets congeal and re-crystallize into spherical solid particles that fall to the bottom of the chamber and subsequently collected as fine powder. The fine powder may then be used for development of solid dosage forms, tablets or direct filling into hard shell capsules. Many types of equipment are available to atomize the liquid mixture and to generate droplets: rotary pressure, two-fluid or ultrasonic atomizers [49, 50].

- **Adsorption to solid carriers:** SEDDS can be adsorbed at high levels (up to 70% (w/w)) onto suitable carriers. Solid carriers can be microporous inorganic substances, high surface area colloidal inorganic adsorbent substances, cross-linked polymers or nanoparticle adsorbents (i.e., silica, silicates, magnesium trisilicate, magnesium hydroxide, talcum, crospovidone, cross-linked sodium carboxymethyl cellulose and crosslinked polymethyl methacrylate). The adsorption technique has been successfully applied to gentamicin and erythropoietin with caprylocaproyl polyoxylglycerides (Labrasol) formulations that maintained their bioavailability enhancing effect after adsorption on carriers [51-53].

- **Melt granulation:** Melt granulation or pelletization is a one step-process allowing the transformation of a powder mix (containing the drug) into granules or spheronized pellets. The technique needs high shear mixing in presence of a meltable binder. This is referred to as "pump-on" technique. Alternatively, the binder may be blended with the powder mix in its solid or semi-solid state and allowed to melt (partially or completely) by the heat generated from the friction of particles during high shear mixing referred to as "melt-in" process. The melted binder forms liquid bridges with the powder particles that shape into small agglomerates (granules) which can, by further mixing under controlled conditions transform to spheronized pellets [48].
- **Melt extrusion/Extrusion spheronization:** It is a solvent-free process that allows high drug loading (60%) as well as content uniformity. Applying extrusion-spheronization, SE pellets of diazepam and progesterone and bi-layered cohesive SE pellets have been prepared [54, 55].

3. Advantages, disadvantages and major challenges of nanoemulsions as drug delivery systems

The advantages of nanoemulsions drug delivery systems include [56-58]:

- The small size of the droplets allows them to deposit uniformly on substrates. Wetting, spreading and penetration may be also enhanced as a result of the low surface tension of the whole system and the low interfacial tension of the o/w droplets.
- The very small droplet size causes a large reduction in the gravity force and the Brownian motion may be sufficient for overcoming gravity. This means that no creaming or sedimentation occurs on storage.
- The small droplet size also prevents any flocculation of the droplets. Weak flocculation is prevented and this enables the system to remain dispersed with no separation. Nanoemulsions are thermodynamically stable system and the stability allows self emulsification of the system
- The small droplets also prevent their coalescence, since these droplets are elastic, surface fluctuations are prevented.
- Nanoemulsions are suitable for efficient delivery of active ingredients through the skin. The large surface area of the emulsion system allows rapid penetration of actives. It is non-toxic and non-irritant so can be easily applied to skin and mucous membranes.
- The transparent nature of the system, their fluidity (at reasonable oil concentrations) as well as the absence of any thickeners may give them a pleasant aesthetic character and skin feel.
- Unlike microemulsions (which require a high surfactant concentration, usually in the region of 20 % and higher), nanoemulsions can be prepared using reasonable surfactant concentration. For a 20 % o/w nanoemulsion, a surfactant concentration in the region of 5 – 10 %

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may be sufficient. Nanoemulsions are usually formulated with surfactants, which are approved for human consumption (GRAS), they can be taken by enteric route.

- Nanoemulsions can be applied for delivery of fragrance, which may be incorporated in many personal care products. This could also be applied in perfumes, which are desirable to be formulated alcohol free.
- Nanoemulsions may be applied as a substitute for liposomes and vesicles (which are much less stable) and it is possible in some cases to build lamellar liquid crystalline phases around the nanoemulsion droplets.
- Nanoemulsions can be formulated in numerous dosage foam such as creams, liquids, sprays and foams.
- They do not damage healthy human and animal cells, so nanoemulsions are suitable for human and veterinary therapeutic purposes [56].
- Increase the rate of absorption, increases bioavailability and eliminates variability in absorption
- Helps solubilize lipophilic drug and masks unpleasant taste of some drugs
- Various routes like topical, oral and intravenous can be used to deliver the product.
- Better uptake of oil-soluble supplements in cell cultures. Improve growth and vitality of cultured cells. It allows toxicity studies of oil-soluble drugs in cell cultures.
- Nanoemulsions could enhance the stability of chemically unstable compounds by protecting them from oxidative degradation and degradation by light.
- Possibilities of controlled drug release and drug targeting, and the incorporation of a great variety of therapeutic actives.

3.1. Major challenges

Although nanoemulsions provide great advantages as a delivery system, however they suffer for some major challenges and limitations which include [56-58]:

- The formulation of nanoemulsions is an expensive process due to size reduction of droplets is very difficult as it required a special kind of instruments and process methods. For example, homogenizer (instrument required for the nanoemulsions formulation) arrangement is an expensive process. More ever micro-fluidization and ultrasonication (manufacturing process) require large amount of financial support.
- One problem associated with nanoemulsion is their stability. Although it is generally accepted that these systems could remain stable even by years, however, due to the small droplet size, it has been reported that the Oswald ripening could damage nanoemulsions, causing their application to be limited. Therefore, in most cases, nanoemulsions are required to be prepared shortly before their use.

- Use of a large concentration of surfactant and cosurfactant necessary for stabilizing the nano droplets.
- Limited solubility capacity for high melting substances.
- Nanoemulsion stability is influenced by environmental parameters such as temperature and pH.
- Lack of understanding of the mechanism of production of submicron droplets and the role of surfactants and cosurfactants.
- Lack of demonstration of the benefits that can be obtained from using nanoemulsions when compared with the classical macroemulsion systems.
- Lack of understanding of the interfacial chemistry that is involved in production of nanoemulsions.

4. Formulation of nanoemulsions

4.1. Materials used in preparation of nanoemulsions

Nanoemulsions are prepared using oils, surfactants and co-surfactants and aqueous phase [59]. Oils used in nanoemulsions preparation include Captex 355, Captex 8000, Witepsol, Myritol 318, Isopropyl myristate, Capryol 90, Sefsol-218, triacetin, isopropyl myristate, castor oil, olive oil, etc. Solubility of the drug in the oil phase is an important criterion for the selection of oils. This is particularly important in the case of oral formulation development, as the ability of nanoemulsion to maintain the drug in solubilized form is greatly influenced by the solubility of the drug in the oil phase. While water-in-oil nanoemulsions are better choice for hydrophilic drugs lipophilic drugs are preferably solubilized in oil-in-water nanoemulsions. Drug loading in the formulation is a very critical design factor in the development of nanoemulsions for poorly soluble drugs, which is dependent on the drug solubility in various formulation components. An understanding of factors influencing drug loading capacity while maintaining the capability of the system to undergo monophasic dilution with water and minimizing the tendency for drug precipitation or crystallization in diluted systems is essential to the design of stable and appropriately low-volume nanoemulsion systems for drug delivery applications [60,61]. Edible oils are not frequently useful due to their poor ability to dissolve large amounts of lipophilic drugs. Moreover, formulation of nanoemulsion with oil of low drug solubility would require incorporation of more oil to incorporate the target drug dose, which in turn would require higher surfactant concentration to achieve oil solubilization, which might increase the toxicity of the system. Novel semi-synthetic medium chain derivatives (as amphiphilic compounds) having surfactant properties are progressively and effectively replacing the regular medium chain triglyceride oils [62,63].

Surfactants used for stabilizing nanoemulsions may be non ionic, zwitterionic, cationic and anionic. The surfactants may include Capryol 90, Gelucire 44/14, 50/13, Cremophor RH 40, Imwitor 191, 742, 780 k, 928, 988, Labrafil CS, M, 2125 CS, Lauroglycol 90, PEG MW > 4000,

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Plurol Oleique CC 497, Poloxamer 124 and 188, Softigen 701, 767, Labrasol, Cremophor EL, Tween 20, Tween 60, and Tween 80, etc. Components of nanoemulsion-based systems are associated with toxicity concerns. Large amounts of surfactants may cause gastrointestinal and skin irritation when administered orally and topically, respectively. Therefore, the proper selection of surfactants is essential. Rational use of the minimum concentration of the surfactant in the formulation is advocated. Nonionic surfactants are relatively less toxic than their ionic counterparts and typically have lower critical micelle concentration (CMCs). Also, o/w nanoemulsion dosage forms for oral or parenteral use based on nonionic surfactants are likely to offer *in vivo* stability [64]. Therefore, proper selection of surfactants is a crucial factor. Another important criterion is the selection of surfactant with proper hydrophile-lipophile-balance (HLB) value. Hydrophilic surfactants and co-surfactants are considered to prefer the interface and to lower the necessary energy to form the nanoemulsions, thereby improving the stability. For instance, the required HLB value to form o/w nanoemulsion is greater than 10 [65]. The right blend of low and high HLB surfactants leads to the formation of a stable nanoemulsion upon dilution with water. The type and nature of the surfactant is also an important factor for consideration; nonionic surfactants are usually selected since they are known to be less affected by pH and changes in ionic strength, are generally regarded as safe, and are biocompatible; ionic surfactants are less commonly used due to toxicological concerns. Solubilization of oil with the surfactant is also an important factor. It is not necessary that the same surfactant that has good solubilizing power for drugs would have equally good affinity for the oil phase. Surfactant–oil miscibility can thus give an initial indication on the possibility of nanoemulsion formation with this system.

Cosurfactants are added to obtain nanoemulsion systems at low surfactant concentration [66]. Short-to medium-chain-length alcohols (C3–C8) are commonly added as cosurfactants, which further reduce the interfacial tension and increase the fluidity of the interface [67,68]. They also increase the mobility of the hydrocarbon tail and allow greater penetration of the oil into this region. Alcohols may also increase the miscibility of the aqueous and oily phases due to its partitioning between these phases. Co-surfactants used in nanoemulsions include Transcutol P, glycerin, ethyleneglycol, ethanol, propanol, ethanol, isopropyl alcohol, n-butanol, PEG 400, Carbitol, and propylene glycol. Nanoemulsion area is often used as the assessment criterion for the evaluation of cosurfactants. The larger the size of the nanoemulsion field, the greater the nanoemulsification efficiency of the system.

Moreover, the most important criterion for selection of all the nanoemulsion components is that all the excipients should be pharmaceutically acceptable for oral administration or topical application, etc., depending upon the requirement and falling under GRAS category.

4.2. Methods of preparation of nanoemulsions

As nanoemulsions are non-equilibrated systems [69-71], and so their preparation involves the input of a large amount of either energy or surfactants and in some cases a combination of both. As a result, high energy or low energy methods can be used in their formulation [70]. Although high energy emulsification method is traditionally used for the preparation of nanoemulsion formulation but low emulsion emulsification method now create an attraction

due to their wide application and advantages as a formulation and stability aspects. Generally, energy is usually required in emulsion formulation because the process may be non-spontaneous. The production of nanoemulsions costs more energy than that required to produce macroemulsions. Presence of surfactants help lower the surface tensions between oil and water. Small molecules such as non-ionic surfactants lower surface tension more than polymeric surfactants such as poly (vinyl alcohol). Another important role of the surfactant is its effect on the interfacial dilatational modulus [72]. During emulsification an increase in the interfacial area takes place and this causes a reduction in surface excess. The equilibrium is restored by adsorption of surfactant from the bulk, but this takes time (shorter times occur at higher surfactant activity). Because of the lack or slowness of equilibrium with polymeric surfactants, dilatational modulus will not be the same for expansion and compression of the interface [72]. In practice, surfactant mixtures are used and these have pronounced effects on surface tension and dilatational modulus. Some specific surfactant mixtures give lower surface tension values than either of the two individual components. Polymer-surfactant mixtures may show some synergistic surface activity. An important role of the emulsifier is to prevent shear-induced coalescence during emulsification. The requirement is that the continuous phase has a significant excess of surfactant. This excess enables new surface area of the nano-scale droplets to be rapidly coated during emulsification, thereby inhibiting shear-induced coalescence. This excess is generally in the form of surfactant micelles in the continuous phase. These micelles dissociate into monomers that rapidly adsorb onto the surfaces of newly created droplets [71].

4.2.1. Low energy methods

As the name suggests, low-energy emulsification methods require low energy for the fabrication of nanoemulsions. These methods are mainly dependent on modulation of interfacial phenomenon/phase transitions and intrinsic physicochemical properties of the surfactants, co-emulsifiers/co-surfactants and oil to yield nano-sized emulsion droplets. The lower energy method, also called the condensation method, is based on the phase transitions taking place during the emulsification process [73,74]. These phase transitions result from changes in the spontaneous curvature of the surfactant and can be achieved (i) at constant composition by changing the spontaneous curvature of non-ionic surfactants with temperature, the well-known Phase Inversion Temperature, PIT, widely used in industry [75,76] or (ii) at constant temperature by varying the composition of the system by the Emulsion Inversion Point (EIP) method [77-79]. In other words, low-energy emulsification method was developed according to the phase behavior and properties of the constituents, to promote the formation of ultra-small droplets [80,81]. These low-energy techniques include self-emulsification, phase transition and phase inversion temperature methods [82]. The low energy method is interesting because it utilizes the stored energy of the system to form small droplets. This emulsification can be brought about by changing the parameters which would affect the hydrophilic lipophilic balance (HLB) of the system like temperature, composition, etc. [83,84]. The limitations include complexity, precise approach required and use of synthetic surfactants. In a nutshell, the most commonly used low-energy emulsification methods include:

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4.2.1.1. Phase Inversion Temperature (PIT) method

This method employs temperature-dependent solubility of non-ionic surfactants, such as polyethoxylated surfactants, to modify their affinities for water and oil as a function of the temperature. It has been observed that polyethoxylated surfactants tend to become lipophilic on heating owing to dehydration of polyoxyethylene groups. This phenomenon forms a basis of nanoemulsion fabrication using the PIT method. In the PIT method, oil, water and nonionic surfactants are mixed together at room temperature. This mixture typically comprises o/w microemulsions coexisting with excess oil, and the surfactant monolayer exhibits positive curvature. When this macroemulsion is heated gradually, the polyethoxylated surfactant becomes lipophilic and at higher temperatures, the surfactant gets completely solubilized in the oily phase and the initial o/w emulsion undergoes phase inversion to w/o emulsion. The surfactant monolayer has negative curvature at this stage [75]. At an intermediate temperature (also termed HLB temperature), the non-ionic surfactant has similar affinity for aqueous and oily phase, and this ternary system has extremely low interfacial tension (in the order of 10^{-2}–10^{-5} mNm^{-1}) and spontaneous curvature typically reaches zero [83,84]. The ternary system at this stage typically consists of a D-phase bicontinuous microemulsion or a mixture of a D-phase bicontinuous microemulsion and lamellar liquid crystalline phases. It has been observed that nanoemulsions with very small droplet size and polydispersity index can be generated by rapid cooling of the single-phase or multiphase bicontinuous microemulsions maintained at either PIT or a temperature above PIT (transitional-phase inversion) [76]. Nanoemulsions can also be generated by rapidly diluting the single bicontinuous microemulsions with the aqueous or oil phase (catastrophic phase inversion) to obtain either o/w nanoemulsion or w/o nanoemulsion. It has been observed that the characteristics of the nanoemulsion are mainly dependent on the structure of the surfactant at HLB temperature (bicontinuous or lamellar) and also on the surfactant/oil ratio. Initially, PIT method was believed to be useful for fabricating o/w nanoemulsions. However, in recent years, the application of the PIT method has been established for fabricating w/o emulsions and nanoemulsions. It is important to note that the use of lipophilic polyethoxylated surfactants and appropriate modifications in the typical PIT protocol are required for obtaining w/o nanoemulsions [82].

4.2.1.2. Solvent displacement method

The solvent displacement method for spontaneous fabrication of nanoemulsion has been adopted from the nano-precipitation method used for polymeric nanoparticles. In this method, oily phase is dissolved in water-miscible organic solvents, such as acetone, ethanol and ethyl methyl ketone. The organic phase is poured into an aqueous phase containing surfactant to yield spontaneous nanoemulsion by rapid diffusion of organic solvent. The organic solvent is removed from the nanoemulsion by a suitable means, such as vacuum evaporation [78-81].

4.2.1.3. Phase Inversion Composition Method (Self-nanoemulsification Method)

This method generates nanoemulsions at room temperature without use of any organic solvent and heat. Forgirani *et al.* observed that kinetically stable nanoemulsions with small droplet size (~50 nm) can be generated by the stepwise addition of water into solution of surfactant in

oil, with gentle stirring and at constant temperature [77]. Although the components used in the aforementioned investigation were not of pharmaceutical grade, the investigation opened doors to design pharmaceutically acceptable nanoemulsions using a similar approach. The spontaneous nano-emulsification has been related to the phase transitions during the emulsification process and involves lamellar liquid crystalline phases or D-type bicontinuous microemulsion during the process [77].

4.2.2. High energy methods

High-energy emulsification methods make use of devices that use very high mechanical energy to create nanoemulsions with high kinetic energy. The high-energy method utilizes mechanical devices to create intensely disruptive forces which break up the oil and water phases to form nano-sized droplets. This can be achieved with ultrasonicators, microfluidiser and high pressure homogenisers [71,85,86]. Particle size here will depend on the type of instruments employed and their operating conditions like time and temperature along with sample properties and composition [87]. These methods include high-pressure homogenization and ultrasonic emulsification. High-pressure homogenization is the most common method used for the fabrication of nanoemulsions. During high-pressure homogenization, the coarse macroemulsion is passed through a small orifice at an operating pressure in the range of 500 to 5000 psi. During this process, several forces, such as hydraulic shear, intense turbulence and cavitation, act together to yield nanoemulsions with extremely small droplet size. The resultant product can be re-subjected to high-pressure homogenization until nanoemulsion with desired droplet size and polydispersity index is obtained [71]. Micro-fluidization employs a high-pressure positive displacement pump operating at very high pressures, up to 20,000 psi. This pump forces macroemulsion droplets through the interaction chamber consisting of a series of micro-channels. The macroemulsion flowing through the microchannels collides with high velocity on to an impingement area resulting in very fine nanoemulsions. The nanoemulsions with desired size range and dispersity can be obtained by varying the operating pressure and the number of passes through interaction chambers like high-pressure homogenization. Ultrasonic emulsification uses a probe that emits ultrasonic waves to disintegrate the macroemulsion by means of cavitation forces. By varying the ultrasonic energy input and time, the nanoemulsions with desired properties can be obtained [85-87]. High-energy emulsification methods can be employed to fabricate both o/w and w/o nanoemulsions. High-pressure homogenization and microfluidization can be used for fabrication of nanoemulsions at laboratory and industrial scale, whereas ultrasonic emulsification is mainly used at laboratory scale. In addition, high-energy methods require sophisticated instruments and extensive energy input, which considerably increases the cost of nanoemulsions fabrication. This is particularly significant in the pharmaceutical sciences [85-87]. High energy methods allow for a greater control of particle size and a large choice of composition, which in turn controls the stability, rheology and colour of the emulsion. Although high-energy emulsification methods yield nanoemulsions with desired properties and have industrial scalability, they may not be suitable for thermolabile drugs such as retinoids and macromolecules, including proteins, enzymes and nucleic acids. Moreover, high energy methods alone normally do not yield oil droplets (<100 nm).

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4.3. Formulation factors that affect the stability of nanoemulsions

Although nanoemulsions enhance the physical as well as chemical stability of drugs, stability of drug product is one of the problems associated with the development of nanoemulsions [88-91]. Stability studies are performed on nanoemulsions by storing them at refrigerator and room temperatures over a number of months. The viscosity, refractive index and droplet size are determined during this period of storage. Insignificant changes in these parameters indicate formulation stability. Accelerated stability studies can also be performed on the nanoemulsions. In this instance, nanoemulsion formulation are kept at accelerated temperatures and samples withdrawn at regular intervals and analyzed for drug content by stability indicating assay methods. The amount of drug degraded and remaining in nanoemulsion formulation is determined at each time interval [92]. Stability of nanoemulsion formulation may be enhanced by controlling factors such as type and concentration of surfactant and co-surfactant, type of oil phase, methods used, process variables and addition of additives [88-93]. Overall nanoemulsion formulation may be considered as effective, safe and patient compliance formulation for the delivery of pharmaceuticals.

Factors to be considered during preparation of nanoemulsion include the following among others [93]:

a. The prime requirement in nanoemulsion production is that an ultra low interfacial tension should be attained at the oil water interface, so surfactants must be carefully chosen.

b. Concentration of surfactant must be high enough to provide the number of surfactant molecules needed to stabilize the nano droplets.

c. The interface must be flexible to promote the formation of nanoemulsion.

5. Characterization of nanoemulsions

Characterization of nanoemulsions involves the physical and chemical tests related to oral liquid dosage forms which includes compatibility of the nanoemulsion components, isotropicity of the formulation, assay, uniformity of content, appearance, pH, viscosity, density, conductivity, surface tension, size and zeta potential of the dispersed phase etc. with respect to the effect of the composition on physical parameters [94-104]. Differential scanning calorimetry (DSC) provides information on the interactions of different components and polarization microscopy using crossed polarizers is employed to confirm isotropicity of the formulation [99]. The process of self-nanoemulsification can be evaluated by visual assessment. Its efficiency would be estimated by determining the rate of emulsification and droplet size distribution. Turbidity measurements are carried out to determine the rapid equilibrium reached by the dispersion and reproducibility of this process. The droplet size of the emulsion is a crucial factor in self-nanoemulsification performance because it determines the rate and extent of drug release as well as absorption. Photon correlation spectroscopy (PCS) and light scattering techniques like static light scattering (SLS), dynamic light scattering (DLS) are a useful method for determination of nanoemulsion droplet size [100]. Viscosity, conductivity

and dielectric methods provide useful information at the macroscopic level. Viscosity measurements for example can indicate the presence of rod-like or worm-like reverse micelles and conductivity measurements provide the means of determining whether a nanoemulsion is oil-continuous or water-continuous, as well as providing a means of monitoring phase inversion phenomena [99]. Dielectric measurements are a powerful means of probing both the structural and dynamic features of nanoemulsion system. Structural features of nanoemulsions have been studied using self-diffusion nuclear magnetic resonance (SD NMR) and small angle x-ray scattering (SAXS). Freeze fracture electron microscopy has also been used to study nanoemulsion structure, however extremely rapid cooling of the sample is required in order to maintain the structure and minimize the possibility of artifacts [101-103]. Nanoemulsion droplet polarity is also a very important factor in characterizing emulsification efficiency. The HLB, chain length and degree of unsaturation of fatty acids, molecular weight of the hydrophilic portion and concentration of the emulsifier have an impact on the polarity of the oil droplets. Polarity represents the affinity of the drug compound for oil and/ or water and the type of forces formed. Rapid release of the drug into the aqueous phase is promoted by the polarity. The charge of the oil droplets of nanoemulsions is another property that should be assessed. Usually it is negative due to the presence of free fatty acids; however, incorporation of a cationic lipid, such as oleylamine at a concentration range of 1-3%, will yield cationic nanoemulsions [104,105].

The following sub-headings could be used to discuss briefly the parameters commonly employed in the assessment of nanoemulsions:

a. **Morphology:** The morphology of nanoemulsions can be determined by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). SEM gives a three-dimensional image of the globules [105]. The samples are examined at suitable accelerating voltage, usually 20 kV, at different magnifications. A good analysis of surface morphology of disperse phase in the formulation is obtained through SEM. Image analysis software may be employed to obtain an automatic analysis result of the shape and surface morphology [106]. In TEM, higher resolution images of the disperse phase are obtained. The sample is negatively stained with 1% aqueous solution of phosphotungstic acid or by dropping 2 % uranyl acetate solution onto a 200 μm mesh size Pioloform™-coated copper grid or a microscopic carbon-coated grid using a micropipette and the sample examined under a transmission electron microscope at appropriate voltage. Qualitative measurements of sizes and size distribution of TEM micrographs can be performed using a digital image processing programme [107]. More sophisticated techniques, such as x-ray or neutron scattering, atomic force microscopy, or cryo-electron microscopy are typically required to explore the structure and behaviour of nanoemulsions [71].

b. **Droplet size, polydispersity and zeta potential:** Dynamic light scattering (DLS) otherwise called photon correlation spectroscopy (PCS) is used to analyze the fluctuations in the intensity of scattering by droplets/particles due to Brownian motion [108]. Nanoemulsion droplet size, polydispersity and zeta potential can be assessed by PCS using a particle size analyzer. This instrument also measures polydispersity index, which is a measure of the broadness of the size distribution derived from the cumulative analysis of dynamic light

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scattering. The polydispersity index indicates the quality or homogeneity of the dispersion [109]. PCS gives z-average particle diameter. Laser diffraction is another technique for measuring particle size. The fundamental particle size distribution derived by this technique is volume based and is expressed in terms of the volume of equivalent spheres (*DN*%) and weighted mean of the volume distribution (mass mean diameter). Since the laser diffraction system is used for this analysis, a rough equivalent of particle polydispersity could be given by two factors/values namely, uniformity (how symmetrical the distribution is around the median point) and span (the width of the distribution). The span value is defined by the expression:

$$\text{Span} = \left(D90\% - D10\%\right) / D50\% \tag{2}$$

Where *DN*% (*N*=10%, 50%, 90%), means that the volume percentage of particles with diameters up to *DN*% equals to *N*%. The smaller the span value the narrower the particle size distribution.

c. **Viscosity:** This is carried out using a viscometer. The viscosity of nanoemulsions is a function of the surfactant, water and oil components and their concentrations. Increasing the water content lowers the viscosity, while decreasing the amount of surfactant and cosurfactant increases interfacial tension between water and oil resulting in increased viscosity. Viscosity is very important for stability and efficient drug release. Nanoemulsion carrier formulations are basically oil-in-water and so in addition to being less greasy than water-in-oil formulations, often possess lower apparent viscosities. They are therefore expected to exhibit faster release of active ingredients and wash out easily after application on the skin surface. Various equipment and methods are available for assessment of rheological properties of nanoemulsion carriers. Monitoring of viscosity change is a method of assessing stability of liquid and semi-solid preparations including nanoemulsion formulations [99].

d. ***In vitro* skin permeation:** Franz diffusion cell is used to obtain the drug release profile of the nanoemulsion formulation in the case of formulations for transdermal application. The extent or depth of skin penetration by the released content can be visualized by confocal scanning laser microscopy. *In vitro* drug release can be determined by dispersing an amount of the preparation in the donor compartment of a Franz cell having a membrane as barrier and monitoring the appearance of the encapsulated drug in the receptor compartment, usually containing phosphate buffer saline (PBS, pH 7.4) and stirring on a magnetic stirrer at 100 rpm at 37 ± 1 °C. Samples (1 ml) of the dispersion are withdrawn from the receptor medium and replaced with an equivalent amount of the medium at definite intervals. The withdrawn sample is then filtered using a 0.22-50 μm filter (e.g., Millipore, USA) and the drug released then analyzed using HPLC or UV-Vis spectroscopy at wavelength of peak absorption of the drug [110]. An alternative and popular method of *ex-vivo* release study is performed using diffusion cell. The skin is cut from the ear or abdomen and underlying cartilage and fats carefully removed. Appropriate size of skin is cut and placed on the diffusion cell which had earlier been filled with receptor solution.

Samples of the vesicular preparation are then applied on the dorsal surface of the skin and the instrument started. At intervals, up to 24 h, samples are withdrawn from the receptor medium and replaced with equal amounts of the medium and the withdrawn samples analyzed for the drug permeated using HPLC or UV spectroscopy [111,112]. Semi-permeable membrane such as regenerated cellulose could be used in place of skin for *in vitro* release studies [113,114]. The flux *J*, of the drug across the skin or membrane is calculated from the formula:

$$J=Ddc/dx \tag{3}$$

Where D is the diffusion coefficient and is a function of the size, shape and flexibility of the diffusing molecule as well as the membrane resistance, c is the concentration of the diffusing species, x is the spatial coordinate [114].

e. ***In vivo*** **bioavailability/pharmacodynamic studies**: *In vivo* release study otherwise referred to as dermatopharmacokinetics, is carried out by applying or administering the preparation to whole live animal. Blood samples are then withdrawn at intervals, centrifuged and the plasma (deproteinated) analyzed for the drug content using HPLC. Results obtained from *in vitro* and *in vivo* studies are extrapolated to reflect bioavailability of the drug formulation. Moreover, the pharmacodynamic properties of nanoemulsion formulations are also assessed depending on the pharmacological properties of the incorporated drug [81-93,95,101].

6. Applications of nanoemulsions in drug delivery

Nanoemulsions could be and have been applied in various aspects of drug delivery including: cosmetics and transdermal delivery of drug, cancer therapy, vaccine delivery, prophylactic in bio-terrorism attack, non-toxic disinfectant cleaner, cell culture technology, formulations for improved oral delivery of poorly soluble drug, ocular and otic drug delivery, intranasal drug delivery, parenteral drug delivery and pulmonary delivery of drugs.

6.1. Applications in cosmetics

Recently importance of nanoemulsions have become increasing as good vehicles for the controlled delivery of cosmetics and for the optimized dispersion of active ingredients in particular skin layers. Due to their lipophilic interior, nanoemulsions are more suitable for the transport of lipophilic drug than liposomes Similar to liposomes, nanoemulsions supports the skin penetration of active ingredients and thus increases their concentration in the skin. Another advantage is the small-sized droplet with its high surface area permit effective delivery of the active to the skin. More ever, nanoemulsions gain increasing interest due to their own bioactive effects. This may reduce the trans-epidermal water loss (TEWL), suggesting that the barrier function of the skin is strengthened. Nanoemulsions are acceptable in

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cosmetics because there is no chance of creaming, sedimentation, flocculation or coalescence, which is observed within microemulsions. The incorporation of potentially irritating surfactants can be avoided by using high-energy equipment during manufacturing process. PEG-free nanoemulsions for cosmetics has also been developed and formulations exhibited good stability [56-58,115,116].

6.2. Antimicrobial nanoemulsions

Antimicrobial nanoemulsions are o/w droplets that range from 200-600 nm. They are made of oil and water and are stabilized by surfactants and alcohol. The nanoemulsions has a broad spectrum of activity against bacteria like *E. coli*, salmonella, *S. aureus*; enveloped viruses like HIV, herpes simplex; fungi like candida, dermatophytes, and spores like anthrax. The nanoemulsions particles are thermodynamically driven to fuse with lipid-containing organisms. This fusion is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge on the pathogen. When enough nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death. In the case of spores, additional germination enhancers are added into the emulsion. Once starting of germination takes place, the germinating spores become susceptible to the antimicrobial action of the nanoemulsions. An aspect of the nanoemulsions is their highly selective toxicity to microbes at concentration range that are non-irritating to skin or mucous membrane. The safety range of nanoemulsions is because of the low amount of detergent in each droplet, yet when acting in concert, these droplets have enough energy and surfactant to destabilize targeted microbes without affecting healthy cells. Nanoemulsions can get a level of topical antimicrobial activity, which can only be previously achieved by systemic antibiotics [56-58,115].

6.3. Prophylactic in bio-terrorism attack

Because of their antimicrobial activity, research has begun on use of nanoemulsions as a prophylactic medicated dosage form, a human protective treatment, to prevent the people exposed to bio-attack such as Anthrax and Ebola. The broad-spectrum nanoemulsions were checked on surfaces by the US Army (RestOps) in Dec 1999 for decontamination of Anthrax spore. It was checked again by RestOps in March 2001 as a chemical decontamination agent. This technology has been tested on gangrene and clostridium botulism spores, and can even be used on contaminated wounds to salvage limbs. The nanoemulsions can be formulated into a cream, foam, liquid and spray to decontaminate a large number of materials, which is marketed as NANOSTAT™ (Nanobio Corp.) [56-58,115].

6.4. Nanoemulsions in vaccines delivery

This medication delivery system uses nanotechnology to vaccinate against human immunodeficiency virus (HIV). There is recent evidence that HIV can infect the mucosal immune system. Therefore, developing mucosal immunity through the use of nanoemulsions may become very important in the future fight against HIV [50]. The oil-based emulsion is admin-

istered in the nose, as opposed to traditional vaccine routes. Recent research results indicate that genital mucosa immunity may be attained with vaccines that are administered into the nasal mucosa [56-58,115]. Nanoemulsions are being used to transport inactivated organisms to a mucosal surface to produce an immune response. The first applications as vaccine, an influenza vaccine and an HIV vaccine, can proceed to clinical trials. The nanoemulsion causes proteins applied to the mucosal surface to be adjuvant and it help uptake by antigen presenting cells. This results in the significant systemic and mucosal immune response due to that the production of specific IgG and IgA antibody as well as cellular immunity. Work in influenza has shown that animals can be prevented against influenza after a single mucosal exposure to the virus mixed with thenanoemulsions. Research has also show that animals exposed to recombinant gp120 in nanoemulsions on their nasal mucosa create significant responses to HIV, thus giving a basis to use of this material as an HIV vaccine. Additional research has been ongoing to complete the proof of concept in animal trials for other vaccines including Anthrax and Hepatitis B. The University of Michigan has licensed this technology to NanoBio [56].

6.5. Nanoemulsions as non-toxic disinfectant cleaner

Nanemulsions have been employed as a disinfectant cleaner. A nontoxic disinfectant cleaner for use in routine markets that include healthcare, travel, food processing and military applications has been developed by EnviroSystems. They have been found to kill tuberculosis and a large spectrum of viruses, bacteria and fungi within 5 to 10 min without any of the hazards posed by other categories of disinfectants. The product requires no warning labels. It does not irritate eyes and can be absorbed through the skin, inhaled or swallowed with harmless effects. The disinfectant formulation is made up of nanospheres of oil droplets less than 100 μm which are suspended in water to produce a nanoemulsions requiring only small amounts of the active ingredient, parachlorometaxylenol. The nanospheres have surface charges that efficiently penetrate the surface charges on microorganisms' membranes like breaking through an electric fence. Rather than 'drowning' cells, the formulation allows parachlorometaxylenol to target and penetrate cell walls. So parachlorometaxylenol is applicable at concentration ranges I-2 times lower than those of other disinfectants, so there are no toxic effects on human, animals or the environment [56-58,115].

Other microbial disinfectants need large doses of their respective active ingredients to surround pathogen cell wall, which causes microbe to disintegrate, ideally 'drowning' them in the disinfectant solution. The disinfectant is not flammable and so safe to store anywhere and to use in unstable conditions. It is non oxidizing, non acidic and nonionic. It will not corrode plastic, metals or acrylic, so it makes the product ideal for use on equipment and instruments. It is environmentally safe so the economical cost and health risks associated with hazardous chemical disposal are removed. The preparation is a broad-spectrum disinfectant cleaner that can be applied to any hard surface, including equipment, walls, fixtures, counters, and floors. One product can now take the place of many other, decreasing product inventories and saving valuable storage space. Chemical disposal costs can be removed, and disinfection and cleaning costs can be reduced. Marketed as EcoTru ™ (EnviroSystems) [56-58,115].

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6.6. Nanoemulsions in cell culture technology

Cell cultures are used for in vitro assays or to produce biological compounds like an antibodies or recombinant proteins. For optimization of cell growth, the culture medium can be supplemented with a large number of molecules or with blood serum. It has been very difficult to provide the media with oil-soluble substances that are available to the cells, and only few amounts of the lipophilic compounds could be absorbed by the cells. Nanoemulsions are a new method for the delivery of oil-soluble substances to human cell cultures. The system is based on a nanoemulsions that is stabilized by phospholipids. This nanoemulsions is transparent and can be passed through 0.1 mm filters for sterilization. Nanoemulsions oil droplets are very easily taken up by the cells. The encapsulated oil-soluble substances therefore have a high bioavailability to cells in culture.

The advantages of using nanoemulsions in cell culture technology include:

- Better uptake of oil-soluble supplements in cell cultures.
- Improve growth and vitality of cultured cells.
- Allows toxicity studies of oil-soluble drugs in cell cultures [52 – 55].

6.7. Nanoemulsion formulations for improved oral delivery of poorly soluble drugs

Nanoemulsions formulation was developed to increase oral bioavailability of hydrophobic drugs. Paclitaxel was selected as a model hydrophobic drug. The o/w nanoemulsions were made with pine nut oil as the internal oil phase, water as the external phase and egg lecithin as the primary emulsifier. Stearylamine and deoxycholic acid were used to give positive and negative charge to the emulsions, respectively. The formulated nanoemulsions had a particle size range of 100-120 nm and zeta potential ranging from 34 mV to 245 mV. After oral administration of nanoemulsions, a significantly higher concentration of paclitaxel was observed in the systemic circulation compare to control aqueous solution. The results of this study suggest that Nanoemulsions are promising novel formulations which can promote the oral bioavailability of hydrophobic drugs [56-58,115].

6.8. Nanoemulsions in ocular and otic drug delivery

Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist [117]. It is a common knowledge that the application of eye drops as conventional ophthalmic delivery systems results in poor bioavailability and therapeutic response because of lacrimal secretion and nasolacrimal drainage in the eye [118,119]. Most of the drug is drained away from the precorneal area in few minutes. As a result, frequent instillation of concentrated solutions is needed to achieve the desired therapeutic effects [120]. But, by the tear drainage, the main part of the administered drug is transported via the nasolacrimal duct to the gastric intestinal tract where it may be absorbed, sometimes causing side effects [121]. In order to increase the effectiveness of the drug, a dosage form should be chosen which increases the contact time of the drug in the eye. This may then increase the bioavailability, reduce systemic absorption, and reduce the need for frequent administration

leading to improved patient compliance. Nanoemulsions could be employed to overcome some of these problems. Dilutable nanoemulsions are potent drug delivery vehicles for ophthalmic use due to their numerous advantages as sustained effect and high ability of drug penetration into the deeper layers of the ocular structure and the aqueous humor. Ammar *et al.* formulated the antiglaucoma drug dorzolamide hydrochloride as ocular nanoemulsion of high therapeutic efficacy and prolonged effect [122]. These nanoemulsions showed acceptable physicochemical properties and exhibited slow drug release. Draize rabbit eye irritation test and histological examination were carried out for those preparations exhibiting superior properties and revealed that they were nonirritant. Biological evaluation of dorzolamide hydrochloride nanoemulsions on normotensive albino rabbits indicated that these products had higher therapeutic efficacy, faster onset of action, and prolonged effect relative to either drug solution or the market product. It was concluded from the study that formulation of dorzolamide hydrochloride in a nanoemulsion form offered a more intensive treatment of glaucoma, a decrease in the number of applications per day, and a better patient compliance compared to conventional eye drops.

6.9. Nanoemulsions as a vehicle for transdermal delivery

Drug delivery through the skin to the systemic circulation is convenient for a number of clinical conditions due to which there has been a considerable interest in this area [123,124]. It offers the advantage of steady state controlled drug delivery over extended period of time, with self administration also being possible, which may not be the case with parenteral route. The drug input can be eliminated at any time by the patient just by removing the transdermal patch. Their transparent nature and fluidity, confers on nanoemulsions a pleasant skin feel. An extra advantage is the total absence of gastrointestinal side effects like irritation and bowel ulcers which are invariably associated with oral delivery. Transdermal drug products have been developed for a number of diseases and disorders including cardiovascular conditions, Parkinsons' and Alzheimer diseases, anxiety, depression, etc. However, the fundamental disadvantage which limits the use of this mode of administration is the barrier imposed by the skin for effective penetration of the bioactives. The three routes by which drugs can primarily penetrate the skin are through the hair follicles, sweat ducts or directly across stratum corneum, which restricts their absorption to a large extent and limits their bioavailability. For improved drug pharmacokinetics and targeting, the primary skin barriers need to be overcome. Also the locally applied drug redistribution through cutaneous blood and lymph vessel system needs to be controlled. Nano sized emulsions are able to easily penetrate the pores of the skin and reach the systemic circulation thus getting channelized for effective delivery [69]. Caffeine has been used for treatment of different types of cancer by oral delivery. Water-in-oil nanoemulsion formulations of caffeine have been developed for transdermal drug delivery. Comparison of *in vitro* skin permeation profile between these and aqueous caffeine solutions showed significant increase in permeability parameters for the nanoemulsion loaded drugs [125]. Use of nanoemulsions in transdermal drug delivery represents an important area of research in drug delivery, which enhances the therapeutic efficacy and also the bioavailability of the drugs without any adverse effects. It is also regarded as a promising technique with many advantages including high storage stability, low preparation cost, thermodynamic stability, absence of

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organic solvents, and good production feasibility. They have also made the plasma concentration profiles and bioavailability of drugs reproducible. These systems are being used currently to provide dermal and surface effects, and for deeper skin penetration [69]. Many studies have shown that nanoemulsion formulations possess improved trans-dermal and dermal delivery properties *in vitro* [66,126-133], as well as *in vivo* [134-136]. Nanoemulsions have improved transdermal permeation of many drugs over the conventional topical formulations such as emulsions and gels [137-141].

Barakat *et al* prepared nanoemulsions by the spontaneous emulsification method for transdermal delivery of indomathacin [142]. A significant increase in the permeability parameters such as steady-state flux, permeability coefficient, and enhancement ratio was observed in nanoemulsion formulations compared with the conventional indomethacin gel. The anti-inflammatory effects of nanoemulsion formulations showed a significant increase in percent inhibition value after 4 hours when compared with conventional indomethacin gel on carrageenan-induced paw edema in rats. Significant increase in permeability parameters was observed in nanoemulsion formulations ($P< 0.05$). The steady-state flux and permeability coefficient for optimized nanoemulsion formulation (were found to be 22.61±3.45 μg/ cm^2/h and $0.22 \times 10^{-2} \pm 0.0003$ cm/h, respectively), which were significant compared with conventional indomethacin gel ($P< 0.001$). Enhancement ratio was found to be 8.939 in optimized formulation compared with indomethacin gel. These results suggested that nanoemulsions can be used as potential vehicles for improved transdermal delivery of indomethacin as an approach to eliminate the side effect of the oral dose.

Singh *et al* developed nanoemulsion formulation for transdermal delivery of carvedilol to enhance the water solubility as well as bioavailability of drug [143]. O/W nanoemulsions were prepared by the spontaneous emulsification method. Post application plasma carvedilol was increased 6.41 fold to marketed dosage form. The study suggested that nanoemulsion significantly enhanced bioavailability of transdermally applied carvedilol and eliminated the first pass metabolism.

Sajid *et al* prepared betamethasone valerate nanoemulsions by aqueous phase titration method, using Sefsol, Tween 20, Transcutol P, and distilled water as the oil phase, surfactant, co surfactant and aqueous phase, respectively and evaluated them based on the induction of contact dermatitis in rats using a dispersion of nickel sulfate in solid vaseline at 5%, carrageenan induce inflammation and their irritation study [144]. The optimized nanoemulsion was converted into hydrogel using Carbopol 934. Drug deposition in skin was found to be 58.46 μg/cm2. *In vivo* anti-inflammatory activity indicated 84.2% and 45.05% inhibition of inflammation in case of developed nanoemulsion gel and marketed cream, respectively. The irritation score was found to be 1.83 which indicates that the optimized nanoemulsion did not cause any irritation. Results of nickel induced dermatitis demonstrate that the nanoemulsion formulation gel did not appear to stimulate an inflammatory or immune response using the contact dermatitis model.

Zhou *et al.* carried out a study to establish a lecithin nanoemulsion without any synthetic surfactant as a topical delivery vehicle and to evaluate its topical delivery potential [145]. Experimental results demonstrated that an increasing concentration of soybean lecithin and

glycerol resulted in a smaller size lecithin nanoemulsion droplet and increasing viscosity, respectively. Lecithin nanoemulsion, incorporated into o/w cream, improved the skin hydration capacity of the cream significantly with about 2.5-fold increase when the concentration of lecithin nanoemulsion reached 10%. Lecithin nanoemulsion was also demonstrated to improve the penetrability of Nile red dye into the dermis layer, when an o/w cream, incorporated with Nile-red-loaded lecithin nanoemulsion, applied on the abdominal skin of rat *in vivo*. Specifically, the arbitrary unit of fluorescence in the dermis layer that had received the cream with a Nile red-loaded lecithin nanoemulsion was about 9.9-fold higher than the cream with a Nile red-loaded general emulsion. These observations suggest that lecithin nanoemulsion could be used as a promising topical delivery vehicle for lipophilic compounds.

Modi *et al* investigated the potential of a nanoemulsion formulation for topical delivery of aceclofenac [146]. The *in vitro* skin permeation profile of optimized formulations was compared with that of aceclofenac conventional gel and nanoemulsion gel. A significant increase in permeability parameters such as steady-state flux, permeability coefficient and enhancement ratio was observed in optimized nanoemulsion formulation consisting of 2 % w/w of aceclofenac, 10 % w/w of Labrafac, 45% w/w surfactant mixture (Cremophor® EL: Ethanol), and 43 % w/w of distilled water. The anti-inflammatory effects of formulation showed a significant increased percent inhibition value after 24 hours when compared with aceclofenac conventional gel and nanoemulsion gel on carrageenan-induced paw edema in rats. These results suggested that nanoemulsions are potential vehicles for improved transdermal delivery of aceclofenac.

Batoota *et al* investigated the potential of nanoemulsion formulations for transdermal delivery of celecoxib [91]. The *in vitro* skin permeation profile of optimized formulations was compared with celecoxib gel and nanoemulsion gel. Significant increase in the steady state flux, permeability coefficient and enhancement ratio was observed in nanoemulsion formulations ($p < 0.05$). The highest value of these permeability parameters was obtained in the formulation that consisted of 2 % (w/w) of celecoxib, 10 % (w/w) of oil phase (Sefsol 218 and Triacetin), 50 % (w/w) of surfactant mixture (Tween-80 and Transcutol-P) and 40 % (w/w) water. The anti-inflammatory effects of the formulation showed a significant increase ($p < 0.05$) in inhibition after 24 hours compared to celecoxib gel and nanoemulsion gel on carrageenan-induced paw edema in rats. These results suggested that nanoemulsions are potential vehicles for improved transdermal delivery of celecoxib.

Harwansh *et al* evaluated an isotropic and thermodynamically stable nanoemulsion formulation for transdermal delivery of glycyrrhizin, with minimum surfactant and cosurfactant concentrations that could improve its solubility, permeation enhancement, and stability [147]. A significant increase in permeability parameters such as steady-state flux and permeability coefficient was observed in the optimized nanoemulsion formulation, which consisted of 1 % w/w of mono ammonium glycyrrhizinate, 32.4 % Span 80, 3.7 % Brij 35, 10 % isopropyl alcohol, 46.5 % soyabean oil and 6.4 % distilled water. No obvious skin irritation was observed for the studied nanoemulsion formulation or the gel. The results indicated that nanoemulsions are promising vehicles for transdermal delivery of glycyrrhizin through human cadaver skin,

without the use of additional permeation enhancers, because excipients of nanoemulsions act as permeation enhancers themselves.

Inayat *et al.* developed a potential of nanoemulsion formulation for transdermal delivery of tamoxifene citrate for breast cancer [148]. Transdermal permeation of tamoxifene citrate through rat skin was determined by Keshary-Chien diffusion cell. A significant increase in permeability parameter such as steady–state flux was observed in optimized nanoemulsion formulation, which consist of 5 % w/w of drug, 4.12 % w/w of oil phase, 37.15 % w/w of surfactant (mix) and 58.73 % w/w of distilled water. It possessed a mean globule size of 68 nm. Transmission electron microscopy demonstrated spherical particle morphology and DSC and FTIR study revealed the compatibility among the ingredient. These results proposed that the prepared system could be promising to improve transdermal efficacy of the tamoxifen citrate.

Shakeel *et al* investigated the potential of a nanoemulsion formulation for transdermal delivery of aceclofenac [149]. Transdermal permeation of aceclofenac through rat abdominal skin was determined by Franz diffusion cell. The *in vitro* skin permeation profile of optimized formulations was compared with that of aceclofenac conventional gel and nanoemulsion gel. A significant increase in permeability parameters such as steady-state flux, permeability coefficient, and enhancement ratio was observed in optimized nanoemulsion formulation, which consisted of 2 % w/w of aceclofenac, 10% w/w of Labrafil R, 5 % w/w of Triacetin R, 35.33 % w/w of Tween 80 R, 17.66 % w/w of Transcutol PR, and 32 % w/w of distilled water. The anti-inflammatory effects of optimized formulation showed a significant increase in percent inhibition value after 24 hours when compared with aceclofenac conventional gel and nanoemulsion gel on carrageenan-induced paw edema in rats. These results suggested that nanoemulsions are potential vehicles for improved transdermal delivery of aceclofenac.

Shakeel *et al* presented an overview of the efforts that have been made in the last decade by various researchers in exploring new types of nanoemulsion-based drug delivery system for dermal and transdermal delivery of many hydrophobic compounds [150]. This area of research would be very advantageous for formulation scientists in order to develop some nanoemulsion-based formulations for their commercial exploitation and clinical applications. Moreover, Harwansh *et al* reviewed efforts made by various researchers in the delivery of phytopharmaceuticals using nanoemulsions [151].

6.10. Nanoemulsion in cancer therapy and in targeted drug delivery

Another interesting application, which is experiencing an active development, is the use of nanoemulsion formulations, for controlled drug delivery and targeting [82]. Because of their submicron size, they can easily be targeted to the tumor area. Although nanoemulsions are chiefly seen as vehicles for administering aqueous insoluble drugs, they have more recently received increasing attention as colloidal carriers for targeted delivery of various anticancer drugs, photosensitizers, neutron capture therapy agents, or diagnostic agents. The development of magnetic nanoemulsions is an innovative approach for cancer therapy. These can deliver photosensitizers like Foscan® to deep tissue layers across the skin thereby inducing hyperthermia for subsequent free radical generation. This methodology can be used for the treatment of cancer in the form of photodynamic therapy [152].

6.11. Nanoemulsions and intranasal drug delivery

Intranasal drug delivery system has now been recognized as a reliable route for the administration of drugs next to parenteral and oral routes. Nasal mucosa has emerged as a therapeutically viable channel for the administration of systemic drugs and also appears to be a favourable way to overcome the obstacles for the direct entry of drugs to the target site [153]. This route is also painless, non-invasive and well tolerated. The nasal cavity is one of the most efficient sites because of its reduced enzymatic activity, high availability of immunoactive sites and its moderately permeable epithelium [154]. There are several problems associated with targeting drugs to brain, especially the hydrophilic ones and those of high molecular weight. This is because of the impervious nature of the endothelium, which divides the systemic circulation and barrier between the blood and brain [155]. The olfactory region of the nasal mucosa provides a direct connection between the nose and brain, and by the use of nanoemulsions loaded with drugs, conditions such as Alzheimer's disease, migraine, depression, schizophrenia, Parkinson's diseases, meningitis, etc. can be treated [156,157]. Preparation of nanoemulsions containing risperidone for its delivery to the brain via nose has been reported [158]. It is inferred that this emulsion is more effective through the nasal rather than intravenous route. Another application of intranasal drug delivery system in therapeutics is their use in development of vaccines. Immunity is achieved by the administration of mucosal antigen. Currently, the first intranasal vaccine has been marketed [158]. Among the possible delivery systems, the use of nano based carriers hold a great promise to protect the biomolecules, promote nanocarrier interaction with mucosae and to direct antigen to the lymphoid tissues. Therefore the use of nanoemulsions in intranasal drug delivery system is set to bring about significant results in targeting drugs to the brain in treatment of diseases related to the central nervous system [159]. Bhanushali *et al* developed intranasal nanoemulsion and gel formulations for rizatriptan benzoate for prolonged action [160]. Various mucoadhesive agents were tried out to form thermo-triggered mucoadhesive nanoemulsions. Mucoadhesive gel formulations of rizatriptan were prepared using different ratios of HPMC and Carbopol 980. Comparative evaluation of intranasal nanoemulsions and intranasal mucoadhesive gels indicated that greater brain-targeting could be achieved with nanoemulsions. Other drugs which have been formulated for nasal delivery are insulin and testosterone [161].

6.12. Nanoemulsions and parenteral drug delivery

This is one of the most common and effective routes of drug administration usually adopted for actives with low bioavailability and narrow therapeutic index. Their capacity to dissolve large quantities of hydrophobics, together with their mutual compatibility and ability to protect the drugs from hydrolysis and enzymatic degradation make nanoemulsions ideal vehicles for the purpose of parenteral transport. Further, the frequency and dosage of injections can be reduced throughout the drug therapy period as these emulsions guarantee the release of drugs in a sustained and controlled mode over long periods of time. Additionally, the lack of flocculation, sedimentation and creaming, combined with a large surface area and free energy, offer obvious advantages over emulsions of larger particle size, for this route of administration [69]. Their very large interfacial area positively influences the drug transport

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and their delivery, along with targeting them to specific sites. Major clinical and pre-clinical trials have hence been carried out with parenteral nanoemulsion based carriers. The advances in these novel drug delivery systems have been reviewed by Patel and Patel [162]. Nanoemulsions loaded with thalidomide have been synthesized where a dose as low as 25 mg leads to plasma concentrations which can be therapeutic [163]. However, a significant decrease in the drug content of the nanoemulsion was observed at 0.01% drug formulation after two months storage which could be overcome by the addition of polysorbate 80. Chlorambucil, a lipophilic anticancer agent has been used against breast and ovarian cancer. Its pharmacokinetics and anticancer activity has been studied by loading it in parenteral emulsions prepared by high energy ultrasonication method. Treatment of colon adenocarcinoma in the mouse with this nanoemulsion leads to higher tumor suppression rate compared to plain drug solution treatment, concluding that the drug loaded emulsion could be an effective carrier for its delivery in cancer treatment [164]. Carbamazepine, a widely used anticonvulsant drug had no parenteral treatment available for patients due to its poor water solubility. Kelmann *et al*. have developed a nanoemulsion for its intravenous delivery, which showed favorable *in vitro* release kinetics [165]. Parenteral nanoemulsion formulations of the following drugs have been documented as well: diazepam, propofol, dexamethasone, etomidate, flurbiprofen and prostaglandin E1 [166].

The high lipophilicity of diazepam (an anxiolytic and sedative) makes the use of solvents (such as propylene glycol phenyl carbinol and ethanol) for the dissolution of the drug in conventional aqueous preparations (Valium® and Stesolid®) necessary, leading to pain and thrombophlebitis on the patient during the injection. The development of a nanoemulsion, commercially available under the name of Diazemuls® (Kabi-Pharmacia) allows for the reduction of these adverse effects, keeping stages of distribution and elimination similar to Valium®. However, higher doses of Diazemuls® are necessary to obtain the same effect as Valium® since this leads to higher free fraction of plasma diazepam [167,168].

The solution for intravenous administration of etomidate (hypnotic short) due to stability problems, its composition contains 35 % propylene glycol (Hypnomidate®) [169,170]. Due to the presence of high osmolarity of the solvent, the administration is associated with various adverse effects such as hemolysis, thrombosis, thrombophlebitis and pain at the site of application [171,172]. A nanoemulsion containing 2 mg/ml Lipofundin® etomidate in medium chain triglyceride named Lipuro-etomidate® (B. Braun) was developed [173]. The emulsion allowed the reduction of the hemolytic and venous sequelae, besides the pain at the time of application [169-171].

The pharmacokinetics and pharmacodynamics of propofol (anesthetic) are complex. It has an initial rapid distribution of about 2-3 minutes, with high variability between patients and reduced concentrations to subtherapeutic levels within minutes. However, due to its high lipophilicity, it has a high volume of distribution and its complete elimination from the body can take days [174]. Due to the occurrence of anaphylactic effects associated with Cremophor EL, present in the original formulation of propofol nanoemulsion as vehicle for this drug-containing composition in soybean oil, glycerol, egg yolk lecithin and disodium edentate, this vehicle helped to reduce the volume of distribution of the drug, accelerating their processes of

clearance by the responsible agencies. This formulation also allowed the use of minimal effective dose need to produce the needed therapeutic effect, allowing a rapid onset and recovery from anesthesia, when compared to a non-lipid (ethanol) solution, thereby generating greater security administration, due to the lower continuous accumulation of the drug, and eliminating the need for constant adjustment of the dose. This product was approved in 1989 in the United States, under the name of Diprivan® 1 or 2 % (AstraZeneca / APP Pharmaceuticals) [175]. In Brazil, the product is available as Lipuro 1% (B. Braun) and Diprivan® 1 and 2% (AstraZeneca), besides the generic 1% (Eurofarma Labs.) [176]. The various generic formulations currently available are constituted by an additional factor of variability in response between individuals in the induction of anesthesia, apart from the pharmacokinetic characteristics of the drug itself [174] and the differences in lipoprotein profile of each patient, due to the high binding of propofol to low density lipoprotein and albumin [177]. Due to related pain at the injection site and increased triglyceride levels after administration for long periods, some changes in the formulation of Diprivan® adverse effects have been proposed, including some already being marketed as Propofol® Lipuro (B. Braun) as oil core which contains a mixture of oils [178]. The addition of more oil to the formulation allowed the reduction of pain on injection due to increased incorporation of the drug in the oily core and the lower amount of free propofol phase the external aqueous emulsion [171,177-180]. Alternative formulations have been developed, for example, the incorporation of higher concentrations of propofol (6 %) in the nanoemulsion [180-182], or the development of a propofol prodrug in solution (Aquavan®) [183].

Furthermore, despite the excellent anti-inflammatory activity of dexamethasone, the clinical use of corticosteroids is limited by numerous side effects [184,185]. To circumvent these drawbacks, lipophilic prodrugs in the body that are gradually hydrolyzed to the active metabolite can be used (thus presenting prolonged anti-inflammatory effect). The advantage is the use of lower doses than those used in conventional water soluble form (dexamethasone phosphate), reducing the risks of adverse effects. Considering that nanoemulsions are picked up by inflammatory cells of the mononuclear phagocytic system, nanoemulsions were used as a vehicle for lipophilic prodrug of dexamethasone (palmitate), which is commercially available as Limethason® (Green Cross Co./Mitsubishi Tanabe Pharma Co.). Limethason® showed excellent results in the treatment of rheumatoid arthritis, West syndrome, inflammatory diseases and other autoimmune diseases. While the solution of dexamethasone phosphate is rapidly distributed in water-rich tissues, such as muscles, the nanoemulsion is accumulated mainly in tissues inflamed organs such as liver and spleen. The biodistribution profile is different even if the elimination pattern is similar between the two. Limethason® removes over 80 % of the phagocytic activity of macrophages at a concentration of 0.03 mg/mL [185].

Flurbiprofen (non-steroidal anti-inflammatory oral use), a lipophilic drug, is used to treat rheumatoid arthritis and other inflammatory diseases associated or not with cancer [186]. The non-availability of oral and/or various gastrointestinal effects caused by this drug often require the use of parenteral route. Considering the severe local irritation caused by the sodium salt of flurbiprofen, it was developed as a prodrug of flurbiprofen (cefuroxime) and because of the lipophilicity of the latter especially in soybean oil, it was incorporated in nanoemulsions for parenteral use (Ropion®, Kaken Pharmaceuticals Co., Lipfen®, GreenCross Co.), and is com-

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mercially available in the Japanese market since 1992. Administration of Ropion® resulted in an increase in area under the concentration-time curve and reduced clearance when compared to the solution. The incorporation of the drug into nanoemulsions containing unesterified ethyl oleate, lecithin and modified egg yolk led to a lower drug accumulation in organs such as the liver and spleen due to the lower uptake by the mononuclear phagocyte system [166].

Prostaglandin E1, which is synthesized in several places of the body, is responsible for various physiological effects such as vasodilatation, lowering of blood pressure, angiogenesis and inhibition of platelet aggregation [187,188]. When administered for the treatment of various diseases, it has a short half-life; high doses are needed, leading to numerous adverse effects such as hypotension, diarrhea, local irritation and pain [187]. In this context, nanoemulsions were made commercially available in 1975, PGE1 complexed to cyclodextrins and, in 1985, prostaglandin E1 incorporated in lipid nanoemulsions (Liple®, Mitsubishi Tanabe Pharma Corporation, Palux®, Taisho Pharmaceutical) [189]. Lipid formulations are used to treat cardiovascular diseases because they accumulate in the walls of injured vessels, transporting the drug to the site of vascular injury and to protect it from rapid inactivation by the lungs [187-190].

6.13. Nanoemulsions and pulmonary drug delivery

The lung is an attractive target for drug delivery due to noninvasive administration via inhalation aerosols, avoidance of first-pass metabolism, direct delivery to the site of action for the treatment of respiratory diseases, and the availability of a huge surface area for local drug action and systemic absorption of drug. Colloidal carriers (ie, nanocarrier systems) in pulmonary drug delivery offer many advantages such as the potential to achieve relatively uniform distribution of drug dose among the alveoli, achievement of improved solubility of the drug from its own aqueous solubility, a sustained drug release which consequently reduces dosing frequency, improves patient compliance, decreases incidence of side effects, and the potential of drug internalization by cells [191]. Until now, the submicron emulsion system has not yet been fully exploited for pulmonary drug delivery and very little has been published in this area [191]. Bivas-Benita *et al.* reported that cationic submicron emulsions are promising carriers for deoxyribonucleic acid vaccines to the lung since they are able to transfect pulmonary epithelial cells, which possibly induce cross priming of antigen-presenting cells and directly activate dendritic cells, resulting in stimulation of antigen-specific T-cells [192]. Therefore the nebulization of submicron emulsions will be a new and upcoming research area. However, extensive studies are required for the successful formulation of inhalable submicron emulsions due to possible adverse effects of surfactants and oils on lung alveoli function (adverse interactions with lung surfactant). A novel pressurized aerosol system has been devised for the pulmonary delivery of salbutamol using lecithin-stabilized microemulsions formulated in trichlorotrifluoroethane [193].

6.14. Nanoemulsions as gene delivery vector

Emulsion systems have been introduced as alternative gene transfer vectors to liposomes [194]. Other emulsion studies for gene delivery (non-pulmonary route) have shown that binding of the emulsion/DNA complex was stronger than liposomal carriers [195]. This stable emulsion

system delivered genes more efficiently than liposomes [196]. Silva *et al* evaluated factors that influence DNA compaction in cationic lipid nanoemulsions [cationic nanoemulsions containing stearylamine (a cationic lipid that presents a primary amine group when in solution, is able to compact genetic material by electrostatic interactions, and in dispersed systems such as nanoemulsions this lipid anchors on the oil/water interface conferring a positive charge to them)] [197]. The influence of the stearylamine incorporation phase (water or oil), time of complexation, and different incubation temperatures were studied. The complexation rate was assessed by electrophoresis migration on agarose gel 0.7%, and nanoemulsion and lipoplex characterization was done by dynamic light scattering (DLS). The results demonstrate that the best DNA compaction process occurs after 120 min of complexation, at low temperature (4 ± 1 °C), and after incorporation of the cationic lipid into the aqueous phase. Although the zeta potential of lipoplexes was lower than the results found for basic nanoemulsions, the granulometry did not change. Moreover, it was demonstrated that lipoplexes are suitable vehicles for gene delivery.

7. Nanoemulsions for phytopharmaceuticals

Recently, considerable attention has been focused on the development of novel drug delivery systems for herbal drugs [198]. However some limitations of plant bioactives like instability in highly acidic pH and liver metabolism led to drug levels below therapeutic concentration in the blood resulting in less or no therapeutic effect [199]. Hence, encapsulation of plant extracts or its bioactives would minimize their degradation or presystemic metabolism, and serious side effects due to accumulation of drugs to the non-targeted areas and improves the ease of administration in the pediatric and geriatric patients [200]. Lipid nanoemulsions containing oil from medicinal plants or hydrophobic drugs have been shown to improve drug solubility, reduce side effects of various potent drugs, increase the bioavailability of drugs, and to prolong the pharmacological effects in comparison to conventional formulations such as conventional emulsions [201]. Formulation of nanoemulsions containing phytoactives have been reported.

The effect of nanoemulsion on intestinal absorption of colchicine was demonstrated *in vivo*. Colchicine nanoemulsion was prepared with isopropyl myristate, eugenol, Tween 80, ethanol and water, with eugenol being the oil phase in the formulation. Result obtained indicated that the intestinal absorption of colchicine was significantly enhanced by the nanoemulsion formulation [202]. Genistein has been shown to possess anticancer activities in different experimental systems, yet the same effects could not be translated in the clinical setting due to its poor bioavailability. Researcher have tried various nano approaches including incorporation of genistein into topical nanoemulsion formulations composed of egg lecithin, medium chain triglycerides or octyldodecanol and water by spontaneous emulsification with improved activity [203]. Oil in water nanoemulsion formulation has also demonstrated increased anti-inflammatory activity of curcumin [204].

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8. Future perspectives

Nanoemulsions are proposed for numerous applications in pharmacy as drug delivery systems because of their capacity to solubilize non-polar active compounds. Future perspectives of nanoemulsion are very promising in different fields of therapeutics or application in development of cosmetics for hair or skin. One of the versatile applications of nanoemulsions is in the area of drug delivery where they act as efficient carriers for bioactives, facilitating administration by various routes. The advantages and applications of nanoemulsions for oral drug delivery are numerous, where the droplet size is related to their absorption in the gastrointestinal tract. Due to the renewed interest in herbal drug formulation, nanoemulsion may be the ideal delivery platform for these difficult-to-formulate phytopharmaceuticals. The prospects of nanoemulsions lie in the ingenuity of formulation experts to utilize the advantages of nanoemulsion carriers in overcoming peculiar problems of drug delivery such as absorption, permeation and stability of both orthodox and herbal drugs.

9. Conclusion

Nanoemulsions offer several advantages for the delivery of drugs and are thus receiving increasing attention as drug carriers for improving the delivery of active pharmaceutical ingredients. They are applicable for almost all routes of delivery and therefore hold promise for different fields, be it cosmetics, therapeutics or biotechnology. This new technology could be developed to overcome the poor absorption of some phytopharmaceuticals and poor miscibility of these compounds with the lipid contents of cell membrane linings.

Author details

S.A. Chime[1], F.C. Kenechukwu[2] and A.A. Attama[2*]

*Address all correspondence to: anthony.attama@unn.edu.ng

1 Department of Pharmaceutical Technology and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria

2 Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria

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Chapter 4

Application of Nanotechnology in Drug Delivery

Joana Silva, Alexandra R. Fernandes and
Pedro V. Baptista

Additional information is available at the end of the chapter

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1. Introduction

1.1. Nanomedicine for cancer

Cancer is one of the leading causes of death worldwide, occupying the second place in developing countries, and showing a growing incidence over time [1]. Current cancer therapy strategies are based in surgery, radiotherapy and chemotherapy, being the chemotherapy the one that shows the greater efficiency for cancer treatment, mainly in more advanced stages [2, 3]. Despite of this great response, anticancer agents are administrated at higher amounts in order to provide a final suitable concentration to the target tissues or organs, and this procedure is repeated in each cycle of chemotherapy [4]. Introduction of new agents to cancer therapy has greatly improved patient survival but still there are several biological barriers that antagonize drug delivery to target cells and tissues, namely unfavorable blood half-life and physiologic behavior with high off-target effects and effective clearance from the human organism [2, 5, 6]. Moreover, in cancer, there is a small subset of cancer cells-cancer stem cells (CSC)-that, like normal stem cells, can self-renew, give rise to heterogeneous populations of daughter cells, and proliferate extensively [7, 8]. Standard chemotherapy is directed against rapidly dividing cells, the bulk of non-stem cells of a tumor, and thus CSC often appear relatively refractory to those agents [7-9]. The development of side effects in normal tissues (e.g. nephrotoxicity, neurotoxicity, cardiotoxicity, etc) and multidrug resistance (MDR) mechanisms by cancer cells leads to a reduction in drug concentration at target location, a poor accumulation in the tumor with consequent reduction of efficacy that may associate to patient relapse [9-13]. To overcome these issues and still improve the efficiency of chemotherapeutic agents there is a demand for less toxic and more target specific therapies towards cancer cells, i.e. novel drugs, drug delivery systems (DDSs) and also gene delivery systems [3, 4, 14-17].

Nanotechnology is the manipulation of matter on an atomic, molecular, and supramolecular scale involving the design, production, characterization and application of different nanoscale materials in several key areas providing novel technological advances mainly in the field of medicine (so called Nanomedicine) [6, 18-20]. The development and optimization of drug delivery approaches based in nanoparticles concerns the early detection of cancer cells and/or specific tumor biomarkers, and the enhancement of the efficacy of the treatments applied [21]. The most important biomedical applications of nanoscale materials can be organized as shown in Figure 1.

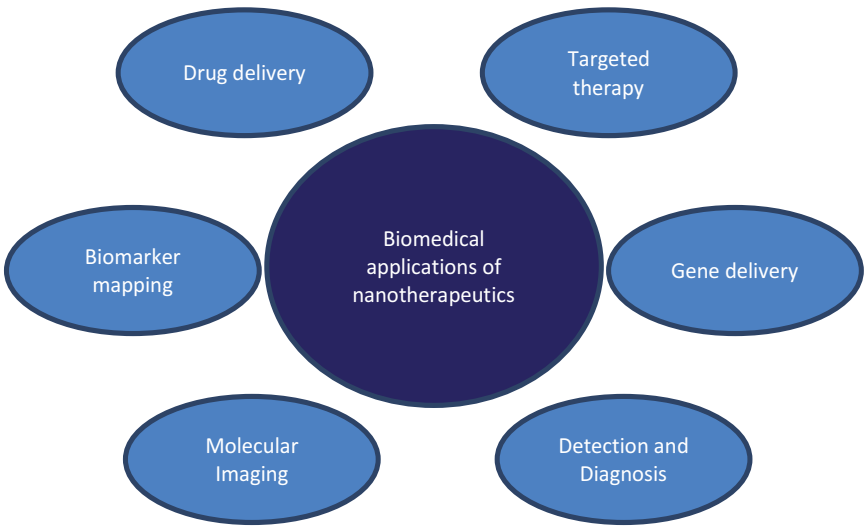

Figure 1. Biomedical application of nanotherapeutics (adapted from [6]).

These nanotherapeutics' potential in cancer relies on i) passive targeting due to the enhance of the permeability and retention (EPR) effect promoted by angiogenic vessels with defective vasculature and improper lymphatic flow surrounding the tumor [18] that can be reinforced by ii) specific targeting based on multifunctional nanomaterials that bypass the biological barriers and reach cancer cells [4]. Nanotechnology for drug vectorization provides for new and more specific drug targeting and delivery platforms that can reduce toxicity and other side effects and also maintain or improve the therapeutic index [9, 22, 23]. In fact, the development of targeting delivery systems is the ultimate goal in cancer therapy, which has been taking the lead in what concerns overcoming the MDR problem [9, 13, 24, 25].

Here, we will discuss recent applications on AuNPs as platforms for anticancer therapy, emphasizing strategies for targeted delivery for gene silencing focusing on the optimal pathways to test these therapeutics *in vitro* and *in vivo*. Also, an overview of the toxicological aspects of these materials will be provided.

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2. Nanoparticles as delivery systems

Nanoparticles have been developed as effective target specific strategies for cancer treatment, acting as nanocarriers and also as active agents [4, 6, 5, 26]. Over the last decades, different types of nanoparticles have been developed based on various components, including carbon, silica oxides, metal oxides, nanocrystals, lipids, polymers, dendrimers, and quantum dots, together with increasing variety of newly developed materials [4, 27-34]. These nanomaterials are capable to provide a high degree of biocompatibility before and after conjugation to biomolecules for specific function so as to translate into nanomedicines and clinical practice. Nanomaterials provide for a favorable blood half-life and physiologic behavior with minimal off-target effects, effective clearance from the human organism, and minimal or no toxicity to healthy tissues in living organisms [35, 36].

In fact, the protection from adsorption to plasma proteins and/or degradation by circulating nucleases allows for an increased availability of effector molecule at site of interest. This is further enhanced by the considerable decrease to clearance from the organism that conjugation to nanoparticles confers. The modulation of pharmacokinetic and pharmacodynamics parameter constitutes a key factor when modifying the mode of administration (and vehicle and route of administration associated) that is usually neglected when compared to the ability of therapeutic nanoconjugates to offer the possibility of enhanced targeting (active and/or passive) and cell uptake. When considering nanoparticles for therapeutics one should also evaluate the effect on cellular metabolism and fate that can be attained via optimal conjugation with (bio)molecules of interest.

DDSs can improve the properties of free drugs by increase their *in vivo* stability and biodistribution, solubility and even by modulation of pharmacokinetics, promoting the transport and even more important the release of higher doses of the drug in the target site in order to be efficient [18, 22, 37, 38].

DDSs can be constructed by direct conjugation with the drugs and further surface modifications can lead to a better delivery for such systems, promoting a targeted delivery to specific types of cells and reaching cell compartments such as nucleus and mitochondria [15, 39]. As far as drug delivery is concerned, the most important nanoparticle platforms are liposomes, polymer conjugates, metallic nanoparticles (for example AuNPs), polymeric micelles, dendrimers, nanoshells, and protein and nucleic acid-based nanoparticles (for a more complete review see [40-42].

Among a wide variety of nanosystems, only a few nanomedicines, such as Doxil® (Janssen Biotech Inc., Horsham, PA, USA), DaunoXome® (Galen US Inc., Souderton, PA, USA), Depocyt® (Pacira Pharmaceuticals Inc., San Diego, CA, USA), Genexol-PM® (Samyang Biopharmaceuticals Corporation, Jongno-gu, Seoul, Korea), Abraxane® (Celgene Corporation, Inc., Berkeley Heights, NJ, USA), Myocet® (Sopherion Therapeutics Inc., Princeton, NJ, USA) and Oncaspar® (Enzon Pharmaceuticals Inc., Bridgewater, NJ, USA), are approved for use in the treatment of cancer (for a review see [6]).

The implementation of nanoparticles towards cancer treatment can be based in certain characteristics as their size, surface properties and the possibility of a variety of specific ligands in their surface [18]. The high surface properties and other physicochemical features of nanoparticles can be modulated for the development of valuable systems that detect tumor cells either qualitatively or quantitatively [10, 19].

Targeting the cancer cells occurs via two different strategies: passive targeting and active targeting [4, 43, 44]. The passive targeting of tumor cells by nanoparticles depends upon an EPR effect promoted by angiogenic vessels with defective vasculature and improper lymphatic flow, reaching a higher accumulation in tumor cells compared to normal cells [15]. The increased accumulation of a drug in the tumor *interstitium* achieved by nanoparticles can be more than ten times higher compared to the drug alone [4]. This type of deliver is based in nanoparticle's half-time of circulation on the bloodstream, size and surface properties, and even depends on the degree of angiogenesis [45]. Despite the increased drug accumulation inside the tumor, this strategy rise some concerns about the targeting specificity of such mechanism based in the controversial influence of the EPR effect on drug externalization, which promotes a widespread distribution all over the tumor [4, 46]. The lack of specificity of such targeting led to further innovation with the implementation of an active targeting, which is achieved by the functionalization of nanoparticle's surface with a plethora of functional moieties such as antibodies and other biomolecules that recognized the specific surface antigens or specific biomarker of tumor cells [4, 44]. The targets choice depends on its high abundance in cell surface and its unique expression, and consequently the capacity of internalization of the nanoconjugate [4, 47, 48]. Although it is considered that active targeting does not have a direct association to the total nanoparticles accumulated within the tumor, it will influence the uptake of nanoparticles via receptor-mediated internalization and improve the efficiency of anti-tumor agents that have intracellular targets [49, 50]. Active targeting can be the potential way of polymeric nanoparticles to deliver chemotherapeutic drugs to cancer cells and is, therefore, one of the main vectors of DDS development at present involving tailoring of nanoparticles to deliver the effective cargo without compromising the selective targeting.

3. Gold Nanoparticles (AuNPs)

Metallic nanostructures are more flexible particles compared to other nanomaterials owed to the possibility of controlling the size, shape, structure, composition, assembly, encapsulation and tunable optical properties [51, 52]. Between the metallic nanostructures possible applied, AuNPs appears of great interest in the medical field, 3showing great efficiency towards cancer therapy [51-54]. The continuous interest in AuNPs is based in their tunable optical properties that can be controlled and modulated for the treatment and diagnosis of diseases [9, 54, 52].

3.1. Synthesis, functionalization, characterization and properties of AuNPs

The synthesis of nanoparticles follows some aspects relying in a high homogeneity of the materials in physical properties that greatly influence the size, shape and surface characteris-

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tics. The main process for nanoparticles development requires chemical administration of capping agents that adsorb in the surface of nanoparticles ([55] and references therein). AuNPs can be synthesized with different sizes through the reduction of gold with different agents such molecules bearing a thiol group, an aliphatic chain and a charged end group, and that can avoid particle aggregation [37]. Furthermore, this dense layer of stabilizing agent promotes a general change in the surface charge of AuNPs allowing ligand exchange with several molecules, promoting AuNPs functionalisation and then an increase in particle stability in physiological environments [55, 56]. AuNPs deliver systems can be formulated based in their capacity to bearing different functional groups, once it can be involved in covalent and non-covalent bindings by a thiol-linker [37, 55]. In fact, robust AuNPs appear by the stabilization with thiolates once the bond between Au and the thiol (S) is very strong [57]. This process enhances the affinity of the AuNPs surface for several types of ligands such as polyethylene glycol (PEG) molecules, nucleic acids (DNA and RNA), peptides, antibodies, and also small drug molecules (Figure 2) [9, 13, 37, 47, 52, 56, 57].

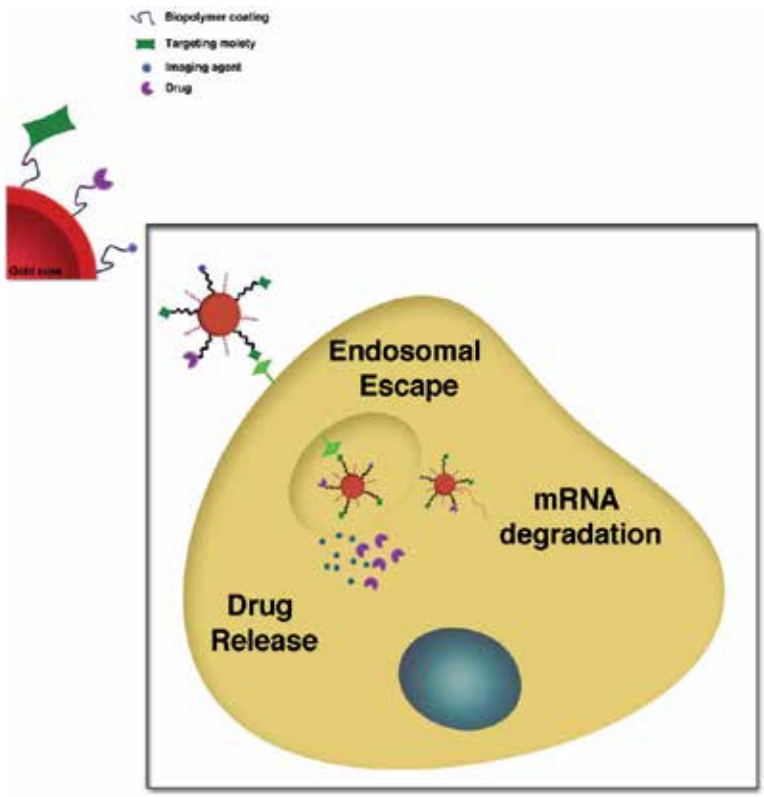

Figure 2. Multifunctional NP-based systems for tumor targeting, delivery and imaging. These innovative NPs comprise a targeting moiety, a silencing moiety and anticancer drug molecules for delivery to the target tissue. Depending on the targeting mechanism, they can be on the surface or inside the NPs. Multifunctional systems can carry reporter molecules tethered to the particle surface and employed as tracking and/or contrast agents.

Most passive targeting AuNPs have a surface coated with PEG for biocompatibility and "stealth" purposes [58]. Importantly, it should be noted that increased hydrophilicity on the AuNPs surface can impede its uptake by cancer cells, thereby hampering efficient drug delivery to tumors by passive targeting nanoparticles [58, 59].

As far as the targeting approach is concerned, one key issue relies on the choice of optimal targeting ligands, possibly by balancing their stoichiometry in comparison with the antibiofouling surface of AuNPs. More specifically, two important ligand properties, ie, affinity and density, can have a key role in effective targeting of nanoparticles to the cell surface membrane. Again, the ligand binding affinity is the result of the equilibrium between enthalpic advantages (for ligand-receptor interaction) and entropic losses (stretching, flexibility, or compressibility of the nanosystem). For example, greater ligand density does not necessarily lead to a higher intracellular concentration, given the decrease in "stealth" surface characteristics. Moreover, although the uptake of AuNPs usually increases with an increasing+/− charge ratio of nanoparticles (in terms of zeta potential values), an excess positive charge can induce toxicity and promote an immunologic reaction. Therefore, the optimal ligand density and charge on the AuNPs surface should be investigated on a case-by-case basis. AuNPs can be incorporated into larger structures such as polymeric nanoparticles or liposomes that deliver large payloads for enhanced diagnostic applications, efficiently encapsulate drugs for concurrent therapy or add additional imaging labels. This array of features has led to their application in biomedical fields, but more recently in approaches where multifunctional gold nanoparticles are used for multiple methods, such as concurrent diagnosis and therapy, so-called theranostics [53, 60-63].

AuNPs characterization is based on UV-Vis spectroscopy for the determination of the surface plasmon resonance (SPR) of the metallic gold, Transmission Electron Microscopy (TEM) for the determination of the average size of the particles, Scanning Electron Microscopy (SEM) for the characterization of the morphological features and Atomic Absorption Spectrometry that quantify the amount of gold [64]. AuNPs biodistribution can be monitored before the delivery of its payload which allows the establishment of treatment plan [65].

The application of AuNPs for *in vitro* diagnosis, *in vivo* imaging, therapy and also as DDSs relies in their chemical stability, high solubility in water, suitable morphology and limited dispersiiity, high surface-to-volume ratio, non-toxicity in biologic systems and an easy synthesis and functionalisation with a plethora of biomolecules (targeting and also silencing moieties) and drugs (Figure 2) [19, 21, 55, 56, 66-68].

3.2. AuNPs in cancer therapy

3.2.1. Photothermal therapy

AuNPs formulations gain a major impact in cancer therapy in different contexts based in their properties that gain particular interest given some cancer specificities. AuNPs presents tunable optical properties that allow the absorption of light at near UV to near infrared, being the last one a characteristic that allows nanoparticles to enter cells, constituting a major breakthrough for its application in photothermal therapy or hyperthermia [57, 69]. This is thought due to the fact that increasing temperature of the cells above 42ºC lead to a loss of cell viability [5]. Thus,

nanoparticles heat up after irradiation of the body or local area with a magnetic field or another source of energy and consequently induce an increase in cancer cells temperature until cell death [5]. Several gold nanostructures are being referred as successful candidates as photothermal agents, such as the case described by Sirotkina and coworkers where AuNPs reach a high concentration in the skin tumor tissue and lead to an apoptotic response [70]. AuNPs compared to the simple irradiation method, the laser hyperthermia (a methodology though to circumvent the side effects associated to the current cancer therapies), has an advantageous of needing less irradiation energy to promote tumor ablation [57].

3.2.2. Radiotherapy

AuNPs have been review in radiotherapy experiments in order to overcome the problems associated to the healthy tissue damage imposed by radiotherapy [5, 57]. This strategy is based in the well-known accumulation of AuNPs in the tumor that will be acting as a decoy to focus the radiation in the tumor and limit its action in normal tumor vicinity, being able to decrease the initial quantity of radiation administrated [5, 71, 72]. A long term study using AuNPs and irradiation in mices bearing implanted tumors in order to eliminate the possibility of tumor regression, results in a reduction of the tumor size until not be detected and 86% long term cure, i.e. for more than a year, which was much higher than the 20% survival for the implementation of just radiotherapy [65].

3.2.3. Angiogenesis inhibition

The inhibition of angiogenesis, i.e. the formation process of new blood vessels, is also a potent mechanism by which AuNPs can operate for cancer therapy [57, 73]. AuNPs have the ability to prevent phosphorylation of the proteins involved in this process of angiogenesis, by their binding to the cysteine residues in heparin-binding growth factors [73]. Complementary, the AuNPs intravenously administrated can be irradiated which leads to endothelium damage and then a break in the oxygen and nutrient supply to the tumors involved, another way of angiogenic therapy [65]. Radiotherapy, once have a major impact in rapidly divided cells, presents reduced activity towards the niche of central cells that become independent of blood supply and then hypoxic and with reduced proliferation capacity, leading to a continuous survival of such cells, which is the main cause of tumor relapse [65]. So the complete abolish of angiogenesis is a potential strategy to eradicate these cancer cells and then eradicate cancer [65].

3.3. AuNPs as delivery systems

The well-known application of AuNPs in cancer therapy described above, lead to further investigation of new potential therapeutic strategies and was verified that AuNPs can be used in the design of delivery systems [74, 75]. The motivation for the implementation of AuNPs as drug delivery platforms is built in their easy to synthesis, functionalisation and also great biocompatibility, demonstrating that functionalisation with specific payloads have a great potential to destroy cancer cells [15, 76]. As described above, AuNPs as a potential nanocarrier have the possibility to carry different payloads, such as small drug molecules for drug delivery or biomolecules like DNA, proteins and RNA (siRNAs), being recognized as an attractive gene

delivery system (Figure 2) [37]. The conjugation of the two types of therapeutic functions in nanoparticles, i.e. a cytotoxic drug and a specific cancer cell target moiety, act as a single platform in a synergetic way to promote a higher affinity to cancer cells in order to signaling them to the efficiently release of the anticancer agent, circumventing the biological and biophysical barriers [23, 5, 77].

3.3.1. Specific targeting

Nanoconjugates for drug delivery can accommodate a myriad of anti-cancer molecules that will be release and have its therapeutic effects in cancer cells, however healthy tissues can also be affected and to avoid this problem a targeting strategy is an important feature for combined therapy [78]. Taking advantage of tumor molecular markers as docking sites to concentrate the therapeutic effect at tumors, it is possible to increase therapeutic efficacy while reducing systemic exposure and off-target effects. Several of these tumor molecular markers are surface proteins/receptors present in cancer cells and in tumor vasculature that are not expressed or are expressed at much lower levels in normal cells, thereby distinguishing tumor masses from the surrounding normal tissues [79]. For a selective and with great potential delivery systems based in nanoparticles it is needed an efficient targeting to uniquely overexpressed receptors in cancer cells [76]. The potency of such systems is achieved by the enhancement of cellular accumulation of AuNPs by an active targeting to cancer cells compared to a free drug that passively enters the cells, which simultaneously avoid the biological response and biophysical barriers *in vivo* [5, 80]. In fact, nanoparticles need to continue in the bloodstream for enough time and cannot be eliminated in order to target the tumor site in the body, and surface modifications can be a useful property to avoid the mononuclear phagocytic system [4].

Based in this specificity, this platform can be a potential methodology for cancer therapy once it can differentiate with high certainty between cancer cells and non-cancer cells, one major concern related to the current cancer therapies [5, 81]. The increase of the surface area of AuNPs associated with other features such as distance-and refractive index-spectroscopic properties appears important for the construction of relevant biodetection molecules [55]. This capacity of AuNPs can be exploited for the improving of the therapeutic capacity of such systems, once the application of diagnostic and therapeutic strategies at the same time, Theranostics, can lead to a greater release at a specific tumor site, the targeting moiety or the drug, and it can be tracking in the whole body [82].

There are several types of tumor-targeting biomolecules such as peptides like RGD [47], proteins (transferrin, epidermal growth factor (EGF)) and carbohydrates [5], oligonucleotides such as aptamers [83], and monoclonal antibodies [85-87]. For example, the anti-epithelial growth factor receptor (EGFR) monoclonal antibody has been used as an active targeting agent, since EGFR and its ligands are commonly overexpressed in a variety of solid tumors [86-88].

Functionalization of AuNPs with specific ssDNA molecules (Au-nanoprobes) concerns a great detection system for specific DNA targets, being a rapid, sensitive, specific and inexpensive system [19]. For example, cancer cells overexpressing the folate receptors can be specifically targeted by AuNPs functionalized with folate ligands, and then the chemotherapeutic agent Doxorubicin (DOX) can be release into them and induce a higher toxicity compared to the one

in healthy cells (do not express these receptors) and when compared to the DOX alone [89]. Additionally, the cell-surface specific markers that characterize the CSC pool constitute a way for targeting those cells in the whole tumor [7, 8]. Therapeutic strategies need to be focused in this specific CSC niche instead of the rapidly divided in order to prevent tumor regression [7, 8].

The targeting of tumor angiogenic vessels is also gaining increased attention, as it may improve therapeutic efficiency in cases where tumor cells are less accessible [79]. In combination with passive targeting strategies, active targeting may enhance receptor-mediated endocytosis of the AuNP conjugates in target cells [80, 90]. The intracellular localization of effector molecules can be further directed with the use of cell-penetrating peptides, which are short peptides that facilitate the delivery of various cargoes to cells, or with nuclear localization sequences, which direct cargos to the nucleus [91, 92]. Decorating AuNPs with proton sponge groups or using photothermal heating can further assist escape from endosomal sequestration/degradation [93]. Active targeting by AuNPs [94-96] has been shown to result in greater tumor accumulation than passive targeting, when AuNPs are administered systemically in vivo (6-13% versus 2-5%) [94, 95].

3.3.2. AuNPs for drug/cargo delivery

The construction of DDSs depends on size, charge and surface functionalities of the AuNPs, once they dictate the uptake capacity of such nanovectorization systems as well as its intracellular fate [5, 75]. The possibility to functionalize AuNPs with a plethora of different cargos allows the development of several distinct approaches for drug delivery [76]. Moreover, stable nanovectorization systems in the blood-stream, drug release rate and clearance of the vector are two other important properties for the use of nanoparticles as DDSs [5, 57]. The use of vectorisation systems based in nanoparticles reveal the capacity to transpose the biological barriers imposed, with the release of low molecular-weight molecules that rapidly diffuse into the body, promoting a selective distribution towards cancer cells [5].

The active release of the drug into cells depends on the interaction between the drug and AuNPs (covalent or non-covalent binding) and even on methods of release after reaching the cells [76]. Non-covalent binding, such as the one used for hydrophobic drugs, does not need further alterations to the drug in order to be released [97] while for covalent interactions, establish for prodrugs, it implies the application of an internal or external mechanisms [98, 99]. The tunable optical properties of AuNPs surface is a prominent feature for the release of a drug either by internal or external stimuli [5]. External stimuli can be administrated by a photo-regulated release, which depends on the administration of light to photo-cleavage of nanoparticles-drug interaction to activate the drug once free [100-102], like was demonstrated with AuNPs functionalized with 5-fluorouracil [103]. You and collaborators [104] reported that up to 60% of a doxorubicin payload could be loaded onto hollow gold nanospheres because the drug molecules were adsorbed to both the inner and the outer surfaces of the hollow gold nanospheres via electrostatic attraction. Owing to the strong SPR absorption of novel gold nanostructures, drug release can be activated by NIR light [104, 105]. Glutathione can be a good internal stimulus of release for covalent interactions, by exchange reactions between

disulfide of AuNPs and the intracellular glutathione [75]. AuNPs loaded with doxorubicin have been shown to be able to reverse cancer cells' resistance to the drug [106].

AuNPs with a high surface area attractive to establish interactions to a plethora of platinum drugs have been described [107]. Brown and coworkers designed a platinum-tethered AuNP system bearing the active compound of oxalipatin as its platinum molecule, and tested its platform in lung and colorectal cancer cell lines, demonstrating a more cytotoxic effect compared to oxaliplatin alone and also a higher accumulation of the active compound in those cancer cells reaching the nucleus for possible DNA interaction, what constitute a good delivery system [107].

3.3.3. AuNPs for gene therapy

Gene therapy is though as a hopeful strategy in cancer therapy being considered as a powerful treatment like chemotherapy and radiotherapy, however the implementation of such systems is based in viral vectors that raise cytotoxic and immune response problems [57, 108]. When conjugated to AuNPs, siRNAs have been shown to exhibit increased stability, cellular uptake and efficacy in physiological conditions, retaining the ability to act through the RNAi pathway [109, 110]. The first demonstration that DNA-AuNP conjugates could be easily internalized into cells, without the need for transfection agents, and induced gene silencing by an antisense mechanism was reported by Rosi and co-workers in 2006 [111]. AuNPs as gene-delivery vectors emerged initially with cationic ligands that appears a good gene delivery system once protects the DNA molecule from degradation by DNAse I [112]. Han and coworkers have identified that cationic AuNPs can trigger DNA release into cells by glutathione intracellular concentrations [113].These remarkable studies prompted others to use AuNPs as siRNA delivery systems and contributed to the development of many strategies to improve intracellular siRNA delivery in vitro and in vivo. These strategies can be grouped into two major categories that are currently used for tethering siRNAs to AuNPs, namely (1) the gold-thiol bond and (2) electrostatic interactions. Both categories involve, in some way, the use of poly(ethylene glycol (PEG) or other passivating agents for stabilization and to promote endosomal escape of the AuNP conjugates into the cell cytoplasm [114].

The silencing of cancer-related molecules can be addressed by this delivery platform, being of major concern the oncogenes that have specific involvement in cell survival and proliferation [115]. siRNAs can be pointed as potential therapeutic molecules once its function relies in the suppression of gene expression [78]. siRNA molecules present limitations when administrated alone: do not cross the cell membrane, are rapidly degradable by endo-and exo-nucleases, have low stability in the blood and induces systemic toxicity [116]. Nanoparticles functionalized with siRNAs that have been tested for targeting reporter genes in *in vitro* cell cultures and recently AuNPs functionalized with siRNAs were investigated for *in vitro* and *in vivo* targeting genes [67, 116]. AuNPs can in fact be a good system for antisense and siRNA delivery since they can protect these molecules from degradation [109]. Thus, in order to improve cytoplasmatic translocation of siRNAs and promoting a complete gene silencing, is of utmost important the formulation of these nanoconjugates with siRNAs once its smaller size can potentiate and improve the interactions between biomolecules in the surface or into a cell [4, 116].

In vivo studies using this system are still scarce, alerting us for the need to overcome remaining barriers that prevent its translation into the clinics. Some recent studies are highlighted. Zhang and co-workers have developed an anti-metastasis therapy consisting of gold nanorods (AuNRs) conjugated electrostatically with siRNAs, which targeted the protease-activated receptor 1 (PAR-1) [117]. These conjugates were then delivered to highly metastatic human breast cancer cells. The authors observed efficient downregulation of PAR-1 mRNA and protein levels and decreased metastatic ability of the cancer cells [117]. By allowing any short nucleic acid to be hybridized to the cargo DNA covalently linked to the AuNP, the former can be designed for a specific purpose, such as gene knockdown, redirection of alternative splicing, and modulation of signal transduction pathways, Ryou and collaborators delivered shRNAs targeting the Mcl-1L mRNA to a xenografted tumor in a mouse model, and showed a ~5% reduction in protein expression which was sufficient to induce apoptosis of the xenograft tumor cells [118]. These studies did not include a targeting strategy because they were performed either in vitro or in vivo by directly injecting the conjugates into tumors. However, for systemic delivery, an additional targeting moiety is generally required to improve treatment efficacy and reduce off-target effects. Lu and co-workers [93] used Au nanocages targeted to folate receptors (overexpressed in many types of cancer) and carrying a siRNA against the NFkBp65, which encodes a transcription factor highly involved in tumor formation and progression. They injected these constructs intravenously into in nude mice bearing HeLa cervical cancer xenografts and observed a significantly higher tumor uptake of the targeted conjugates compared to the non-targeted ones. They additionally took advantage of the photothermal properties of the Au nanocages to achieve a controlled cytoplasmic delivery of siRNA upon NIR light irradiation and observed efficient NF-kappaB p65 downregulation only when tumors were irradiated with NIR light [93].

AuNPs functionalized with c-myc siRNAs were studied in a cervix adenocarcinoma cell line and demonstrate a great accumulation in the cytoplasm of the tumor cells and an evident ability to silencing the *c-myc* oncogene [67]. They functionalized AuNPs with PEG, cell penetration (TAT) and cell adhesion peptides (RGD, which binds to the integrin $\alpha V\beta 3$ receptor family) [119], and c-myc targeting-siRNAs [5]. They have also shown in a more recent study that these same nanoconjugates are capable of targeting tumor cells in a lung cancer murine model and of inducing significant downregulation of the c-myc oncogene, followed by tumor growth inhibition and prolonged survival of lung tumor bearing mice [116].

Furthermore, miRNAs can appear with aberrant patterns of expression in tumors and then be related to its development, progression and tumor differentiation [47]. miRNAs can act as oncogenes or tumor suppressor genes accounting to its deregulation in cells, then it's down-regulation or up-regulation respectively, can be a major breakthrough for cancer therapy [120]. Conde and coworkers revealed a platform based in Au-nanobeacons to targeting and efficiently silencing miR-21, an oncogenic miRNA commonly up-regulated in almost all types of cancers [47, 121].

This targeting approached can also become a potential strategy to overcome the problem of multidrug resistance of cancer cells to the application of several drugs (Fernandes and Baptista, 2014). One of the major mechanisms of multidrug resistance in cancer is associated to ATP-

binding cassette (ABC) membrane transporters, such as P-glycoprotein (P-gp), and others efflux pumps such as BCRP, which imply these as potential targets of silencing for cancer therapy [122, 123]. Cancer stem cells (CSCs) can also express these membrane proteins which confer to this subpopulation resistance to the current chemotherapeutic agents [123, 124]. Thus, the implication of a silencing strategy towards these cancer related genes evolve in order to minimize cancer resistance barriers to the actual therapy and then obtain an efficient response towards the chemotherapeutic agents applied [115, 125]. It was demonstrated by a system of lipid-modified dextran nanoparticles bearing siRNAs towards *ABCB1* gene (P-gp), that this approach can efficiently deliver the siRNA molecule and reduces the expression of P-gp although at the same order of greatness as the siRNA alone [126]. This reveals the necessity to continuously develop nanoparticles systems that can target and silencing these genes and proteins.

Another multidrug resistance mechanism is associated to the capacity of cancer cells to evade apoptotic response, when resistance induced by efflux pumps is not seen [127]. Apoptosis is the major cellular process induced by chemotherapeutic agents, so cancers bearing apoptosis defects cannot be efficiently treated by those agents, then discovery of the molecular basis of such system can formulate novel therapeutic approaches [127, 128]. For example, the anti-apoptotic protein Bcl2 is considered a proto-oncogene, and nano-based vector delivery systems has been establish with great efficacy towards this molecule [127, 129].

4. Toxicity of AuNPs

One major concern regarding AuNPs application in medical field relies in its toxicity in the biological systems, i.e. the production of a general toxicity response not only in cancer cells but also reaching healthy cells at the vicinity [78]. Taken into account the size, surface modifications and solubility in promoting biocompatibility of the nanovectorization systems, they can be safer to apply in the medical field to the treatment of cancer [130]. In fact, nanoparticles size is an important feature because it turns possible to circumvent the immune response and renal clearance, which maintains the therapeutic capacity of such systems [5].

Toxicity of AuNPs is generally accepted to be dependent on particle size, shape, and surface charge and chemistry [131-134]. However, it is thought that once AuNPs have a smaller size, approximately the size of biomolecules, it can be taken like one and then evade cellular barriers, with access to different tissues, and in the end can lead to the disruption of cell biological processes [75, 135]. A control of the size dependent cytotoxicity of AuNPs, revealed that AuNPs with a 1-2 nm size represents more toxicity towards four cancer cells lines compared to AuNPs with 15 nm that do not display any toxicity (Pan *et al.*, 2007). Additionally, the main organs affected by AuNPs are the liver and the spleen (Sun *et al.*, 2011). Also, very small particles (1.4 and 5 nm in diameter) seem to be capable to enter the nucleus, where they can interact with DNA and cause molecular disturbance [136, 137]. Larger particles (16 nm and 33 nm) are retained in endosomes and accumulate in the periphery of the nuclear region [138, 139]. At least three different studies reported that cellular uptake of AuNPs reach maximum levels for

a particle size of about 50 nm [140-142]. Also, surface functionalisation seem to be capable of inducing higher level of apoptotic cell death, probably related to increased cell uptake when compared to unmodified 40 nm AuNPs [141]. According to data from in vitro studies, AuNPs' toxicity is believed to result mainly from the induction of oxidative stress [143-145]. Indeed, up-regulation of stress related genes was found to result from cell exposure to AuNPs, which also promoted the down-regulation of cell cycle related genes [145-147]. Nevertheless, most of these studies paid little attention to genome damage, such as DNA strand breaks and nuclear abnormalities, or characterization of protein markers for toxicity. An integrated toxicology evaluation encompassing DNA damage, stress related enzymes and a proteome profiling approach showed no significant cytotoxicity of PEGylated AuNPs and no up-regulation of proteins related to oxidative damage [148]. Nevertheless, previous studies using metallic nanoparticles showed acute toxicity, mainly by the introduction of damages to the DNA molecule and also by oxidative damage [146, 149, 150].

AuNPs are however generally considered a system that do not cause acute or adverse toxicity, and then are been taken as safer systems for therapeutic use [135]. AuNPs demonstrate to be a safe system due to their easy of functionalization [151]. This ideal is based in the assumption that gold nanoparticles do not lead to any effect in the cell, and instead, the function moiety in its surface promote the cytotoxic effect expected [139]. In the other way, expression studies revealed an overexpression of stress and inflammation related genes after AuNPs treatment, being associated to the action of AuNPs in oxidative stress induction [75]. A decrease in cell cycle genes expression was simultaneously observed, which symbolizes an irreversible damage that leads to cell death by necrosis [75].

Nanoparticles surface composition is another relevant point when talking about toxicity of nanoparticles systems [5]. The ligands and surface capping agents of AuNPs as the first line of contact with the different actors in the cell pathways can promote toxicity that in the end represents the overall toxicity associated to these nanoconjugates [5]

Also, both positive and negatively charged AuNPs were found to be similarly more cytotoxic against human keratinocytes (HaCaT cells) when compared to neutral AuNPs, with LD50 values of roughly half of those determined for the latter [152]. Despite the disruption in cell morphology and the dose-dependent toxicity observed for all three types of AuNPs, both anionic and cationic AuNPs induce mitochondrial stress and apoptosis in opposition to the necrotic cell death caused by neutral particles [152]. Another in vitro study comparing positive and negatively charged AuNPs reported that cationic NPs were far more toxic to Cos-1 cells, human red blood cells and E. coli than anionic NPs, possibly as a result of cell lysis, as shown by a dye leakage technique [133]. However, Alkilany and co-workers clearly showed that serum proteins become readily adsorbed to the surface of charged NPs, inducing an inversion of surface charge in particles that were originally cationic [153]. This would reduce electrostatic interaction between the original positive NPs and the negative cell membrane, the first step towards cell lysis mediated toxicity of cationic NPs [133].

Regarding in vivo experiments, several studies have demonstrated that AuNPs of 50 nm and larger were non-toxic to mice, conversely to what has been observed for AuNPs <40 nm [54, 55]. In fact, there are concordant data from different studies on the biodistribution and

accumulation of AuNPs in mice showing that most of the intravenously injected nanoparticles are retained in the liver, regardless of their size [156-158]. There is also an agreement in that AuNPs have the ability to transpose the blood-brain barrier and thus reach the brain, with a cut-off limit in diameter of around 20 nm [159], and that smaller particles have the most widespread organ distribution [156-158]. Organ distribution seems to be ruled by a more or less complex relationship with nanoparticle size. For instance, it is known that renal excretion of AuNPs is maximized for a narrow size range of 6-8 nm, resulting in an accelerated clearance rate [160]. Despite the valuable use of animal models, the effect of size on the toxicity of AuNPs in humans is difficult to predict since the size of endothelial cells' fenestrae is highly variable between individuals, thereby affecting nanoparticle clearance [75]. Therefore, more consistent data on the toxicological profile of AuNPs in vivo is necessary. For a more complete review on biodistribution, encompassing earlier studies and administration routes other than intravenous injection, see Khlebtsov and Dykman [159]. Furthermore, core size, charge and surface chemistry of AuNPs seems to correlate to toxicity on the development of zebrafish embryos, with positive and negatively charged AuNPs causing mortality and malfunctions to the embryos, respectively [161]. Adverse effects were also found in the model system Drosophila melanogaster after exposure to citrate-capped AuNPs, which were shown to reduce fertility in a dose-dependent manner and also the life span [144, 162].

Nonetheless, long-term studies in higher organisms are necessary to further characterise the safety of AuNPs as therapeutic agents, so they can be safely administrated to humans without concerns about late toxicity symptoms.

5. Conclusions & future

Cancer is a complex disease with a plethora of cell types and differentiation stages that trigger standard molecular mechanisms towards recruitment of cells and nutrients to enhance survival and proliferation. Cancer complexity is also dependent from the specific and multi-faceted umor microenvironment. All these different molecular pathways, mechanism and markers can be used as potential targets for therapeutics. However, current therapeutics (drugs and molecules) show serious cell toxicity that is not merely directed at the cancer cells but instead promote off-target cellular disarray and cell death, usually reported ad undesirable side effects and systemic toxicity.

Nanomedicine has been putting forward several therapeutic concepts that disrupt the way we have been dealing with cancer therapy, i.e. nanoparticles as drug delivery agents, minimising side effects and toxicity of the drugs. Furthermore, these nanoparticle platforms allow for selective targeting of cancer cells or tumor vessels either by incorporating novel or standard anticancer drugs and/or the delivery of therapeutic genetic modulators. These approaches, often based on the robustness and chemical properties of AuNPs, have shown great promise in preclinical models. Some recent advances in ligand-targeted NPs have begun to demonstrate improvement in cancer therapy.

What is more, many tumors become resistant to drugs, requiring that novel strategies involving drug targeting vehicles that deliver high concentrations of combinatorial therapeutics to the selected targets. For this to happen, it is crucial that these nanoconjugates are capable to withstand the body's clearance and reaction to non-self particulates. The robustness of AuNPs as target delivery platforms will be achieved when reticuloendothelial system clearance is avoid and occur an enhance of the endothelial penetration, once the first one can lead to a longer time in circulation and the second leads to an increase of targeting and drug accumulation (Kumar *et al.*, 2013).

The use of multiple nanoparticles that can be used together may overcome current limitations of each individual nanoformulation alone. For example, AuNPs have proven to be outstanding vectorisation systems for gene delivery and can be used to target molecular pathways, including those involved in drug resistance and in survival of cancer cells. These NPs may be used in combination with any other polymeric and/or metallic nanoparticles in therapeutic approaches that include drug and thermal ablation, selective delivery via out of the boy triggering (light source).

All of these applications of AuNPs in therapeutics still lack enough toxicology and pharmacology studies and data that can support the effective translation into the clinics. However, the efficacy in fighting cancer cells shows that the effort to push forward with the needed regulatory requirements and compliance is worth pursuing since the enhanced properties allow for outstanding improvements to biocompatibility, circulation and therapeutic response.

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Author details

Joana Silva[1], Alexandra R. Fernandes[1,2] and Pedro V. Baptista[1,3*]

*Address all correspondence to: pmvb@fct.unl.pt

1 Department of Life Sciences, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal

2 Centro de Química Estrutural, Instituto Superior Técnico, Lisboa, Portugal

3 CIGMH, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, Portugal

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Chapter 5

Nanoparticle based Drug Delivery Systems for Treatment of Infectious Diseases

Mojtaba Salouti and Azam Ahangari

Additional information is available at the end of the chapter

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1. Introduction

The chemotherapy of infections caused by bacteria that inhabit intracellularly presents a number of uncommon challenges. Many bacteria have found the way to produce a "silent" infection inside the cells and to avoid from their bactericidal mechanisms. However many methods for diagnosing and treating these and other bacterial infections presently exist, there is an essential need for new and improved approaches for bacterial destruction. Although the therapeutic efficacy of drugs has been well recognized, inefficient delivery could result in insufficient therapeutic index. It is now clear that a nanotechnology-driven approach using nanoparticles to selectively target and destroy pathogenic bacteria can be successfully implemented. Nanotechnology is one approach to overcome challenges of conventional drug delivery systems based on the development and fabrication of nanostructures. Some challenges associated with the technology are as it relates to drug effectiveness, toxicity, stability, pharmacokinetics and drug regulatory control. Localized diseases such as infection and inflammation not only have perforated vasculature but also overexpress some epitopes or receptors that can be used as targets. Thus, nanomedicines can also be actively targeted to these locations. Various types of nanoparticulate systems have been tried as potential drug delivery systems, containing biodegradable polymeric nanoparticles, polymeric micelles, nanocapsules, nanogels, fullerenes, solid lipid nanoparticles (SLN), nanoliposomes, dendrimers, metal nanoparticles and quantum dots. Nanoparticles have been found useful in the development of systemic, oral, pulmonary, transdermal and other administration routes to study drug targeting, the enhancement of drug bioavailability and protection of drug bioactivity and stability. In recent years, encapsulation of antimicrobial drugs in nanoparticle systems has emerged as an innovative and promising alternative that enhances therapeutic effectiveness

and minimizes the undesirable side effects of drugs. The major goals in designing nanoparticles as delivery systems are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site-specific action at the therapeutically optimal rate and dose regimen. This chapter focuses on nanoparticle-based drug delivery systems and clinical applications to treat a variety of bacterial infectious diseases and their potential applications in the field of medicine and biology.

2. Types of infections

Infectious disease is a clinically obvious disorder resulting from the presence of a pathogenic agent which can either be a virus, bacterium, fungus or parasite. These diseases are also called communicable diseases due to their ability to get transferred from one person to another (malaria, tuberculosis) and also sometimes from one species to another (flu, influenza). Infectious diseases can be vastly classified as: 1) known diseases which are insistently there (e.g., dengue, malaria, tuberculosis); 2) new, previously unknown diseases (e.g., severe acute respiratory syndrome); and 3) diseases which threaten to enhance in the near future (e.g., avian influenza). These diseases own a great risk as more than half of the deaths happening worldwide can be attributed to these diseases, particularly in developing countries [1]. Parasitism is based on the benefits acquired by a pathogenic bacterium invading the host and causing an infection. A bacterial infection is the process occurring when the microbe manifests its pathogenicity, and thus its capacity of inducing disease, by invading and causing a damage (locally or systemically) of the host organism. Consequently, the infectious disease could result in an acute infection, with a short and severe course, or a chronic, low-grade and long lasting infection [2].

3. Classification of bacterial pathogens

The classification of infectious agents inregards to their infective lifestyles in the host and corresponding pathogenic indications must be precisely described [3]. In the life of a microbe, the intracellularity and extracellularity are unclear designations unless obviously related to the situation where it is living. For a microbial pathogen, what matters is whether intra-or extracellularity is in the basis of the *in vivo* life and in relationship with pathogenicity. Classically, infectious agents are indicated as extracellular and intracellular pathogens [4-6].

3.1. Extracellular pathogens

Staphylococcus aureus, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* are typical examples of bacteria which have been considered extracellular pathogens, and lesion infections, osteomyelitis, scarlet fever, specified forms of pneumonia, urinary tract infections are examples of infections caused by these pathogens [7]. To produce disease, extracellular

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pathogens utilize any portal of entry provided a satisfactory fluid medium be recognized at the site of lesion [4]. Extracellular pathogens utilize virulence mechanisms to avoid the antimicrobial capabilities of humoral immunity and phagocytosis thus advancing extracellular reproduction [8], in contrast with intracellular pathogens that promote the entry in to host cells containing macrophages and non-professional phagocytes such as epithelial cells [9].

3.2. Intracellular pathogens

Classical examples of intracellular pathogens are *Brucella abortus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Salmonella enterica*, and typical infectious diseases caused by them include brucellosis, listeriosis, tuberculosis, and salmonellosis [10]. Intracellular pathogenic bacteria have the ability to establish a relationship in the sensitive host which includes a stage of intracellular reproduction [11]. To establish an infection, these pathogens have to make contact with the appropriated type of host cell that provides suitable intracellular conditions for growth [4]. Bacteria such as *Mycobacterium, Legionella, Brucella* or *Listeria* have extended the ability to resist and replicate inside various mammalian cells including the aggressive phagocytic cells, which establish the first-line defense against invading pathogens [12].

4. Targeted therapy of infections using nanoparticles

The hydrophilic nature of some antibiotics prevents thier capacity to penetrate the cells and, furthermore, the internalized molecules are mostly accumulated in lysosomes, where the bioactivity of the drug is low. Therefore, limited intracellular activity against sensitive bacteria is often found [13, 14]. Thus, the use of drug delivery systems (DDS) has been suggested for passive targeting of infected cells of the mononuclear phagocytic system to enhance the therapeutic index of antimicrobials in the intracellular environment, while minimizing the side effects associated with the systemic administration of the antibiotic [15]. The pathophysiological and anatomical changes of the affected tissues in a disease state offer many possibilities for the delivery of various nanotechnology-based products [16]. Bacteria gains antibiotic resistance due to three reasons namely: 1) modification of active site of the target resulting in reduction in the efficiency of binding of the drug, 2) direct destruction or modification of the antibiotic by enzymes produced by the organism or, 3) efflux of antibiotic from the cell [17]. Nanoparticles (NPs) can target antimicrobial agents to the site of infection, so that higher doses of drug can be given at the infected site, thereby overcoming existing resistance mechanisms with fewer harmful effects upon the patient [18]. As with nanoparticles targeting intracellular bacteria, nanoparticles targeting the site of infection can release high concentrations of antimicrobial drugs at the site of infection, while keeping the total dose of drug administered low. Nanoparticles can be targeted to sites of infection passively or actively. Passively targeted nanoparticles selectively undergo extravasation at sites of infection, where inflammation has led to enhanced blood vessel porousness. Actively targeted nanoparticles contain ligands (e.g. antibodies) that bind receptors (e.g. antigens) at sites of infection [19]. Passive targeting with nanoparticles, however, faces multiple barriers on the way to their target; these include

mucosal barriers, nonspecific uptake of the particle and non-specific delivery of the drug (as a result of uncontrolled release) [20]. Passive nanoparticulate targeting of chemotherapeutics to the cells and organs of the reticuloendothelial system (RES) has been a significant area of research for the treatment of chronic infectious diseases. The RES comprises monocyte-lineage immune cells such as macrophages and dendritic cells, as well as the spleen, liver, and kidneys. These components of the RES are consistently implicated as sites of nanoparticle clearance and localization [21]. The few studies that have compared targeted and nontargeted systems have demonstrated that the role of targeting ligands in localization at the target site is application dependent. Targeted delivery to atherosclerotic lesions is greatly enhanced by targeting ligands which impart an improved ability to accumulate at the target site [22]. Many active targeting strategies use the enhanced permeability and retention (EPR) effect, so that active and passive targeting mechanisms act synergistically that lead to higher concentration of nanostructures in the infected region than that in healthy tissues [23]. Targeted antimicrobial drug delivery to the site of infection, particularly intracellular infections, using NPs is a sensational prevision in treating infectious diseases [24, 25]. Intracellular microorganisms are taken up by alveolar macrophages (AMs), intracellulary survive or reproduce, and are persistent to the antimicrobial agents. Antibiotics loaded NPs can enter host cells through endocytosis, followed by releasing the payloads to delete intracellular microbes [26, 27]. The need to target drugs to specific sites is increasing day by day as a result of therapeutic and economic factors. Nanoparticulate systems have shown enormous potential in targeted drug delivery, specially to the brain [28].

5. Challenges in treating infectious diseases using nanotechnology

Use of antibiotics began with commercial production of penicillin in the late 1940s and claimed to be a great success until the 1970–1980s when newer and even stronger antibiotics were additionally improved [29]. Resistance to antimicrobial drugs becomes a threatening problem not only in hospitals but also in communities, resulting in fewer effective drugs available to control infections by “old” well-known bacteria [30]. Carrier systems allow antibiotics to be delivered selectively to phagocytic cells and to increase their cellular penetration in order to treat intracellular infections, particularly in the case of antibiotics active against microorganisms that produce this type of infection but that have a low intracellular penetration capacity [31]. Nevertheless, significant challenges remain for implementation of clinically viable therapies in this field. New challenges in the development of nanotechnology-based drug delivery systems include: the possibility of scale-up processes that bring innovative therapeutic techniques to the market rapidly, and the possibility of obtaining multifunctional systems to carry out several biological and therapeutic requirements [32]. Thus, a drug delivery system should be multifunctional and possess the ability to switch on and switch off specified functions when urgent. Another important requirement is that different properties of the multifunctional drug delivery systems are harmonized in an optimal fashion [33]. Therefore, design, discovery, and delivery of antimicrobial drugs with improved efficacy and avoidance of resistance are extremely requested [34].

5.1. Advantages of nanoantibiotics

The use of NPs as delivery vehicles for antimicrobial agents suggests a new and promising model in the design of effective therapeutics against many pathogenic bacteria [35]. Antimicrobial NPs propose several clinical advantages. First, the surface properties of nanoparticles can be changed for targeted drug delivery for *e.g.* small molecules, proteins, peptides, and nucleic acids loaded nanoparticles are not known by immune system and efficiently targeted to special tissue types [36]. Second, nanocarriers may overcome solubility or stability issues of the drug and minimize drug-induced side effects [37]. Third, using nanotechnology, it may be possible to achieve co-delivery of two or more drugs or therapeutic modality for combination therapy [33]. Fourth, NP-based antimicrobial drug delivery is promising in overcoming resistance to common antibiotics developed by many pathogenic bacteria [38]. Five, administration of antimicrobial agents using NPs can progress therapeutic index, extend drug circulation (i.e., extended half-life), and achieve controlled drug release, increasing the overall pharmacokinetics [30]. Six, the system can be used for several routes of administration including oral, nasal, parenteral, intra-ocular etc [39]. Thus, antimicrobial NPs are of great interest as they provide a number of benefits over free antimicrobial agents [35].

5.2. Disadvantages of nanoantibiotics including nanotoxicology

Although nanoantibiotics promises significant benefits and advances in addressing the key obstacles in treating infectious diseases, there are foreseeable challenges in translating this exciting technology for clinical application [40]. Profound knowledge about the potential toxicity of nanoantibiotics is also needed to guarantee successful clinical translation [41]. The toxic effects of antimicrobial NPs on central nervous system (CNS) are still unknown, and the interactions of NPs with the cells and tissues in CNS are poorly understood [42]. Furthermore, NPs represent size-specific properties that limit the use of currently available *in vitro* experiments in a general way, and there is no standardized definition for NP dose in mass, number, surface area, and biological samples (e.g., blood, urine, and inside organs) [43, 44]. This means that there is a high request to develop new characterization techniques that are not affected by NP properties as well as biological media [45]. NPs usually have short circulation half-life due to natural defense mechanism of human body for eliminating them after opsonization by the mononuclear phagocytic system. Therefore, the particles surfaces need to be changed to be hidden to opsonization [46]. A hydrophilic polymer such as polyethylene glycol is prevalently utilize for this purpose because it has worthwhile characteristics such as low degree of immunogenicity and antigenicity, chemical inertness of the polymer backbone, and availability of the terminal primary hydroxyl groups for derivatization [47].

6. Nanotechnology-based drug delivery systems

Perfectly, nanoparticulate drug delivery system should selectively accumulate in the necessary organ or tissue and at the same time, penetrate target cells to deliver the bioactive agent [48]. It has been proposed that, organ or tissue accumulation could be achieved by the passive or

antibody-mediated active targeting, while the intracellular delivery could be mediated by specified ligands or by cell-penetrating peptides [49-53]. The purpose of drug delivery is to carry out sustained (or slow) and/or controlled drug release and therefore to improve efficacy, safety, and/or patient comfort [54]. Thus, the use of drug delivery systems has been suggested for passive targeting of infected cells of the mononuclear phagocytic system to enhance the therapeutic index of antimicrobials in the intracellular environment, while minimizing the side effects related with the systemic administration of the antibiotic [55]. These systems propose many advantages in drug delivery, mainly focusing on improved safety and efficacy of the drugs, e.g. providing targeted delivery of drugs, improving bioavailability, extending drug or gene effect in target tissue, and improving the stability of therapeutic agents against chemical/enzymatic degradation [56]. The nanoscale size of these delivery systems is the basis for all these advantages [57]. It is therefore assumed that, DDS with enhanced targeting property is highly promising in increasing the efficiency and efficacy of therapy while at the same time minimizing side effects [33].

7. Types of drug carriers in medicine

7.1. Polymeric nanoparticles

Polymer-based nanoparticles are submicron-sized polymeric colloidal particles in which a therapeutic agent of interest can be embedded or encapsulated within their polymeric matrix or adsorbed or conjugated onto the surface [59]. The drugs may also be sensitive to gastrointestinal degradation by digestive enzymes. The advantage of using polymeric nanoparticles is to permit encapsulation of bioactive molecules and protect them against enzymatic and hydrolytic degradation [60]. Therapeutically used polymeric nanoparticles are composed of biodegradable or biocompatible materials, such as poly (ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), alginic acid, gelatin and chitosan [61-64]. Polymeric nanocarriers (NCs) may suggest an opportunity to target chlamydial organism within the contents, as NCs have been shown to be excellent intracellular carriers, and can be appropriate to encapsulate a variety of therapeutics containing biomacromolecules. Compared to free drugs, polymeric NCs have many other advantages including improved drug bioavailability, high carrier capacity, the ability to release the payload in a controlled behavior and to adapt to different routes of administration and to concentrate in inflammatory and infectious locations by virtue of their enhanced permeability and preservation. Conjugating NCs with specific moieties have also been shown to enhance their targeting to specific cells and tissues [65]. Polymeric nanoparticles have been extensively explored as means for drug solubilization, stabilization and targeting [66]. Polymeric nanoparticles possess several unique characteristics for antimicrobial drug delivery. Firstly, polymeric nanoparticles are structurally stable and can be synthesized with a sharper size distribution. Secondly, particle properties such as size, zeta potentials, and drug release profiles can be accurately tuned by selecting different polymer lengths, surfactants, and organic solvents during the synthesis. Thirdly, the surface of polymeric nanoparticles typically contains functional groups that can be chemically changed with either drug moieties or targeting ligands [67]. For targeted antimicrobial delivery, polymeric

nanoparticles have been repeatedly ornamented with lectin, which is a protein that binds to simple or complex carbohydrates present on most bacterial cell walls. For example, lectin-conjugated gliadin nanoparticles were studied for treating *Helicobacter pylori* related infection diseases. It has been found that lectin-conjugated nanoparticles bind specially to carbohydrate receptors on cell walls of *H. pylori* and release antimicrobial agents into the bacteria [30, 67]. Rifampicin-loaded polybutylcyanoacrylate nanoparticles have also shown enhanced antibacterial activity both *in vitro* and *in vivo* against *S. aureus* and *Mycobacterium avium* due to an effective delivery of drugs to macrophages [68].

7.2. Hydrogels

A hydrogel is a network of hydrophilic polymers that can swell in water and hold a large amount of water while maintaining the structure [69]. Drugs can be loaded into the polymer matrix of these materials and controlled release is dependent on the diffusion coefficient of the drug across the hydrogel network [70]. Amongst the several types of drug delivery systems that have been developed in order to improve effectiveness and biocompatibility, hydrogels are extremely promising. Hydrogels are biocompatible hydrophilic networks that can be constructed from both synthetic and natural materials [71]. In an overall view, hydrogels can be classified based on a variety of characteristics, containing the nature of side groups (neutral or ionic), mechanical and structural features (affine or phantom), method of preparation (homo-or co-polymer), physical structure (amorphous, semicrystalline, hydrogen bonded, supermolecular, and hydrocollodial), and responsiveness to physiologic environment stimuli (pH, ionic strength, temperature, electromagnetic radiation, etc.) [72-75]. Classically, hydrogels have been used to deliver hydrophilic, small-molecule drugs which have high solubilities in both the hydrophilic hydrogel matrix and the aqueous solvent swelling the hydrogel [76]. Hydrogel-based hydrophobic drug delivery is in many respects a more difficult problem given the innate incongruity of the hydrophilic hydrogel network and the hydrophobic drug. A variety of strategies for introducing hydrophobic domains directly into otherwise hydrophilic hydrogel networks have permitted significant improvements in the loading of hydrophobic drugs [76]. Hydrogel/glass composite (Nitric oxide-releasing nanoparticles) NO NPs have also been shown to have a high degree of effectiveness against (Methicillin-resistant *Staphylococcus aureus*) MRSA infection in several different mouse models. In one mouse study by Martinez et al., administration of topical hydrogel/glass composite NO NPs into skin wounds infected with MRSA reduced bacterial burden significantly compared to controls [77]. Despite these many advantageous properties, hydrogels also have several limitations. The low elastic force of many hydrogels limits their use in load-bearing applications and can result in the precocious decomposition or flow away of the hydrogel from a targeted local site. This limitation may not be important in many typical drug delivery applications (e.g. subcutaneous injection) [78].

7.3. Metal nanoparticles

Metal-based nanoparticles of different shapes, sizes (between 10 to 100 nm) have also been investigated as diagnostic and drug delivery systems. Most common metallic nanoparticles contain gold, nickel, silver, iron oxide, zinc oxide, gadolinium, and titanium dioxide particles

[79]. Metal nanoparticles, which have a high specific surface area and a high fraction of surface atoms, have been studied extensively because of their unique physicochemical characteristics including catalytic activity, optical properties, electronic properties, antimicrobial activity, and magnetic properties [80-82]. Even though metallic nanoparticles are biocompatible and immobile carriers, a significant fraction of metal particles can be retained and accumulated in the body after drug administration, probably causing toxicity. Consequently, the use of metallic nanoparticles for drug delivery is a concern [83].

7.3.1. Gold nanoparticles

Gold nanoparticles (GNPs) have found many applications in many fields such as cancer diagnosis and therapy, drug and gene delivery, DNA and ptotein determination, etc. Due to their unique properties of small size, large surface area to volume ratio, high reactivity to the living cells, stability over high temperatures and translocation into the cells [84]. GNPs are suitable for the delivery of drugs to cellular destinations due to their ease of synthesis, functionalization and biocompatibility. GNPs functionalized with targeted specific biomolecules can effectively destroy cancer cells or bacteria [85]. The efficacy of GNPs conjugated to several antibiotics has also been the subject of some studies by Grace and Saha et al. They discovered that GNPs conjugates were more efficient in inhibiting the growth of Gram-positive and Gram-negative bacteria in comparison with the same dosage of antibiotics utilized alone. Their results suggest that GNPs can act as an effective drug carrier in a drug delivery system [86, 87]. Conjugates of gold nanoparticles with antibiotics and antibodies also have been used for selective photothermal killing of protozoa and bacteria [88]. Gu et al. synthesized stable gold nanoparticles covered with vancomycin and showed significant enhancement of antibacterial activity, in comparison with the activity of the free antibiotic [89]. In another report, Selvaraj et al. utilized the anticancer compound 5-fluorouracil bound to GNPs and found that the resulting conjugate was significantly more effective against a range of bacterial and fungal organisms in comparison with alone [90]. Recently, it has been reported that the gentamicin conjugated with gold nanospheres was significantly more effective against *S. aureus* in comparsion with free gentamicin [91]. Each GNP surrounded by a number of drug moieties acts as a single group against the microbial organisms [92]. The greater antibacterial effect of the GNPs conjugates has been ascribed to their ability to bind to and/or penetrate the cell wall and, in doing so they are able to deliver a large number of antibiotic molecules into a highly localized volume [93].

7.3.2. Silver nanoparticles

Silver nanoparticles of size smaller than 100 nm contain about 10000–15000 silver atoms [94, 95]. They are prepared by engineering the metallic silver into ultrafine particles by numerous physical methods, which include spark discharging, electrochemical reduction, solution irradiation and cryochemical synthesis [96]. The most widely used and known application of silver nanoparticles is in the medical sciences. These include topical ointments and creams containing silver to prevent infection of burns and open wounds [97]. Among the many different types of metallic and metal oxide NPs, silver nanoparticles have demonstrated to be

the most effective against bacteria, viruses, and other eukaryotic microorganisms [98, 99]. Antibacterial properties inhibit the reproduction of bacteria, which is a microbe. The silver nanoparticles can "inactivate proteins, blocking respiration and electron transfer, and subsequently inactivating the bacteria" [100]. The antibacterial properties of the silver nanoparticles depend on the size of the particles; the smaller the particles the better the effect. The particle size is a major factor because the smaller the particle the greater the surface area, which allows for greater interaction with the bacteria [100]. It has been reported that combined use of silver nanoparticles with antibiotics, such as penicillin G, amoxicillin, erythromycin, and vancomycin, resulted in enhanced and synergistic antimicrobial effects against Gram-positive and Gram-negative bacteria (e.g., *E. coli* and *S. aureus*) [80, 101, 102]. Although beneficial as antimicrobial agents, silver nanoparticles have adverse effects on cells such as the production of reactive oxygen species which are toxic to both bacteria and eukaryotic cells [103, 104]. In contrast, the cytotoxicity of gold nanoparticles is quite low, and they have been used for medical imaging and have served as scaffolds for drug delivery [105, 106].

7.3.3. Magnetic nanoparticles

Magnetic nanoparticles engineered as drug delivery devices retain the ability to track their movement through the body. This is significant because it allows clinicians to monitor the effectivity of injected therapeutics to reach their target sites [107]. Iron oxide nanoparticles (IONPs) are magnetic Fe_3O_4 or Fe_2O_3 nanocrystals which can interact with external magnetic fields, offering different opportunities in nanomedicine, e.g., as contrast agents in MRI, for magnetic hyperthermal therapies, or as magnetically triggerable drug delivery systems [108]. There are some studies on evaluating the toxicity of magnetite nanoparticles on eukaryote cells, which their results showed negligible toxicity in eukaryote cells of the modified magnetite nanoparticles with different surfactants such as glycine or oleic acid. But the toxicity of magnetite nanoparticles on bacteria cells has not been reported [109]. However, in most of the cases where magnetic nanocarriers have been used, difficulties in achieving these objectives appeared. In turn, magnetic force may not be strong enough to overcome the force of blood flow and to accumulate magnetic drugs only at target site [110]. Therefore, designing magnetic drug delivery systems requires taking into consideration many factors, e.g., magnetic properties and size of particles, strength of magnetic field, drug loading capacity, the place of accessibility of target tissue, or the rate of blood flow [111]. The vancomycin functionalized magnetic nanoparticles for pathogen detection have been investigated by Gu et al. [112]. Vancomycin can be attached to the magnetic nanoparticles surface by activating the–COOH group of vancomycin followed by reaction with the amine groups on the surface of the iron oxide nanoparticles. The vancomycin conjugated iron oxide nanoparticles were utilized as probes to selectively entrap *S. saprophyticus* (a pathogen that usually infects the urinary tract of young women) and *S. aureus* bacteria from urine specimen using a magnetic field [1, 112]. It has been reported that the various nanoparticles, Al_2O_3, Fe_3O_4, CeO_2, ZrO_2 and MgO were subjected to evaluate its antibacterial potential against ophthalmic pathogens such as *Pseudomonas aeruginosa, Acinetobacter* sp., *Klebsiella pneumoniae, E. coli, Streptococcus viridans* and *Streptococcus pyogenes*. Among the nanoparticles, Fe_3O_4 showed maximum activity against

Pseudomonas aeruginosa. The reactive oxygen species (ROS) generated by Fe_3O_4 nanoparticles could kill bacteria without harming nonbacterial cells [113].

7.4. Silica nanoparticles

Silica materials are suitable for several important biological applications, such as drug delivery, imaging, oxygen carrier or controlled release [114]. Silica materials have been proved to be efficient carriers for the local release of antibiotics, which could be of interest in the context of biofilm associated infections, which are a real challenge for the modern medicine [115]. Moreover, mesoporous silica has been found to be relatively "non-toxic" and biocompatible, however of course depending on dose and administration route [116]. Nanoporous silica materials possess large pore volumes and high surface areas, allowing the absorption of large amounts of drugs, thus providing sufficient concentrations for local treatment. The surface of silica materials is reactive due to the presence of silanol groups. This allows for facile modification by silanization reactions and thus opens possibilities for enhancing the drug loading and for controlling the drug release [117]. Till present there are only few reports concerning the application of silica materials, crystalline or amorphous, in the antimicrobial therapy [115]. Zhang et al. suggested a highly-sensitive fluoroimmunoassay for the determination of *staphylococcal* enterotoxin C_1 (SEC_1). This method utilizes anti-SEC_1 coated NPs for detection which is possible in food samples and enables fluorescence microscopy imaging for the determination of SEC_1 [118]. Recently, Grumezescu et al. reported that silica nanostructures have significantly improved the anti-*staphylococcal* activity of bacitracin and kanamycin sulfate, as revealed by the drastic decrease of the minimal inhibitory activity of the respective antibiotics loaded in the SiO_2 nanopowder. These results, correlated with the high biocompatibility of the porous silica structure recommend it as an efficient vehicle for the local delivery of antibiotics in lower active doses, reducing thus their cytotoxicity and side effects [119].

7.5. Micelles

Micelles are submicroscopic aggregates of surfactant molecules assembly of amphiphillic block copolymers or polymer-lipid conjugates or other surface-active molecules that self-assemble in aqueous media to form structures with a hydrophobic core [120, 121]. The ability to functionalise the micelles as well as tailor the disintegration behaviour by varying the co-polymer composition are beneficial parameters in making them drug carriers of choice. Their small size (1-50 nm) makes them ideal for intravenous delivery. In addition they are also more stable, when compared to liposomes due to be ability to design them to be chemically stable and biocompatible [122]. One specific feature of micelles is that the amount of drug released can be controlled by an external stimulus like pH, temperature, ultrasound or certain enzymes [123]. Other unique properties of polymeric micelles are that they are easily altered with small functional groups that enhance their targeting potential [124]. Generally, polymeric surfactants are known to be less toxic than low-molecular-weight surfactants, such as sodium dodecyl sulfate. Furthermore, in theory, polymeric micelles are considered very safe in relation to chronic toxicity [125]. The disadvantage for the polymeric micelle systems is the immature technology for drug incorporation in a physical manner. The another disadvantage is much

slower extravazation of polymeric carrier systems than that of low molecular weight drugs. This results from a difference in extravazation mechanisms between polymeric carrier systems and low molecular weight drugs [126].

7.6. Liposomes

Liposomes are small spherical vesicles in which one or more aqueous parts are completely surrounded by molecules that have hydrophilic and hydrophobic functionality. Liposomes change with composition, size, surface charge and method of preparation. They can be single or in multiple bilayers. Those including one bilayer membrane are called small unilamellar vesicles or large unilamellar vesicles based on their sizes [127]. Nanoparticulate DDS, such as liposomes, are mostly used to enhance the efficacy of drug and DNA delivery and targeting [128, 129]. Liposomes are also the most broadly used antimicrobial drug delivery vehicles because their lipid bilayer structure imitators the cell membrane and can readily fuse with infectious microbes [30]. One of the disadvantages of liposomal antibiotics is the short shelf-lives of lipid vesicles, which limits drug stability. Short shelflives can be conditioned by both physical and chemical processes [130]. There are many advantages of liposomes as antibiotic carriers: improved pharmacokinetics and biodistribution; decreased toxicity; enhanced activity against intracellular pathogens; target selectivity; enhanced activity against extracellular pathogens, in particular to overcome bacterial drug resistance [131]. The ability of liposomes to alter drug distribution depends mostly on their size and surface properties [132]. Thus, liposomal encapsulation of antibiotics helps to increase their therapeutic index with mode of action related to increasing the drug concentration at the site of infection and/or reducing its toxicity [133]. For instance, encapsulation of vancomycin and teicoplanin in liposomes resulted in significantly improved elimination of intracellular methicillin resistant *S. aureus* (MRSA) infection [35]. Netilmicin liposomes showed an increase in pharmacological activity in a peritonitis model of mice infected with *E. coli*, in terms of survival both prophylactically and therapeutically [134]. Recently, Deol and Khuller produced lung-specific liposomes made of phosphatidylcholine, cholesterol, dicetylphosphate, O-steroyl amylopectin and monosialogangliosides/distearylphosphatidylethanolamine-poly (ethylene glycol) 2000 for the targeted delivery of anti-Tuberculosis (TB) drugs to the lung [135].

7.7. Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles (SLN) were developed at the beginning of 1990s as an alternative carrier system to emulsions, liposomes and polymeric nanoparticles as a colloidal carrier system for controlled drug delivery [20]. SLNs are sub-micron colloidal carriers, ranging from 50 nm to 1 μm, that are composed of physiological lipid dispersed in water or in aqueous surfactant solution [136]. In the last decade SLNs have gained considerable interest as novel particulate drug delivery systems. SLNs are suitable for the incorporation of lipophilic and hydrophilic drugs within the lipid matrix in considerable amounts [137]. SLN consist of a solid lipid matrix at room and body temperature, where the drug is normally incorporated in the submicron size range (below 1 μm) [35]. Some advantages of SLNs are

possibility of controlling drug release and drug targeting, increased drug stability, high drug payload, possibility of the incorporation of lipophilic and hydrophilic drugs, lack of biotoxicity of the carrier, no problems with respect to large-scale production, sterilization possibility, and good tolerability [138]. Common disadvantages of SLN are their particle growing, their unpredictable gelation tendency, their unexpected dynamics of polymorphic transitions and their inherent low incorporation rate due to the crystalline structure of the solid lipid [139]. SLNs are considered good drug carriers to obtain sustained release of antibiotics [140]. SLNs can act as promising carriers for sustained ciprofloxacin release in infections or to enhance the bioavailability of tobramycin from antibiotic-loaded SLN in the aqueous humor for topical ocular delivery [141, 142]. Nimje et al. (2009) reported the selective delivery of rifabutin, another antituberculosis drug, to alveolar tissues, using drug-loaded solid lipid nanoparticles, increasing the therapeutic margin of safety and reducing side effects [143]. Another prominent example of SLNs-based drug delivery is pulmonary delivery of antimicrobials to treat tuberculosis, a serious lung infection caused by *Mycobacterium tuberculosis*. In some severe cases, tuberculosis infection spreads from the lungs and affects the lymphatic systems. SLNs can facilitate the delivery of anti-tuberculosis drugs such as rifampin, isoniazidand pyrazinamide to the lungs as well as to the lymphatic systems [144]. Even though the development history of SLN-based antimicrobial drug delivery systems is relatively shorter than other nanoparticle systems such as liposomes and polymeric nanoparticles, SLNs have shown great therapeutic potentials [145].

7.8. Fullerenes

Fullerenes are a new form of carbon, other forms being diamond, graphite, and coal. They can take three forms of a hollow sphere, ellipsoid, or tube. Their small size, spherical shape, and hollow interior all provide therapeutic opportunities [146]. The most abundant form of fullerenes is buckminsterfullerene (C60) with 60 carbon atoms arranged in a spherical structure [147]. The shape of the molecule, recognized as truncated icosahedron, resembles that of a football ball, containing 12 pentagons and 20 hexagons, in which every carbon atom forms bond to three other neighbor atoms through sp^2 hybridization [148]. Friedman et al and Schinazi et al distinguished that the hydrophobic cleft of the human immunodeficiency virus (HIV)-1 protease can seamlessly host a C60 molecule [149]. This discovery was the first piece of evidence that fullerenes could have pharmaceutical significance through interactions with biological targets, highlighting the great potential of fullerenes in medicinal applications. Since fullerenes possess unique geometrical shapes, as well as novel photophysical properties, in addition to being efficient radical scavengers, a wide variety of biological applications have been considered [150-152]. Some studies asserted that C60 could be also utilized for the photodynamic inactivation of bacteria, as persuasively demonstrated in studies examining the effects of water-soluble and nanoparticulate C60 on various bacterial strains [153]. The effects were significantly more pronounced in Gram positive (*Staphylococcus* spp., *Streptococcus* spp.) than in Gram negative bacteria (*Klebsiella Pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Streptococcus pyogenes*), indicating that the bactericidal action was dependent on the fullerene insertion into the microbial cellwall, the structure of which differs between

Gram positive and Gram negative bacteria [154]. Additionally, the quinazolin–fullerene conjugate 18 was reported to have an inhibitory potential of 98.83% at a minimal inhibitory concentration of 1.562 μg/mL when treating *M. tuberculosis* [155].

7.9. Dendrimers

First discovered in the early 1980's by Tomalia and co-workers, such hyperbranched molecules were called dendrimers [156]. Dendrimers are globular repeatedly branched macromolecules that exhibit controlled patterns of branching with multiple arms extending from a central core [157]. The well defined structure, monodispersity of size, surface functionalization capability, and stability are properties of dendrimers that make them attractive drug carrier candidates [20]. Asymmetric dendrimers are synthesized by coupling dendrons of different generations (G1-G4) to a linear core, which yields a branched dendrimer with a nonuniform orthogonal architecture. This asymmetry allows for tunable structures and molecular weights, with precise control over the number of functional groups available on each dendron for attachment of drugs, imaging agents, and other therapeutic moieties [158]. Dendrimers also possess many unique properties that make them a good nanoparticle platform for antimicrobial drug delivery. They are highly arranged and regularly branched globular macromolecules, with a core, layers of branched repeat units emerging from the core and functional end groups on the outer layer of repeat units [159]. Dendrimer biocides may contain quaternary ammonium salts as functional end groups displaying greater antimicrobial activity against bacteria than small drug molecules, due to a high density of active antimicrobials on the dendrimer surfaces [160]. Dendrimers can be made from a wide variety of biocompatible materials, the most frequently used are polyamidoamine (PAMAM), polyethylene oxide (PEO), polypropylene imine (PPI), polyethyleneimine (PEI), polyethylene glycol (PEG) etc [161]. PAMAM dendrimers are dendritic polymers characterized by regular branching and radial symmetry. PAMAM dendrimers have illustrated useful drug delivery and antimicrobial applications with amino-terminated dendrimers showing high antibacterial efficacy [162]. It is well known that PAMAM dendrimers with primary amine surface functional groups may enter the cellular membrane. Sulfomethoxazole (a sulfonamide derivative poorly soluble and thus presenting low bioavailability) was administered with PAMAM dendrimers *in vitro* [163]. Sulfamethoxazole (SMZ)-encapsulating PAMAM dendrimers led to sustained release of the drug *in vitro* and 4–8 folds increased antibacterial activity against *E. coli,* compared to free SMZ [163].

7.10. Zeolites

Zeolites are solid hydrated crystalline materials with frame-works comprising silicon, aluminum and oxygen and featuring nano-channels and cages of regular dimensions [164]. Silica is a neutral regular tetrahedronin in which positive charge of silicon ion is balanced by oxygen [165]. The capacity of cation exchange depends on the ratio of silica/alumina in the structure. Generally, zeolits with a low silica/alumina (Si/Al) ratio have higher ion exchange capacity. According Si/Al ratio, there are several types of natural and synthetic zeolites including zeolite-β, zeolite A, zeolite X and zeolite Y, which are the most common commercial adsorbents [165]. Zeolites are minerals with selective pores that can be used to sieve molecules

having certain dimensions [166]. Several recent studies showed that the potential of zeolites in medical applications is due to their structural properties and stability in biological environments [167]. Zeolites have also been explored as suitable hosts for the encapsulation of drug molecules, in search for efficient drug delivery sysytems. Both zeolites and drugs have been administrated simultaneously to a patient without loss of the individual pharmacological effect of the drugs [164, 167]. Coating or impregnating zeolite with metallic silver nanoparticles to prepare zeolite composites can enhance the antibacterial ability of materials, and these materials can inhibit bacterial growth effectively [168]. It has been reported that silver embedded zeolite A was found to be antibactrerial against *E. coli*, *Bacillus subtilis* and *staphylococcus aureus* [165]. Moreover, polymer composites of plasticized poly (vinylchloride) pellets with silver zeolites demonstrated activity against *S. epidermidis* and *E. coli*, while polyurethane composites with silver zeolites showed antimicrobial action against *E. coli* and polylactid acidpolylactide (PLA)/silver zeolite composites also presented activity against *S. aureus* and *E. coli*, with silver being effectively released from the films [169].

7.11. Quantum dots

Quantum dots (QDs) are nanocrystals formed by semiconductor materials, showing attractive photophysical properties, containing high quantum yield, resistance to photobleaching, and harmonic photoluminescence, making them potentially powerful tools in a range of biomedical applications [170, 171]. QDs are typically in the size range between 1 nm and 10 nm, composed of groups II–VI (e.g., CdSe) or II–V (e.g., InP) elements of the periodic table. QDs are highly bright, photostable and possess high quantum yield [172]. Due to their very small size, they possess unique properties and behave in different way than crystals in macro scale [173]. Water-soluble QDs may be cross-linked to biomolecules such antibodies, oligonucleotides, or small molecule ligands to render them specific to biological targets [174]. A variety of techniques have been explored to label cells internally with QDs, using passive uptake, receptor-mediated internalization, chemical transfection, and mechanical delivery. QDs have been loaded passively into cells by exploiting the innate capacity of many cell types to uptake their extracellular space through endocytosis [175, 176]. Krauss group utilized CdSe/ZnS streptavidin-coated QDs to detect solitary pathogenic *E. coli* O157:H7 in phosphate buffer saline solution [177]. Biotinylated anti-*E. coli* O157:H7 distinguished streptavidin-coated QDs via famous avidin–biotin binding. Once treated, QD labeled antibody selectively targeted pathogenic *E. coli* O157:H7 over common lab strain *E. coli* DH5α. This assay represented 2 orders of magnitude more sensitivity than using an organic dye with minimal non-specific binding between the QDs and the bacterial cells [178]. Recently, Luo et al. reported that CDTe QDs coupled to a rocephin antibiotic complex exhibited antibacterial activity against *Escherichia coli* [179]. The mechanism for the antimicrobial activity of QDs is unclear, but it is possible that QDs can produce singlet O_2, a source of free radicals, under irradiation. Heavy metal ion oxides can also form the QDs core and result in antimicrobial activity [180]. A recent and excellent review emphasized the application of bioconjugated quantum dots for the detection of food contaminants such as pathogenic bacterial toxins like botulinum toxin, enterotoxins produced by *Staphylococcus aureus* and *Escherichia coli* [181].

8. Antibacterial activity of carrier systems for intracellular infection

Treatment of intracellular bacterial infection remains both a medical and economic challenge. Pathogens thriving or maintaining themselves in cells, or simply taking transient refuge therein, are indeed shielded from many of the humoral and cellular means of defense. They also seem more or less protected against many antibiotics [182]. Various infectious diseases are caused by facultative organisms that are able to survive in phagocytic cells. The intracellular location of these microorganisms protects them from the host defence systems and from some antibiotics with poor penetration into phagocytic cells. Intracellular infections are especially difficult to eradicate because bacteria fight for their survival using several ingenious mechanisms: inhibition of the phagosome–lysosome fusion, resistance to attack by lysosomal enzymes, oxygenated compounds and defensins of the host macrophages, escape from the phagosome into the cytoplasm [183]. Thus, the need for the development of improved antimicrobial chemotherapeutics and prophylaxis strategies is increasing [4]. In spite of the availability of a wide variety of *in vitro* active antibiotics, therapeutic deficiencies are reported, mainly because of the inability of the drugs to reach the bacteria harboring intracellular compartments or to perform their activity in the intracellular environments [182, 183]. However, the poor cellular penetration limits these use in the treatment of infections caused by intracellular pathogens [183]. One strategy utilized to improve the penetration of antibiotics into phagocytic cells is the use of carrier systems that deliver these drugs directly to the target cells [185]. Several *in vivo* and *in vitro* studies have reported the potential applications of various carrier systems to enhance the selectivity of antibiotics for phagocytic cells and sustain therapeutic efficiency in the treatment of intracellular infections [31].

8.1. Infections due to mycobacteria

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a ordinary lung infection that is even endemic to specified regions. Its prevalence has increased recently because it is often associated with AIDS. The *Mycobacterium avium* complex (MAC) complex is the main cause of hardships in immunodepressed patients [186]. There are drugs that are efficient against tuberculosis, but these are used in extended treatment, increasing the risk of side effects [187]. Moreover, tuberculosis has emerged as an occupational disease in the health care set-up. Although an effective therapeutic regimen is available, patient non-compliance (because of the need of taking antitubercular drugs daily or several times a week) results in treatment failure as well as the emergence of drug resistance [188]. The use of delivery systems facilitates the selective shuttling of antibiotic to the site of infection and such systems provide slow and prolonged drug release, which permits administration over longer intervals of time [189]. The encapsulation of antitubercular drugs in polymeric particles is another strategy to improve the current therapeutic regimen of tuberculosis. In the last few years several antitubercular drugs-containing PLGA and PLA microparticles and mainly nanoparticles have been comprehensively studied [190]. Fawaz et al. encapsulated the synthetic drug ciprofloxacin in polyisobutylcyanoacrylate (PIBCA) nanoparticles. When testing these nanoparticles against a *M. avium* infection in a human macrophage culture, it was found that though nanoparticle associated ciprofloxacin was more effective than unbound ciprofloxacin, it was much less so

than anticipated [191]. Rifampicin-loaded polybutylcyanoacrylate nanoparticles have shown enhanced antibacterial activity both *in vitro* and *in vivo* against *S. aureus* and *M. avium* due to an effective delivery of drugs to macrophages [192]. The encapsulation of different antibiotics in liposomes has shown good antibacterial efficacy in both macrophage cell lines and in animal models of MAC-due disease [193]. Ciprofloxacin efficiently inhibits the growth of *M. avium in vitro* in a murine macrophage-like cell line using negatively charged liposomes and *in vivo* using specific stealth liposomes in a mouse model of tuberculosis infection [194]. Similar results have been obtained using stealth liposomes of isoniazid and rifampicin, which show controlled release and reduce toxicity *in vivo* in mice infected with *M. tuberculosis* [195].

8.2. Brucellosis

Brucellosis is an infectious disease caused by *Brucella* spp. Four species, *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*, have been recognized as human pathogens each associated with a different natural host animal [196]. These small coccobacilli are mainly localized intracellularly within phagocytic cells making treatment difficult, since most antibiotics, although highly active *in vitro*, do not actively pass through cellular membranes [197]. However in the last two decades many experiments have provided good evidence criteria for its antibiotic treatment, the most suitable antimicrobial therapy for human brucellosis continues to be a controversial subject [198]. Because of its intracellular location, long treatments with several antibiotics are required. Relapses are frequent owing to the low efficacy of many drugs and the lack of patient agreement [199]. Thus, alternative methods such as drug delivery systems to achieve high intracellular bactericidal activity should be considered [198]. Gentamicin, encapsulated in different types of liposomes, has been evaluated against murine monocytes infected with *B. abortus*. All such liposomes reduced the number of bacteria, the most effective being SPLVs (stable plurilamellar vesicles) [200]. Rifampicin-loaded mannosylated dendrimers have indicated specific pH-dependent delivery of this antibiotic to rat alveolar macrophages [201]. Recently, gentamicin loaded poly (D, L-lactide-co-glycolide) (PLGA) have been obtained by the several emulsion solvent evaporation method for the treatment of brucellosis [202]. Thus, alternative methods such as DDS to achieve high intracellular bactericidal activity seem promising. The possible use of drug delivery systems containing aminoglycosides may be one of the most appropriate therapeutic advances in human brucellosis treatment in the recent years [203].

8.3. Salmonellosis

Salmonellosis is one of the most serious food-borne diseases affecting humans. It may be considered the most important pandemic zoonosis under natural conditions [204]. Bacteria of the genus *salmonella* are facultative intracellular parasites that cause salmonellosis and typhoid fever. Antibiotics effective against this type of bacteria have limitations owing to the problems of formulation, low penetration, or the appearance of side effects; these can be solved using carrier systems [205]. Several studies using antibiotic-loaded nanoparticles have been performed in order to recognize the suitability and efficacy of these carriers in experimental models of salmonellosis [204]. In order to recognize whether polyalkycyanoacrylate nanopar-

ticles were also effective against non-dividing bacteria, Page-Clisson *et al.* studied the effectiveness of these carriers in a model of persistent *Salmonella typhimurium* infection [206]. They found that although at early stages of the infection, when bacteria are actively dividing, there was an antibacterial effect, neither free nor nanoencapsulated ciprofloxacin or ampicillin could significantly reduce infection in the liver or the spleen at later stages [206]. Liposomal ciprofloxacin, administered intravenously and intraperitoneally to mice infected with intracellular *S. typhimurium*, has increased habitation time in plasma and the concentration of drug in the liver, spleen, lungs and kidneys is also increased, while when administered intratracheally its pulmonary retention is increased. Compared with free ciprofloxacin, it extends survival and reduces the number of bacteria in the liver and spleen [207]. Therefore, alternative methods such as DDS which achieve high protective and bactericidal activity should be taken into account in the future as suitable treatments for *Salmonella*-induced infections [203].

8.4. Lysteriosis

Lysteria monocytogenes is a facultative intracellular parasite able to cause meningitis and septicaemia. The encapsulation of ampicillin in liposomes decreases the survival of *L. monocytogenes* in mouse peritoneal macrophages to different extents, depending on the composition of the liposomes [208]. Chitosan-coated plastic films, alone or loaded with antimicrobial agents, were evaluated for their effect against *L. monocytogenes*. These chitosan-coated films inhibited this pathogen growth in a concentration-dependent manner whereas chitosancoated films impregnated with antibiotics were significantly more effective against *L. monocytogenes* [209]. Formulation of gentamicin in liposomes containing DOPE (dioleylphosphatidylethanolamine) and sensitive to pH has been reported to increase the concentration of drug in mouse macrophages infected with *L. monocytogenes*, increasing its bactericidal activity. This formulation is more effective against *L. monocytogenes* than against other bacteria owing to its location in the cytosol [210]. Furthermore, the efficacy of liposomes and free antibiotic were distinguished in *Listeria*-infected mice. Seven days after the treatment, ampicillin-loaded liposomes had reduced the infection by 3.2 logs in the liver and 2.8 logs in the spleen, while free ampicillin was ineffective [208]. In another example, ampicillin-encapsulated polyisohexylcyanoacrylate nanoparticles have been investigated against *L. monocytogenes* in mouse peritoneal macrophages [211].

9. Specific applications of biodegradable NPs

Attractive features, such as increased dissolution velocity, increased saturation solubility, improved bioadhesivity, versatility in surface modification and ease of post-production processing, have widened the applications of nanosuspensions for various routes. One major problem with the intravenous administration of colloidal particles is their interaction with the reticulo-endothelial system [212]. The applications of nanosuspensions in parenteral and oral routes have been very well investigated and applications in pulmonary and ocular delivery

have been discovered. However, their applications in buccal, nasal and topical delivery are still awaiting exploration [213].

9.1. Oral delivery

In recent years, significant research has been done using nanoparticles as oral drug delivery vehicles. Oral delivery of drugs using nanoparticles has been shown to be far superior to the delivery of free drugs in terms of bioavialability, residence time, and biodistribution [214]. Oral drug delivery is the choicest route for drug administration because of its non-invasive nature [215]. The drugs may also be susceptible to gastrointestinal degradation by digestive enzymes. The advantage of using polymeric nanoparticles is to permit encapsulation of bioactive molecules and maintain them against enzymatic and hydrolytic degradation [214] The use of submicron-size particular systems in oral drug delivery, especially peptide drugs, has attracted considerable pharmaceutical interest [216]. The efficacy or proficiency of the orally administered drug commonly depends on its solubility and absorption through the gastrointestinal tract. Therefore, a drug candidate that represents poor aqueous solubility and/or decomposition-rate limited absorption is believed to possess low and/or highly variable oral bioavailability [212]. Despite numerous studies providing evidence that oral delivery of encapsulated antigens can efficiently elicit immune responses, up to now, less studies report a protection induced by antigen loaded particles administrated by the oral route against a challenge with the pathogen [217]. Fattal et al. achieved the protection of mice against *S. typhimurium* following oral administration of *S. typhimurium* phosphorylcholine antigen encapsulated in PLGA particles [218]. Pinto and Muller (1999) incorporated SLN into spherical pellets and investigated SLN release for oral administration [219]. Orally administered antibiotics such as atovaquone and bupravaquone replicate this problem very well. Nanosizing of such drugs can lead to a dramatic increase in their oral absorption and consequently bioavailability [212].

9.2. Pulmonary delivery

Besides its non-invasive nature, pulmonary drug delivery has many other advantages compared to alternative drug delivery strategies, containing a large surface area for solute transport, rapid drug uptake, and improved drug bioavailability [220, 221]. Delivery of antimicrobial agents to the lung via systemic NP administration is persistent and potentially harmful upon systemic exposure to the drugs. Alternatively, various NPs exhibiting preferential accumulation in the lung and other organs have been tried. It was reported that intratracheally administered antibiotics loaded NPs were able to penetrate through the alveolar-capillary barrier into the systemic circulation and accumulate in extrapulmonary organ containing liver, spleen, bone, and kidney [222]. Micronization of drugs plays an important role in improving the drug dosage form and therapeutic efficiency today. If a drug is micronized into microspheres with suitable particle size, it can be addressed directly to the lung by the mechanical prevention of capillary bed in the lungs [223]. Nanosuspensions may demonstrate to be an ideal approach for delivering drugs that display poor solubility in pulmonary secretions [212]. Furthermore, because of the nanoparticulate nature and uniform size distribution of nanosuspensions, it is very likely that in each aerosol droplet at least one

drug nanoparticle is contained, leading to even distribution of the drug in the lungs as compared to the microparticulate form of the drug. In regular suspension aerosols many droplets are drug free and others are highly filled with the drug, directing to uneven delivery and circulating of the drug in the lungs. Nanosuspensions could be utilized in all available types of nebulizer [224]. In a recent study, antitubercular drugs (rifampicin, isoniazid and pyrazinamide) were incorporated into various formulations of solid lipid particles ranged from 1.1–2.1 μm and formulations were nebulized to guinea pigs by mouth for direct pulmonar delivery [212]. Similarly, conditions such as pulmonary aspergillosis can easily be targeted by using suitable drug candidates, such as amphotericin B, in the form of pulmonary nanosuspensions instead of using stealth liposomes [225].

9.3. Ocular delivery

Nanosuspensions can assay to be a advantage for drugs that show poor solubility in lachrymal fluids. For delivery of such drugs, approaches such as suspensions and ointments have been proposed. Although suspensions present advantages such as extended residence time in a cul-desac (which is desirable for most ocular diseases for effective treatment) and avoidance of the high tonicity produced by water-soluble drugs, their actual performance depends on the native solubility of the drug in lachrymal fluids. Thus, the intrinsic decomposition rate of the drug in lachrymal fluid governs its release and ocular bioavailability [226]. An approach that has recently been investigated to achieve the desired duration of action of the drug is the formulation of polymeric nanosuspensions loaded with the drug [212]. Ocular drug administration via SLN has been reported several times. Ocular drug administration via SLN has been reported several times [227]. Cavalli et al (2002) evaluated SLN as carriers for ocular delivery of tobramycin in rabbit eyes. As a result SLN significantly enhanced the drug bioavalability in the aqueous humor within 6 hours [228]. In addition, poly-cationic polymers may be useful penetration enhancers for ocular drug delivery [229]. De Campos et al. discovered the potential of cyclosporin-A loaded nanoparticles for the management of extraocular disorders, i.e. keratoconjunctivitis sicca or dry eye disease. They reported that the advantages of these systems in ocular drug delivery contain their ability to contact intimately with the corneal and conjunctival surface, thereby increasing delivery to external ocular tissues without compromising inner ocular structures and systemic drug exposure, and to provide these target tissues with long term drug level [230]. De Salamanaca et al. have reported that chitosan nanoparticles readily penetrate conjunctival epithelial cells and are well suffered at the ocular surface of rabbits [231].

9.4. Brain delivery

There is a great interest in the development of drug delivery systems that could allow an efficient and sitespecific transport of drugs to the target tissues affected by the disease. One of the most challenging barriers in the body is the blood–brain barrier (BBB) [232]. Endothelial cells of the BBB limit the solute movement into the brain by regulating transport mechanisms at the cell surface. These transport mechanisms help to keep the harmful substances out of the brain in order to maintain homeostasis [233]. Besides the development of simple prodrugs, an

emerging approach to circumvent the BBB is the use of liposomes, polymeric nanoparticles or solid lipid nanoparticles, in which the therapeutic drugs can be adsorbed or entrapped [234]. A drug can passively spread through the BBB in a more efficient manner after it is transformed into a more lipophilic prodrug. The same principle can be applied to brain targeting by delivering drugs on nanocarriers with enhanced lipophilicity. Fenart et al demonstrated that when polysaccharide nanoparticles were coated with a lipid bilayer, a 3 to 4-fold improvement in brain uptake without disruption of the BBB integrity was observed [235]. It has been reported that poly (butylcyanoacrylate) nanoparticles were able to deliver hexapeptide dalargin, doxorubicin and other agents into the brain which is significant because of the great difficulty for drugs to cross the BBB [236]. Recently dendrimers have been evaluated for CNS delivery of antiretrovira (ARVs) too. Polyamidoamine dendrimers loaded with lamivudine, a nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) commonly utilized in HIV treatment, were evaluated for their *in vitro* antiviral activity inMT2 cells infected with HIV-1. When loaded on dendrimeric nanocarriers, a 21-fold increase in cellular lamivudine uptake and 2.6-fold reduction in the viral p24 levels were observed when compared to the group treated with free drug solution [237]. In summary, nanoparticles are a very useful and universal method to deliver drugs to the brain. Industrial applications of the nanosphere technology would have several benefits: 1) Nanoparticles deliver drugs to the brain that normally do not cross the blood-brain barrier. 2) They reduce peripheral side effects of (approved) drugs that cross the BBB by increasing the relative dose of drugs reaching the brain; 3) Nanoparticles can also be used as a screening tool. Delivering drug candidates to the brain by nanosphere technology for initial screening of CNS activity obviates direct CNS injections [238].

10. Conclusion

In many healthcare facilities around the world, bacterial pathogens that express multiple resistance mechanisms are becoming the norm, complicating treatment and increasing both human morbidity and financial costs. Until now, no antibiotic therapy has been reported to eliminate most intracellular bacteria such us *Brucella* or *Mycobaterium* too. Furthermore, a prolonged exposure to combined antibiotics is required to reduce the disease relapses down to 5-15%. In this sense, drug delivery scientists are searching for the ideal nanovehicle for the ideal nanodrug delivery system; one that would dramatically reduce drug dosage, improve in the drug absorption so that the patient can take a smaller dose, and yet have the same benefit, deliver the drug to the right place in the living system, increase the local concentration of the drug at the favorite site and limit or eliminate side effects. Compared with other colloidal carriers, polymeric particles, mainly nanoparticles, have appeared more recently as attractive carriers for the delivery of drugs to infected cells. Synthetic biodegradable and biocompatible polymers have been shown to be effective for encapsulating a great variety of antibiotics. In addition, these polymeric particles powerfully enhance phagocytosis and are suitable for intracellular delivery of antibacterial agents. With the continuous attempts in this field, there is no doubt that nanoparticle-based drug delivery systems will continue to improve treatment to bacterial infections, particularly in life-threatening diseases such as tuberculosis infections.

Today the application of nanotechnology in drug delivery is widely expected to change the scenery of pharmaceutical and biotechnology industries for the foreseeable future. Target-specific drug therapy and methods for early diagnosis of pathologies are the precedency research areas where nanotechnology would play a prominent role.

Author details

Mojtaba Salouti[1*] and Azam Ahangari[2]

*Address all correspondence to: saloutim@yahoo.com

1 Biology Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran

2 Department of Microbiology, Hidaj Branch, Islamic Azad University, Hidaj, Iran

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Chapter 6

Nanoparticles for Dermal and Transdermal Drug Delivery

Okoro Uchechi, John D. N. Ogbonna and
Anthony A. Attama

Additional information is available at the end of the chapter

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1. Introduction

The term "nanoscale" refers to particle size range from ~ 1 to 100 nm [1], but for the purpose of drug delivery, nanoparticles in the range of 50 – 500 nm are acceptable depending on the route of administration. The method by which a drug is delivered can have a significant effect on its efficacy. Some drugs have an optimum concentration range within which maximum benefit is derived and concentrations above or below this range can be toxic or produce no therapeutic benefit. The slow progress in the efficacy of the treatment of several diseases has suggested a growing need for a multidisciplinary approach to the delivery of therapeutics to target tissues [2]. Transdermal drug delivery systems (TDDS) or patches are controlled-release devices that contain the drug either for localized treatment of tissues underlying the skin or for systemic therapy after topical application to the skin surface [3]. TDDS are available for a number of drugs, although the formulation matrices of these delivery systems differ. They differ from conventional topical formulations in the following ways:

- they have an impermeable occlusive backing film that prevents intensive water loss from the skin beneath the patch;
- the formulation matrix of the patch maintains the drug concentration gradient within the device after application so that drug delivery to the interface between the patch and the skin is sustained; and
- TDDS are kept in place on the skin surface by an adhesive layer ensuring drug contact with the skin and continued drug delivery [4].

Topical or transdermal drug delivery is challenging because the skin acts as a natural and protective barrier. TDDS were introduced into the US market in the late 1970s [5], but transdermal delivery of drugs had been used for a very long time. There have been previous reports about the use of mustard plasters to alleviate chest congestion and belladonna plasters as analgesics. The mustard plasters were homemade as well as available commercially where mustard seeds were ground and mixed with water to form a paste, which was in turn used to form a dispersion type of delivery system. Several methods have been examined to increase the permeation of therapeutic molecules into and through the skin and one such approach is use of nanoparticulate delivery system.

The skin has been an important route for drug delivery when topical, regional, or systemic effects are desired. Nevertheless, skin constitutes an excellent barrier and presents difficulties for the transdermal delivery of therapeutic agents, since few drugs possess the characteristics required to permeate across the stratum corneum in sufficient quantities to reach a therapeutic concentration in the blood [6]. In order to enhance drug transdermal absorption, different methodologies have been investigated, developed, and patented. Improvement in physical permeation-enhancement technologies has led to renewed interest in transdermal drug delivery. Some of these novel advanced transdermal permeation-enhancement technologies include iontophoresis, electroporation, ultrasound, microneedles to open up the skin, and more recently the use of transdermal nanocarriers.

2. The human skin

The potential of using the intact skin as the port of drug administration to the human body has been recognized for several decades. However, the skin is a very difficult barrier to the ingress of materials allowing only small quantities of a drug to penetrate over a period of time. In order to design a drug delivery system, one must first understand the skin anatomy and its implication of drug-of choice and method of delivery.

The human skin is the largest organ in our body with surface area of 1.8-2.0 m^2. It is composed of three main layers; the epidermis, dermis and hypodermis (subcutaneous layer) (Fig. 1). The skin is a well energized organ that protects the organism against environmental factors and regulates heat and water loss from the body.

3. Routes of drug penetration through the skin

The permeation of drugs through the skin involves the diffusion through the intact epidermis through the skin appendages (hair follicles and sweat glands). These skin appendages form shunt pathways through the intact epidermis, occupying only 0.1% of the total human skin. It is known that drug permeation through the skin is usually limited by the stratum corneum (Fig. 2). Three main penetration routes are recognized (Fig. 3).

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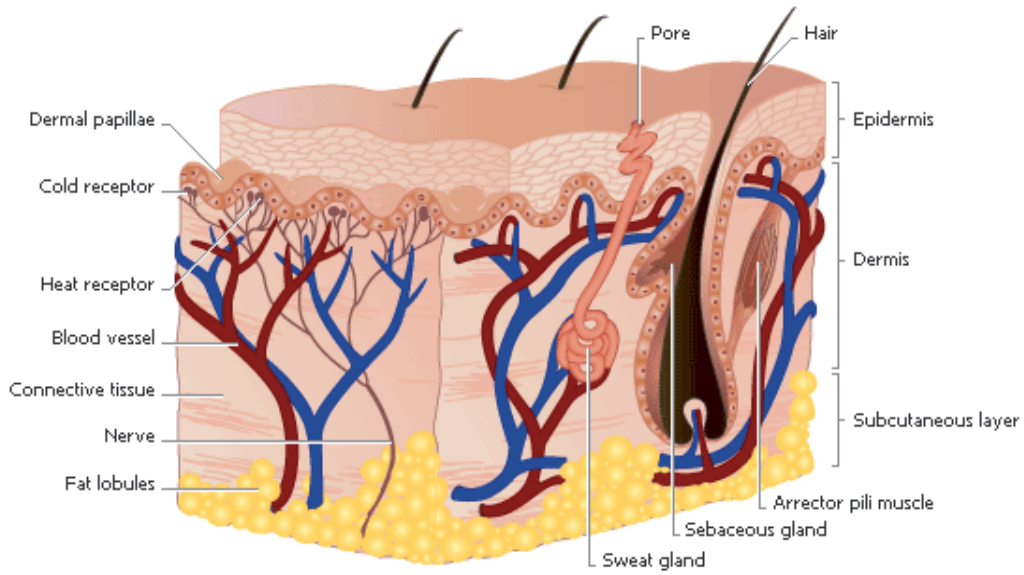
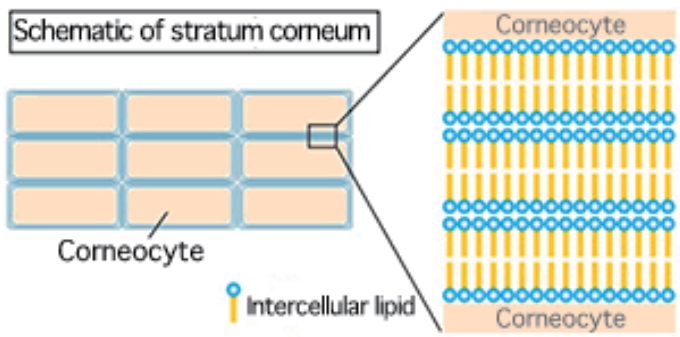


Figure 1. Structure of the skin (http://www.naturalrussia.com/natural/skin/structure.html. Downloaded on April 26, 2014)



(http://www.spring8.or.jp/en/news_publications/press_release/2011/110406/. Downloaded April 26, 2014).

Figure 2. The stratum corneum

3.1. The intercellular lipid route

Interlamellar regions in the stratum corneum, including linker regions, contain less ordered lipids and more flexible hydrophobic chains. This is the reason for the nonplanar spaces between crystalline lipid lamellae and their adjacent cells' outer membrane. Fluid lipids in skin barrier are crucially important for transepidermal diffusion of the lipidic and amphiphilic molecules, occupying those spaces for the insertion and migration through intercellular lipid

layers of such molecules [7]. The hydrophilic molecules diffuse predominantly "laterally" along surfaces of the less abundant water-filled interlamellar spaces or through such volumes; polar molecules can also use the free space between a lamella and a corneocyte outer membrane to the same end.

3.2. The transcellular route

Intracellular macromolecular matrix within the stratum corneum abounds in keratin, which does not contribute directly to the skin diffusive barrier but supports mechanical stability and thus intactness of the stratum corneum. Transcellular diffusion is practically unimportant for transdermal drug transport [8]. The narrow aqueous transepidermal pathways have been observed using confocal laser scanning microscopy. Here, regions of poor cellular and intercellular lipid packing coincide with wrinkles on skin surface and are simultaneously the sites of lowest skin resistance to the transport of hydrophilic entities. This lowest-resistance pathway leads between clusters of corneocytes at the locations where such cellular groups show no lateral overlap. The contribution to transdermal drug transport can increase with pathway widening or multiplication, e.g., that which is caused by exposing the stratum corneum to a strong electrical (electroporation/iontophoresis), mechanical (sonoporation/sonophoresis), or thermal stimulus, or suitable skin penetrants.

3.3. Follicular penetration

Recently, follicular penetration has become a major focus of interest due to the fact that drug targeting to the hair follicle is of great interest in the treatment of skin diseases. However, follicular orifices occupy only 0.1% of the total skin surface area. For this reason, it was assumed to be a nonimportant route for drug penetration. But a variety of studies have shown that hair follicles could be an interesting option for drug penetration through the skin [6]. Such follicular pathways have also been proposed for topical administration of polystyrene nanoparticles. They were investigated in porcine skin (*ex vivo*) and human skin (*in vivo*). Surface images revealed that polystyrene nanoparticles accumulated preferentially in the follicular openings. This distribution was increased in a time-dependent manner, and the follicular localization was favored by the smaller particle size. The study also confirmed similarity in the penetration between both membranes (porcine and human skin). In other investigations, the influence of microparticle size in skin penetration has been shown by differential stripping. Nanoparticles can act as efficient drug carriers through the follicle or can be utilized as follicle blockers to stop the penetration of topically applied substances.

4. Main factors for nano-based delivery system

4.1. Particle size, size distribution and zeta potential

Particle size and shape affect drug release, physical stability and cellular uptake of the nanoparticulate materials. The yield and size distribution of each system are affected by certain

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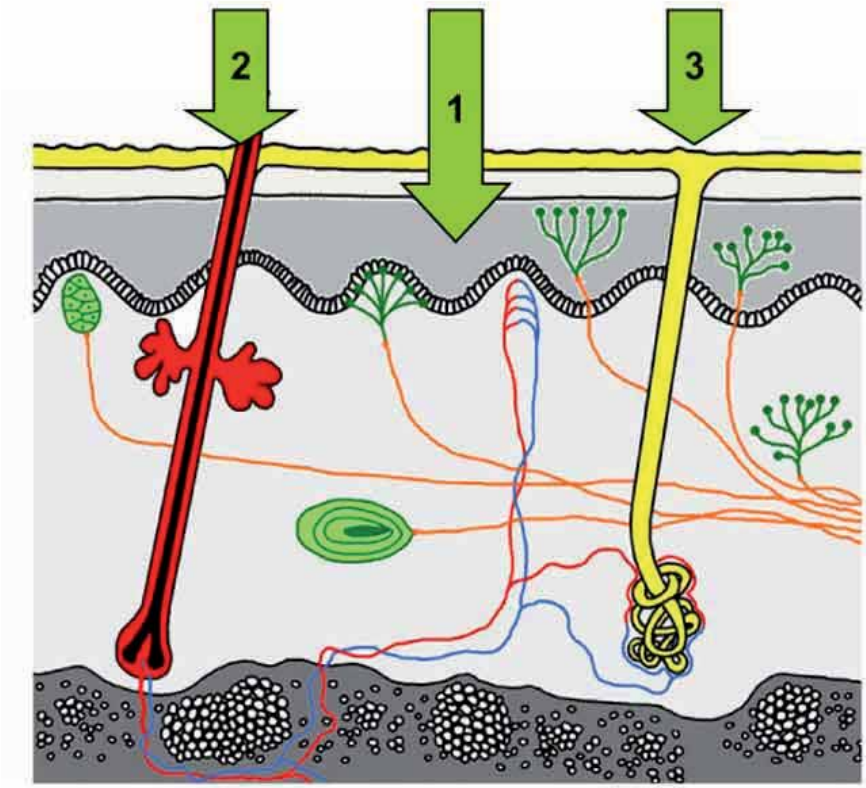

Figure 3. Structure of the skin showing routes of penetration: (1) across the intact horny layer, (2) through the hair follicles with the associated sebaceaous glands, or (3) via the sweat glands (http://www.skin-care-forum.basf.com/en/author-articles/strategies-for-skin-penetration-enhancement/2004/08/12?id=5b9a9164-6148-4d66-bd84-6df76bd6d111&mode=Detail. Downloaded April 26, 2014).

in-process operations and conditions such as stirring rate, temperature, type and amount of dispersing agent as well as the viscosity of the organic and aqueous phases [9,10]. Zeta potential of a dispersion is necessary for dispersion stability [11].

4.2. Surface properties

The attachment of nanoparticles to cell membrane is affected by the surface charge of the particles. Variation of the particle surface charge could potentially control binding to the tissue and direct nanoparticles to cellular compartments both *in vitro* and *in vivo*. Cellular surfaces are dominated by negatively charged sulphated proteoglycans molecules that play pivotal roles in cellular proliferation, migration and motility [12]. Cell surface proteoglyans consist of a core protein anchored to the membrane and linked to one or more glycosaminoglycan side chains (heparan, dermatan, keratan or chondrotine sulfates) to produce a structure that extends away from the cell surface.

Nanoparticles show a high affinity for cellular membrane mainly due to electrostatic interactions [12]. It is known that cell membranes have large negatively charged domains, which should repel negatively charged nanoparticles. The high cellular uptake of negatively charged nanoparticles is related first to the non-specific process of nanoparticles adsorption on the cell membrane and second to formation of nanoparticle clusters [13]. The adsorption of the negatively charged particles at the positively charged sites via electrostatic interaction can lead to localized neutralization and a subsequent bending of the membrane favouring in turn endocytosis for cellular uptake [14]. Thus the formulation of nanoparticles with different surface properties can influence their cellular uptake and intracellular distribution and it is possible to localize the nanoparticles to specific intracellular targets (lysosomes, mitochondria, cytoplasm, etc) by modifying their surface charge [15].

There are some investigations that showed the effect of surface charge, for example polymer charge density of dendrimers was found to significantly impact membrane permeability. The most densely charged polymer facilitates the transport of dye molecule across the membrane [16]. Other investigation showed that lipid coating of ionically charged nanoparticles was able to increase endothelial cell layer crossing 3 or 4 fold compared with uncoated particles, whereas nanoparticles coating of neutral particles did not significantly alter their permeation characteristics across the endothelial cell monolayer [13]. Transdermal drug administration systems have been limited to certain drugs of a range of molecular weight and lipophilicity, and of certain charge preference. For instance, cationic compounds have a positive effect on skin permeation, since the skin carries a negative surface charge due to phosphatidylcholine [17] and carbohydrates found in mammalian cells contain negatively charged groups. Therefore, nanoparticles with predominant positive charge would promote transdermal permeation.

5. Dermatopharmacokinetics

Dermatopharmacokinetics describe the pharmacokinetics of topically applied drugs in the stratum corneum with pharmacodynamic effects. The smart techniques (tape stripping and microdialysis) use in dermatopharmacokinetic methodology assesses the cutaneous drug concentration at the site of application. Various studies have shown dermatopharmacokinetics to be a reliable and reproducible method for determining bioequivalence, and have indicated that it is applicable for all topical dermatological drug products. Dermatopharmacokinetics refer to the determination of stratum corneum concentration-time curves for topical actives. This is analogous to plasma/urine concentration-time curves for systemically or orally administered drugs, and the concept is clearly adaptable to microdialysis, where drug is determined in the skin compartment in which the microdialysis fibre is positioned (Fig. 4).

Although, this procedure is invasive, it is a method of great potential offering information of high value and relevance. There could be sampling in a compartment within the skin. It is a technically demanding procedure, however, requiring experimental dexterity of high order. The potential for use on diseased skin is a unique and considerable advantage over other techniques, but real challenges remain with respect to reproducibility, sensitivity, applicable drugs, etc.

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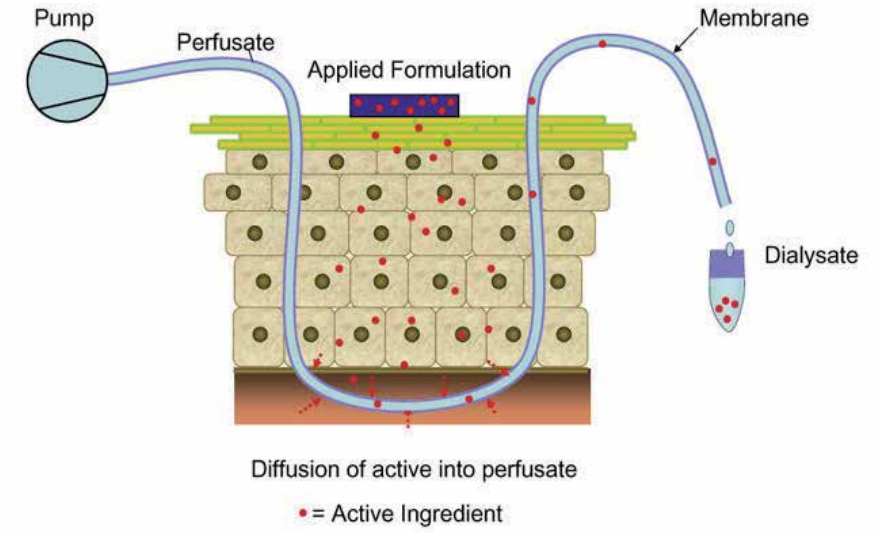

Figure 4. Sampling in the skin by microdialysis (http://www.skin-care-forum.basf.com/en/author-articles/strategies-for-skin-penetration-enhancement/2004/08/12?id=5b9a9164-6148-4d66-bd84-6df76bd6d111&mode=Detail. Downloaded April 26, 2014)

Stratum corneum tape-stripping is a minimally invasive method for determining drug levels in human stratum corneum *in vivo*. It involves repeated application of adhesive tapes on a site that has been treated with a topical formulation and determination of drug levels in stratum corneum collected on tape strips.

The dermatopharmacokinetics approach suggested by the Food and Drug Administration (FDA) proposes to evaluate the level of a topically applied drug in the stratum corneum during its uptake and clearance so as to calculate classic pharmacokinetic parameters [18]. The assumption is that stratum corneum concentration-time curves are directly related to concentration-time curves in the epidermis and dermis.

When applied to diseased skin, topical drug products induce one or more therapeutic responses, where onset, duration, and magnitude depend on the relative efficiency of three sequential processes, namely:

- the release of the drug from the dosage form
- penetration of the drug through the skin barrier, and
- generation of the desired pharmacological effect.

Because topical products deliver the drug directly to or near the intended site of action, measurement of the drug uptake into and drug elimination from the stratum corneum can provide a dermatopharmacokinetics means of assessing the bioequivalence of two topical drug products [19,20]. Presumably, two formulations that produce comparable stratum corneum concentration-time curves may be bioequivalence, just as two oral formulations are judged

bioequivalent if they produce comparable plasma concentration-time curves. Even though the target site for topical dermatologic drug products in some instances may not be the stratum corneum, the topical drug must still pass through the stratum corneum, except in instances of damage, to reach deeper sites of action [21]. In certain instances, the stratum corneum itself is the site of action. For example, in fungal infections of the skin, fungi reside in the stratum corneum and therefore dermatopharmacokinetic measurement of an antifungal drug in the stratum corneum represents direct measurement of drug concentration at the site of action [22]. In instances where the stratum corneum is disrupted or damaged, *in vitro* drug release may provide additional information toward the bioequivalent assessment. In this context, the drug release rate may reflect drug delivery directly to the dermal skin site without passage through the stratum corneum. For antiacne drug products, target sites are the hair follicles and sebaceous glands. In this setting, the drug diffuses through the stratum corneum, epidermis, and dermis to reach the site of action. The drug may also follow follicular pathways to reach the sites of action. The extent of follicular penetration depends on the particle size of the active ingredient if it is in the form of a suspension [21, 23-25]. Under these circumstances, the dermatopharmacokinetic approach is still expected to be applicable because studies indicate a positive correlation between the stratum corneum and follicular concentrations. Although the exact mechanism of action for some dermatological drugs is unclear, the dermatopharmacokinetic approach may still be useful as a measure of bioequivalence because it has been demonstrated that the stratum corneum functions as a reservoir, and stratum corneum concentration is a predictor of the amount of drug absorbed [26].

For reasons thus cited, dermatopharmacokinetic principles should be generally applicable to all topical dermatological drug products including antifungal, antiviral, antiacne, antibiotic, corticosteroid, and vaginally applied drug products. The dermatopharmacokinetic approach can thus be the primary means to document bioavailability/bioequivalence. Generally, bioequivalence determinations using dermatopharmacokinetic studies are performed in healthy subjects because skin where disease is present demonstrates high variability and changes over time. Use of healthy subjects is consistent with similar use in bioequivalence studies for oral drug products.

A dermatopharmacokinetic approach is not generally applicable when:

- a single application of the dermatological preparation damages the stratum corneum
- for otic preparations except when the product is intended for otic inflammation of the skin; and
- for ophthalmic preparations because the cornea is structurally different from the stratum corneum.

6. Ideal drugs for dermal and transdermal delivery

Owing to the selective nature of the skin barrier, only a small pool of drugs can be delivered systemically at therapeutically relevant rates [27]. Few drugs constitute the whole segment of

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the transdermal drug market. Besides great potency, the physicochemical drug characteristics often evoked as favourable for percutaneous delivery include moderate lipophilicity and low molecular weight [28]. However, a large number of pharmaceutical agents do not fulfill these criteria. This is especially true for macromolecules, such as insulin, human growth hormone or cyclosporine, which are very challenging from the drug delivery point of view. The physicochemical properties of ideal drug for transdermal delivery include:

- Molecular weight less than approximately 1000 Daltons.
- Affinity for both lipophilic and hydrophilic phases. Extreme partitioning characteristics not ideal.
- Low melting point.
- Should be potent, with short half life and be non-irritating.

Overcoming low skin permeability to xenobiotics can be achieved by a variety of approaches, and is an active field of research. Their effectiveness and applicability will vary from drug to drug depending on the physicochemical nature of the compound. New drug discovery is still a complicated process and generally requires substantial time and monetary investment. Technologies for formulation change provide the benefit of improving pharmaceutical product efficacy and safety as well as patient convenience; these technologies provide a relatively simple approach to creating new pharmaceuticals compared with new drug discovery because the active compounds used in the formulation have already been approved [29-31]. Nnamani *et al* [32] developed and evaluated the antimicrobial activities of an alternative non-invasive, convenient and cost-effective transdermal drug delivery system (TDDS) containing gentamicin in biodegradable polyester-based matrices. Other drugs which have been formulated for dermal and transdermal delivery are nitroglycerin, nicotine, scopolamine, clonidine, fentanyl, 17-β-estradiol, testosterone, Boswellic acid (*Boswellia serrata*) and curcumin (*Curcuma longa*).

7. Advantages of dermal and transdermal drug delivery

Transdermal delivery provides convenient and pain-free self-administration for patients. It eliminates frequent dosing administration and plasma level peaks and valleys associated with oral dosing and injections to maintain constant drug concentrations, and a drug with a short half-life can be delivered easily. All this leads to enhanced patient compliance, especially when long-term treatment is required, as in chronic pain treatment and smoking cessation therapy [3,33,34].

- Avoidance of hepatic first-pass metabolism and the gastrointestinal (GI) tract for poorly bioavailable drugs is another advantage of transdermal delivery. Elimination of the first-pass effect allows the amount of drug administered to be lower, and hence, safer in hepato-compromised patients, resulting in the reduction of adverse effects.
- Transdermal systems are generally inexpensive when compared with other therapies on a monthly cost basis, as patches are designed to deliver drugs from 1 to 7 days.

- The other advantage of transdermal delivery is that multiple dosing, on-demand or variable-rate delivery of drugs is possible with the latest programmable systems, adding more benefits to the conventional patch dosage forms.
- The general acceptability of transdermal products by patients is very high, which is also evident from the increasing market for transdermal products.
- Transdermal route permits the use of a relatively potent drug with minimal risk of system toxicity [35,36].
- In case of toxicity, the transdermal patch can easily be removed by the patient [37].

8. Disadvantages of dermal and transdermal delivery systems

Even though dermal and transdermal delivery systems have a lot of advantages over conventional topical formulation, it still suffer from a lot of limitations. The disadvantages of dermal and transdermal delivery systems according to Ranade and Cannon [38] are that:

- Not all drugs are suitable for transdermal delivery.
- Drugs that require high blood levels cannot be administered.
- The adhesive used may not adhere well to all types of skin.
- Drugs or drug formulation may cause sensitization or irritation which must be evaluated fairly early in the development process.
- The patches may/can be uncomfortable to wear.
- The manufacture requires specialized equipments which results in the formulation being more expensive to manufacture than conventional dosage forms thus the formulation will not be economical for most patients.
- There is always a lag time for drug to penetrate through the skin to the systemic circulation, therefore TDDS is not suitable for drugs requiring rapid onset of action.
- There is a requirement for low dose/high permeable drug. In general a drug with molecular weight less than 400, $\log P_{o/w}$=2-3 and dose less than 10 mg will be the best candidate for transdermal delivery.

9. Characterization of dermal and transdermal delivery systems and their performance

Dermal and transdermal delivery systems are characterized using different methods.

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9.1. Drug solubility determination

The determination of solubility of the drug in the transdermal/dermal matrix early in the formulation process can avoid crystallization problem, which is one of the instabilities in transdermal drug delivery systems (TDDS). This instability in the matrix which could be due to supersaturation makes the formulation metastable and upon storage results in changes in the liberation/release rate of the drug from the formulation.

9.2. Micromeritic measurements

9.2.1. Particle-size, shape and zeta potential analysis

Light scattering is an important way of characterizing colloidal and macromolecular dispersions and could be useful in assessing properties of particulate TDDS e.g. ethosomes. The particle size and size distribution are primarily measured using wet laser diffraction sizing otherwise called dynamic light scattering (DLS) [39]. Size of formulation can also be determined using dynamic light scattering (e.g. using a Zetasizer). This is necessary to ascertain the possible effect of the size on drug release and penetration across barriers in transdermal and dermal delivery as well as to monitor stability over time. The zeta potential of a formulation is very important. It is determined using Zetsizer or by other means, and gives information on the charge of the particles and the tendency of the particles in a formulation to aggregate or to remain discrete.

9.2.2. Specific surface area

An important parameter of bulk powders is the specific surface area expressed per unit weight. The specific surface area measurement includes the cracks, crevices, nooks, and crannies present in the particles. To include these features in the surface-area measurement, methods have been developed to probe these convoluted surfaces through adsorption by either a gas or a liquid [40-42]. The most widely used surface area measurement technique is the adsorption of a monolayer of gas, typically krypton or nitrogen as the adsorbate gas in helium as an inert diluent, using the method developed by Brunauer, Emmett, and Teller known as the BET method. Surface area affects spreading and occlusivity of TDDS.

9.3. Visualization by transmission electron microscopy

A combination of transmission electron microscopy (TEM) and freeze fracturing otherwise referred to as freeze fracture electron microscopy (FFEM) could be used to visualize skin structures and certain perturbations in the skin. A micrograph image is generated by transmitting a beam of electrons through a specimen appropriately treated to enhance the visualization of skin structural details. High resolution of TEM makes it possible to visualize both structures and transition processes in the epidermis. Using different techniques, epidermal granules [43], Langerhans cells [44] and the lipids in stratum corneum and epidermis [45], amongst others, have been observed. Samples preparation in FFTEM involves freezing the sample and subsequent longitudinal fracturing approximately parallel to the original skin

surface under high vacuum [46]. Further treatment could be done on the sample after which the fracture is viewed under high voltage. This visualization method can provide information on the interaction between the nanoparticle formulation and the skin. Since the fracture will always run along the plane of least resistance, FFEM micrographs of treated stratum corneum often show the lipid coated surfaces of corneocytes or the lipid lamellae.

9.4. Stability

Physical and chemical instabilities of carrier systems often limit their widespread use in medical applications [47]. Instabilities in ethosomes and other nanocarrier formulations are caused by hydrolysis or oxidation of the phospholipid molecules and are indicated by leakage of the encapsulated drug and alterations in vesicle size due to fusion and aggregation [48,49]. Changes in size and size distribution, entrapment efficiency and aggregation of vesicles are very important parameters in monitoring stability. These parameters can be assessed by EM or DLS repeatedly over time at varying storage conditions. It has recently been found that although multilamellar and large unilamellar benzocaine-loaded ethosome vesicles remained substantially stable with time, in terms of drug entrapment yield and particle dimensions, small unilamellar vesicles showed high tendency to form aggregates due to increased surface area exposed to the medium [10]. Such vesicle aggregation indicates instability. In addition, changes in storage conditions led to marked decrease in particle dimensions and drug-entrapping yield with less regular morphology for frozen-and-thawed multilamellar ethosome dispersions, while the untreated multilamellar and unilamellar vesicular dispersions remained homogenous and stable with regard to those parameters assessed over the period [50]. Temperature of formulation and storage conditions affect physical stability of nanoparticle preparations [10,51].

Optical characteristics, viscosity and physical changes such as cracking or creaming are also important in assessing stability of ethosomes. Ethosomes are colloidal disperse systems therefore, cracking and creaming may be observed during storage as in water-in-oil emulsions. The use of an innovative optical analyzer, Turbiscan Lab® Expert, in studying the influence of optical characteristics on long-term stability of vesicular colloidal delivery systems has been advocated [52]. The principle of this measurement is based on the variation of the droplet volume fraction (migration) or mean size (coalescence), thus resulting in the variation of backscattering and transmission signals as a function of time. No variation of particle size occurs when the backscattering profile is within the interval ± 2 %. Variations greater than 10 % either as a positive or negative value in the graphical scale of backscattering are representative of an unstable formulation.

9.5. High-pressure liquid chromatography (HPLC)

It is used to monitor the stability of pure drug substance and drugs in formulation with quantitation of degradation product. A liquid mobile phase is pumped under pressure through a stainless steel column containing particles of stationary phase with a diameter of 3-10 μm. The analyte is loaded onto the head of the column via a loop valve and separation of a mixture occurs according to the relative lengths of time spent by its components in the stationary phase.

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All components in a mixture spend more or less the same time in the mobile phase in order to exit the column. The column effluent can be monitored with a variety of detectors.

The combination of high-pressure liquid chromatography (HPLC) with monitoring by UV/Visible detection provides an accurate, precise and robust method for quantitative analysis of pharmaceutical products and is the industry standard method for this purpose. The two principal mechanisms which produce retardation of a compound passing through a column are straight-phase packing where adsorption of polar groups of a molecule onto the polar groups of a stationary phase occur and reverse-phase packing which is due to partitioning of the lipophilic portion of a molecule into the stationary phase.

9.6. Liquid chromatography–mass spectrometry (LC/MS)

Mass spectrometry in conjunction with liquid chromatography provides a method for characterizing impurities in drugs and formulation excipients [53]. It provides highly sensitive and specific methods for determining drugs and their metabolities in biological fluids and tissues.

9.7. Fourier Transform infra red (FTIR) spectroscopy

FTIR spectroscopic properties are used to determine the chemical stability of the drug in a TDDS. FTIR spectra of formulations, the starting materials and pure drug sample are normally obtained at a range of 4000-400 cm^{-1} and the spectra obtained on infrared spectrophotometer using potassium bromide of spectroscopic grade.

Detailed insights into the organization of the stratum corneum can be gained through the study of the vibrations of amide, amine and carboxylic groups and the frequencies of the methylene stretching, scissoring and rocking vibrations. FTIR is used to study the lateral lipid organization of the intercellular lipid matrix in stratum corneum, which is essential for the barrier function of stratum corneum, as more densely organized membranes are less permeable to substances. The stretching vibrations are used to determine whether lipids are in an ordered (hexagonal or orthorhombic lateral packing) or disordered packing (liquid phase), while the scissoring and rocking vibration provide detailed information on the presence of orthorhombic phases. By performing measurements at different temperatures, also the thermotropic behaviour of the lipids can be determined.

9.8. Attenuated Total Reflectance FTIR (ATR-FTIR)

Attenuated total reflectance FTIR (ATR-FTIR) is a modification of FTIR. In this technique, IR radiation is not transmitted through the sample but reflected by the sample. With this technique, it is possible to perform measurements on stratum corneum *in vivo*, because the skin can be placed on the ATR crystal. The IR radiation beam penetrates only to a limited extent into stratum corneum. In order to detect substances in the stratum corneum, it is necessary to remove stratum corneum layers, by tape-stripping, which makes it also possible to generate a penetration profile of an applied substance in stratum corneum [54-56]. ATR-FTIR has been used to determine effects of topically applied substances on the lipid organization in the

stratum corneum [54,57]. ATR-FTIR can be combined with tape-stripping to determine the penetration profile of hydrophilic and lipophilic substances in stratum corneum in addition to the water profile of the stratum corneum.

9.9. Differential scanning calorimetry (DSC)

This technology is used to evaluate the degree of perturbation of the skin lipids as a result of penetration of a formulation or drug through skin. The free intercellular lipid bilayers of the stratum corneum have a unique composition compared to other epithelial lipid bilayers and consist of ceramides (50%), cholesterol (25%), and fatty acids (10-20%, highly enriched in linoleic acid). These common skin lipids are detected at different transition temperatures when the skin is subjected to DSC studies.

9.10. Small angle X-ray diffraction (SAXD)

This technique is used to analyse the long range order of the crystalline structure of lipids. Stratum corneum is a very thin layer of about 10 μm and composed of corneocytes and an intercellular lipid matrix. The ordered structure of the intercellular lipid matrix plays an important role in skin barrier function. Structural analyses of intercellular lipids in mammalian stratum corneum by X-ray diffraction have shown more detailed lipid structure models. The X-ray pattern of a lamellar phase is characterized by a series of sequential maxima, which are positioned at equal interpeak distances at increasing scattering angle [58]. The sequential peaks are referred to as the 1st order (positioned at distance Q1), the 2nd order (Q2), the 3rd order (Q3), etc, in which Q is directly related to the scattering angle. The repeat distance (d) of a lamellar phase can be directly calculated from the peak positions $d=2\pi/Q1=4\pi/Q2=6\pi/Q3$, etc. In skin research SAXD is used to study the lamellar organization of the lipids in the intercellular matrix of stratum corneum of humans and other mammals. Furthermore, SAXD measurements using lipid mixtures of ceramides, cholesterol and free fatty acids have revealed the role of the various lipid classes in the lamellar phases. Additionally, it has been used to study effects of topically applied substances [59] or physical stratum corneum perturbation methods [54]. SAXD is also used to study the effects of hydrophilic and lipophilic agents like nanoparticles on the lamellar organization of isolated stratum corneum.

9.11. Dermal irritation assay

If a new drug is intended to be applied to the skin or eyes, one of the first tests to be conducted would be to determine if the drug, or the formulation containing the drug, will cause irritation of the skin or eyes. Even if a drug is intended only for dermal application, eye irritation testing may also be required because of the possibility of inadvertent exposure to the eyes. The test is conducted as follows: Six male albino rabbits are to be clipped free of hair on the back. One area of skin is left intact, whereas another is abraded in a tic-tac-toe pattern with the point of a hypodermic needle so as to incise the superficial epidermis layer without causing bleeding. The test material, 0.5 ml of liquid or 0.5 g of solid or semisolid is applied to each site under a 1 × 1 inch gauze pad. The entire trunk of the animal is wrapped with an impervious material and held in place with tape for 24 h. The patches are then removed and excessive material

wiped off. The skin reactions are scored at 24 and 72 h after the initial application according to a scheme such as that listed in Table 1.

Skin reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Table 1. Dermal irritation scoring system

The mean values of the six rabbits for erythema and eschar formation at 24 and 72 h for both intact and abraded skin (four values) are added. The mean values of the six rabbits for edema at 24 and 72 h (four values) are also added. The total of eight values is divided by 4 to give the primary irritation index. Values of 5 or greater are considered indicative of a positive irritant [60].

9.12. Occlusivity

It is usually the aim of cosmetic chemists to maintain the skin's softness and freshness and it is considered important to retain moisture in the stratum corneum. The degree to which a formulation retains or promotes the loss of moisture from the stratum corneum is termed the occlusivity. The occlusivity of formulations for topical application is determined *in vivo* by measuring the suppression of transepidermal water loss (TEWL) of the skin. The occlusive effect of the formulation also depends on the characteristics of the skin such as the lipid level and prevailing environmental condition. The occlusivity of films formed by nanoparticles varies with time, type of formulation, coating amount, physical form, size of particles etc. It is necessary to determine the occlusivity of a nanoparticle formulation for topical application as it directly affects liberation and penetration of the encapsulated drug. Under occlusive conditions, the skin is more hydrated and transport of drug could be higher.

9.13. Spreadability

Pharmaceutical semisolid preparations include ointments, pastes, creams, emulsions, gels, and rigid foams. Their common property is the ability to cling to the application surface for a reasonable period of time before they are washed off or worn off [61]. They usually serve as vehicles for topically applied drugs, as emollients, or as protective or occlusive dressings, or they may be applied to the skin and membranes such as the rectal, buccal, nasal, and vaginal mucosa, urethral membrane, external ear lining, or the cornea [62]. These preparations are widely used as a means of altering the hydration state of the substrate (i.e., the skin or the mucous membrane) and for delivering the drugs (topical or systemic) by means of the topical–mucosal route. Nanoparticles for transdermal application could be formulated as gels, creams, emulsions, foams etc, or dispersed in ointment bases. This makes the spreadability characteristics of the formulation very pertinent in achieving the desired objective.

The efficacy of topical therapy depends on the patient spreading the formulation in an even layer to deliver a standard dose. The optimum consistency of such a formulation helps ensure that a suitable dose is applied or delivered to the target site. This is particularly important with formulations of potent drugs. A reduced dose would not deliver the desired effect, and an excessive dose may lead to undesirable side effects. The delivery of the correct dose of the drug depends highly on the spreadability of the formulation. Spreadability, in principle, is related to the contact angle of the drop of a liquid or a semisolid preparation on a standardized substrate and is a measure of lubricity, which is directly related to the coefficient of friction [63]. Spreadability is subjectively assessed at shear rates varying from 10^2 to 10^5 s^{-1}. The rate of shear during spreading, γ s^{-1}, is calculated using the following equation for plane laminar flow between two parallel plates:

$$\gamma = \frac{v}{d} \tag{1}$$

in which v is the relative velocity of the plates (cm s^{-1}) and d is the distance between them (cm); that is, a measure of thickness of the film between the skin surfaces [64].

To assess the spreadability of a topical or a mucosal semisolid preparation, the important factors to consider include hardness or firmness of the formulation, the rate and time of shear produced upon smearing, and the temperature of the target site [64]. The rate of spreading also depends on the viscosity of the formulation, the rate of evaporation of the solvent, and the rate of increase in viscosity with concentration that results from evaporation [65].

9.14. Rheology

Rheology is the science that studies how materials deform and flow under the influence of external forces. Characterization of the rheological properties of the system is important not only in the design of the product and its application, but during its processing and to ensure long shelf-life [66]. It is thus necessary to explore the rheological changes that our formulations would experience when subjected to external forces during manufacture and in use. To that effect, measurements of the shear stress, strain, viscosity are done on the formulations. This

property can also be used to assess the stability of the formulation over time. To obtain information about viscous and elastic behaviour as well as microstructure of the topical gels, flow viscometry, oscillatory rheometry, and transient measurements are conducted.

10. Novel technologies for dermal and transdermal application

Nanoparticles for dermatological applications such liposomes and other vesicular systems as well as other types of nanosized drug carriers such as solid lipid nanoparticles, nanostructured lipid carriers, polymer-based nanoparticles and magnetic nanoparticles have been developed. These have in one way or the other, addressed the shortcoming of the traditional TDDS such as ointments, gels etc. Different carrier systems have been proposed in an attempt to favor the transport of drugs through the skin, enabling drug retention and in some cases allowing a controlled release [6]. Skin penetration is essential to a number of current concerns, e.g. contamination by microorganisms and chemicals, drug delivery to skin (dermatological treatments) and through skin (transdermal treatments), and skin care and protection (cosmetics) [6].

Physicochemical properties of nanocarrier systems determine the interaction with biological systems and nanocarrier cell internalization. The main physicochemical properties that affect cellular uptake are size, shape, rigidity, and charge in the surface of nanoparticles. The most used and investigated nanocarriers for dermal/transdermal drug delivery in the pharmaceutical field include liposomes, transfersomes, ethosomes, niosomes, dendrimers, nanoparticles-lipid and polymer nanoparticles, and nanoemulsions. In general, the advantages and limitations of using nanocarriers for transdermal drug delivery are their tiny size, their high surface energy, their composition, their architecture, and their attached molecules. Table 2 summarizes the advantages and disadvantages of common transdermal nanocarriers.

10.1. Microemulsions

Microemulsions are dispersions with droplet size from 10 to 100 nm and do not have the tendency to coalesce [67-69]. Microemulsions form spontaneously with appropriate amounts of a lipophilic and a hydrophilic ingredient, as well as a surfactant and a co-surfactant [70]. Microemulsions have several specific physicochemical properties such as transparency, optical isotropy, low viscosity and thermodynamic stability [70,71]. As efficient drug carriers, microemulsions have been widely employed in both transdermal and dermal delivery of drugs [72,73].

Most of the microemulsions have very low viscosity, which may restrict their application to the transdermal delivery field due to inconvenient use [74]. The main mechanisms to explain the advantages of microemulsions for the transdermal delivery of drugs include the high solubility potential for hydrophilic drugs of microemulsion systems, permeation enhancing effect of the ingredients of microemulsions, and the increased thermodynamic activity of the drug in the carriers [68,70,71].

10.2. Nanoemulsions

Nanoemulsions are isotropic dispersed systems of two nonmiscible liquids, normally consisting of an oily system dispersed in an aqueous system, or an aqueous system dispersed in an oily system but forming droplets or other oily phases of nanometric sizes. They are thermodynamically unstable systems, in contrast to microemulsions, because some nanoemulsions need high energy to produce them. They are susceptible to Oswald ripening, and as a consequence susceptible to creaming, flocculation, and other physical instability problems associated with emulsions. Despite this, they can be stable (metastable) for long periods due to their extremely small size and the use of adequate surfactants. Hydrophobic and hydrophilic drugs can be formulated in nanoemulsions. They are nontoxic and nonirritant systems, and they can be used for skin or mucous membranes and parenteral and non-parenteral administration in general, and they have been utilized in the cosmetic field. Nanoemulsions can be prepared by three methods mainly: high-pressure homogenization, microfluidization, and phase-inversion temperature. Transdermal delivery using nanoemulsions has decreased due to the stability problems inherent to this dosage form. Some examples of drugs using nanoemulsions for transdermal drug delivery are gamma tocopherol, caffeine, plasmid DNA, aspirin, methyl salicylate, insulin and nimesulide [75].

Presently, transdermal nanoemulsion formulations are not developed as much as nanoparticles or liposomes due to the stability problems inherent to this dosage form. Nevertheless, gamma tocopherol, caffeine, plasmid DNA, aspirin, methyl salicylate, insulin, and nimesulide have been included in nanoemulsions. The use of these nanocarriers to deliver analgesics, corticosteroids, anticancer agents, etc, is very important, as these drugs are able to act immediately because they do not need to cross extra barriers [76-82].

10.3. Vesicular systems

10.3.1. Liposomes

Liposomes (Fig. 5) are spherical, self closed vesicles of colloidal dimensions, in which phospholipid bilayer sequesters part of the solvent, in which they freely float, into their interior [83]. Their advantage, with respect to pharmaceutical application as drug carriers, is the wide variety of drugs to be incorporated as well as the biocompatibility, inherently connected with natural phospholipids. Regarding the penetration behavior of liposomes it is still under discussion if such objects might penetrate intact skin [84-87].

Liposomes have become one of the pharmaceutical nanocarriers of choice for many applications. Currently, many liposome-based drugs and biomedical products have been approved for use in clinic. They were used to study membrane processes and membrane-bound proteins. Liposomes were also proposed as drug carriers that reduce toxicity and increase efficacy. The nature of liposomes makes them one of the best alternatives for drug delivery because they are nontoxic and remain inside the bloodstream for a long time. They are being successfully used in cancer therapy and in skin melanoma [6]. However, to date many liquid-type nanocosmetic carriers, such as liposomes, are structurally unstable. Specifically, when passing through the skin, they adhere to the inside walls of the skin cells, causing the collapse of

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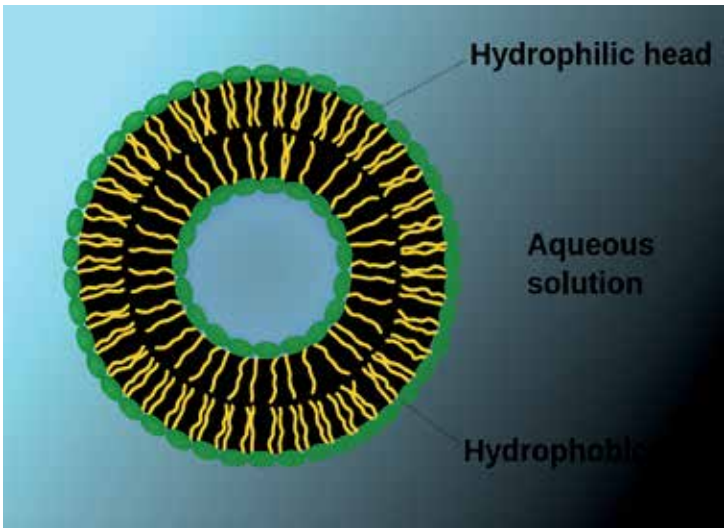

Figure 5. Structure of a liposome (http://en.wikipedia.org/wiki/Liposome. Downloaded on April 26, 2014)

phospholipid-association bodies and the leak of their encapsulated ingredients. As a result, their ability to transport active ingredients to deep skin is not likely good. For this reason, the use of flexible liposomes (transformable liposomes or transfersomes) has emerged as an invaluable strategy to reach the objective of drug delivery via the transdermal route. Examples of drugs delivered throughout the skin by using liposomes are melatonin, indinavir, methotrexate, amphotericin B, ketoprofen, estradiol, clindamicyn hydrochloride, and lignocaine, while examples of transdermal drug delivery using transformable liposomes (transfersomes) are diclofenac, insulin, tetanus toxoid, corticosteroids, superoxide dismutase, DNA, triamcinolone-acetonide, ketoprofen, interleukin-2, and ketotifen fumarate [88-94].

10.3.2. Niosomes

These are non-ionic surfactant vesicular novel drug delivery system in which the medication is encapsulated in a vesicle. Niosomes are unilamellar or multilamellar vesicles capable of entrapping hydrophilic and hydrophobic solutes. From a technical point of view, niosomes are promising drug carriers as they possess greater stability and lack of many disadvantages associated with liposomes, such as high cost and the variable purity problems of phospholipids [95]. Another advantage is the simple method for the routine and large scale production of niosomes without the use of unacceptable solvents. One alternative of phospholipids is the hydrated mixture of cholesterol and non-ionic surfactants such as alkyl ethers, alkyl esters or alkyl amides [95,96]. This type of vesicle formed from the above mixture has been known as niosomes or non-ionic surfactant vesicles. Niosome surfactants are biodegradable, biocompatible and non-immunogenic.

Niosomes are versatile carrier systems that can be administered through various routes, including transdermal delivery [97,98]. Particular efforts have been aimed at using niosomes

as effective dermal and transdermal drug-delivery systems [99,100]. In particular, niosomes are considered an interesting drug-delivery system in the treatment of dermatological disorders. Examples of transdermal drug delivery using niosomes are minoxidil and ellagic acid. Niosomes have been reported to enhance the residence time of drugs in the stratum corneum and epidermis, while reducing the systemic absorption of the drug, and improve penetration of the trapped substances across the skin. In addition, these systems have been reported to decrease side effects and to give a considerable drug release [101]. Niosomes formed from sorbitan monoesters (Spans) with cholesterol molar ratios of 1:1 are a promising approach for the topical delivery of minoxidil in hair-loss treatment [102]. Junyaprasert et al demonstrated that the Span 60 and Tween 60 niosomes may be a potential carrier for dermal delivery of ellagic acid [103].

10.3.3. Transfersomes

Transfersomes have been defined as specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. Accordingly, transfersomes resemble lipid vesicles, liposomes, in morphology but, functionally, transfersomes are sufficiently deformable to penetrate pores much smaller than their own size [104].

They are metastable, which makes the vesicle membrane ultraflexible, and, thus, the vesicles are highly deformable. It is chiefly the unusually strong membrane adaptability that allows the transfersomes vesicles to accommodate to a confining pore and thus trespass such a pore. Typical transfersomes are, therefore, characterized by at least one order of magnitude more elastic membrane than that of conventional lipid vesicles, liposomes. In order to change liposomes into transfersomes, one can incorporate one or more edge-active substance(s) into the vesicular membrane, surfactants were suggested as examples of such edge-activators [105-107]. Another specific difference between transfersomes and liposomes is the higher hydrophilicity of the former, which allows transfersome membrane to swell more than conventional lipid vesicle bilayers.

10.3.4. Ethosomes

Ethosomes are lipid vesicular carriers embodying ethanol in relatively high concentrations for enhanced skin permeation of drugs [108]. They are composed mainly of phospholipids, ethanol and water. The high concentration of ethanol, which essentially differentiates ethosomes from other vesicular carriers, acts to enhance skin permeation in order to release the entrapped drug particles into deeper layers and systemic circulation. Ethosome was developed by Touitou in 1996, in the course of studying the use of lipid vesicles in drug delivery systems for skin treatment [109].

Structurally, an ethosomal vesicle is composed a phospholipid bilayer and an aqueous inner core containing the entrapped active ingredient. They are soft and malleable. The size of an ethosome vesicle lies within the nanometer range [110]. In addition, the size of ethosome vesicle is smaller than that of a liposome when prepared under the same condi-

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tions, due to the high alcohol content. The size decreased as alcohol increased from 20 to 45 % [39]. This reduction in size was attributed to the conferment of a net negative charge on the vesicle surface by ethanol. Other excipients usually added in ethosome formulation include cholesterol, for vesicle membrane stabilization; permeation enhancers, marker dyes (if required) such as rhodamine, for characterization study. The stabilizing effect of cholesterol is due to prevention of vesicle aggregation and enlargement during storage [111]. The small size and malleability of ethosomes enable them to pass through the skin or membrane barrier and also influence the extent of transdermal permeation. The smaller the size, the greater the extent of penetration [108].

Ethosomes permeate through the stratum corneum barrier and posses significantly high transdermal flux unlike classical liposomes. These effects of combined phospholipids and high concentration of ethanol in vesicular formulations have been suggested to be responsible for deeper distribution and penetration in the skin lipid bilayers [110]. Application of ethosomes in drug delivery has numerous advantages [112-114]: simplicity of the technology, non-invasive means of application (e.g., topical), enhanced transdermal drug delivery, and avoidance of first-pass effect. Non-invasiveness enhances patient's compliance hence, better therapeutic outcome. Ethosomes have been shown to exhibit high encapsulation efficiency for a wide range of molecules including lipophilic drugs due to the multilamellarity of the vesicles as well as the presence of ethanol, which allows for better solubility of many drugs [39,115]. Unlike liposomes and transfersomes, ethosomes were able to improve skin delivery of drugs both under occlusive [117] and non-occlusive conditions [111,118,119].

The application of transformable liposomes, which are prepared using alcohol (ethosomes) in the lipid bilayer of stratum corneum, able to deform and penetrate throughout the skin when pressure is applied, has been increased. For example, tacrolimus-loaded ethosomes may be useful as a therapeutic agent for atopic dermatitis [120]. Skin permeation of ethosomal formulations assessed by confocal microscopy revealed enhanced permeation of Rhodamine 123-loaded formulation in comparison to the hydroalcoholic solution. Another ethosomal formulation has proved to be a potentially useful vehicle for transdermal delivery of ketoprofen [121]. Furthermore, an ethosomal carrier (phosphatidylethanolamine) is an optional treatment for psoriasis that provides long-term therapeutic effects, is nontoxic, and has better compliance with patients. Application of ethosomal carriers with 5-aminolevulinic acid (ALA) in hyperproliferative murine skin can improve the penetration of ALA and the formation of protoporphyrin IX and significantly reduce tumor necrosis factor in this disordered skin compared to an ALA aqueous solution [122].

Ethosomes were used efficiently to enhance the anti-inflammatory activity of ammonium glycyrrhizinate compared to the ethanolic or aqueous solutions of this drug [123]. Moreover, the ethosomal system dramatically enhanced the skin permeation of minoxidil *in vitro* compared with either ethanolic or hydroethanolic solution or phospholipid ethanolic micellar solution of minoxidil. In addition, the transdermal delivery of testosterone from an ethosomal patch was greater both *in vitro* and *in vivo* than from commercially available patches [124]. Examples of transdermal drug delivery using ethosomes are tacrolimus [120], clotrimazole [125], trihexyphenidyl HCl [126], ketoprofen [121] and testosterone.

10.4. Dendrimers

Dendrimers are nonpeptidic fractal 3-D structures made of numerous small molecules. The structure of these molecules results in relatively uniform shapes, sizes, and molecular weights. The permeability of dendrimers through the skin depends on physicochemical characteristics like generation size, molecular weight, surface charge, composition, and concentration. These nanocarriers have been used to transport photosensitizers for photochemical therapy and antifungal molecules.

Dendrimers have been utilized for transdermal drug delivery, as shown in Table 1. The main problems with this kind of transdermal carrier are their poor biodegradation and inherent cytotoxicity [127]. The main advantage of dendrimers is that they have multivalency [128] and it is possible to precisely control the functional groups on the surface [129]. Due to their form and size, these molecules can carry drugs, imaging agents, etc. Dendrimers interact with lipids present in membranes, and they show better permeation in cell cultures and intestinal membranes. Dendrimers also act like solubility enhancers, increasing the permeation of lipophilic drugs. However, they are not good carriers for hydrophilic drugs. Examples of drugs delivered throughout the skin by using dendrimers are tamsulosin [130], indomethacin [131], ketoprofen, diflunisal [132], 5-fluorouracil [133] and peptides [134].

Nanocarrier	Advantages	Disadvantages
Nanoparticles	• They can be made of a lot of biodegradable materials.	• Not enough toxicological assessment has been done.
	• There are many ways to prepare them.	• It is difficult to develop an analytical method for drug delivery.
	• They can include antibodies in their surface to reach target organs.	• Some processes are difficult to scale up.
	• Both hydrophilic and hydrophobic drugs can be loaded in ananoparticle.	• Sometimes, the size they reach is not enough to avoid the immune system.
	• They are able to avoid the immune system due to their size.	
Nanoemulsions	• They can be formulated as foams, liquids, creams, and sprays.	• They are susceptible to Oswald ripening.
	• They are nontoxic and nonirritant.	• Surface charge has a marked effect on stability.
	• Easily applied to skin and mucous membranes.	• Variable kinetics of distribution processes and clearance.
Liposomes	• Control release based on natural lipids.	• When high-pressure homogenization is used, decreased stability of high molecular weight molecules.

Nanocarrier	Advantages	Disadvantages
	• High biocompatibility.	• Lipid crystallization leads to a lot of polymorphic issues.
	• Simple manufacture.	• Variable kinetics of distribution processes.
	• Protein carriers increase their stability.	• They are susceptible to physical instability.
	• High drug loads.	
Dendrimers	• They increase stability of therapeutic agents.	• They have shown cellular toxicity.
	• They are easily prepared and functionalized.	• Elimination and metabolism could be a problem depending on the generation and materials.
	• They increase bioavailability of drugs.	• Their synthesis costs are higher than other nanocarriers.
	• They covalently associate drugs.	• Hemolytic effects can be found.
	• Dendrimers also act like solubility enhancers, increasing the permeation of lipophilic drugs.	• They are not good carriers for hydrophilic drugs.
Niosomes, transfersomes, ethosomes	• Biodegradable and low toxicity.	• Predisposition to oxidative degradation.
	• Easy to prepare.	• Purity of natural phospholipids.
	• Softness, malleability.	• Formulations may be expensive.
	• They can encapsulate both hydrophilic and lipophilic moieties.	
	• Ability to target organs for drug delivery.	
	• Extremely high flexibility of their membrane.	

Table 2. Advantages and disadvantages of nanocarrier systems for transdermal drug delivery [6, 75].

10.5. Lipid nanoparticles

Lipid nanoparticles include solid lipid nanoparticles (SLN), nanostructured lipid carriers, lipid drug conjugates and are colloidal drug carrier systems [135-137]. They are very much like nanoemulsions, differing in lipid nature. The liquid lipid used in emulsions is replaced by a lipid solid at room temperature in SLN including high-melting point glycerides or waxes [136,138,139]. Lipid nanoparticles are good candidates for transdermal delivery. They can be prepared in different sizes and it is possible to modify surface polarity in order to improve skin penetration. From the upper skin, nanoparticles can reach deeper skin regions because they exhibit mechanical flexion [6].

10.5.1. Nanostructured lipid carriers (NLC)

NLC are colloidal carriers characterized by a solid lipid core consisting of a mixture of solid and liquid lipids, and having a mean particle size in the nanometer range. They consist of a

lipid matrix with a special nanostructure [140]. This nanostructure improves drug loading and firmly retains the drug during storage. NLC system minimizes some problems associated with SLN such as low payload for some drugs; drug expulsion on storage and high water content of SLN dispersions.

The conventional method for the production of NLC involves mixing of spatially very different lipid molecules, i.e. blending solid lipids with liquid lipids (oils). The resulting matrix of the lipid particles shows a melting point depression compared with the original solid lipid but the matrix is still solid at body temperature. Depending on the method of production and the composition of the lipid blend, different types of NLC are obtained. The basic idea is that by giving the lipid matrix a certain nanostructure, the payload for active compounds is increased and expulsion of the compound during storage is avoided. Ability to trigger and even control drug release should be considered while mixing lipids to produce NLC. Newer methods of generating NLC have been developed [141].

10.5.2. Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are formed by a matrix of lipids which are biodegradable raw materials that are physiologically well tolerated [142]. The main advantages of these systems include protection of labile substances from chemical degradation, control of the release of substances due to the solid state of the lipid matrix, and formation of films over the skin showing occlusive properties [140]. Additional features are the avoidance of organic solvents during the preparation and amenability to large scale production and sterilization. Furthermore, the great ability of SLNs to facilitate the contact of active substances with the stratum corneum, because of the small size of the particles and consequently the high surface area, leads to the high permeation of the carried substances through the viable skin [143].

The degree of crystallinity of lipid nanoparticles has a great impact on the extent of occlusion by the formulation. With increasing crystallinity, the occlusion factor increases as well [142]. This explains why liquid nanoemulsions in contrast to SLNs do not show an occlusive effect and why the extent of occlusion by nanostructured lipid carrier (NLC) compared to SLN is reduced. Other parameters influencing the occlusion factor are the particle size and the number of particles. The occlusion factor decreases with increase in particle size but increases with increase in number of particles. The occlusive effect leads to reduced water loss and increased skin hydration. Highly crystalline SLNs can be used for physical sun protection due to scattering and reflection of the ultra violet (UV) radiation by the particles. A high crystallinity was found to enhance the effectiveness and was also synergistic with UV absorbing substances used in conventional sunscreens. Similarly, synergism was observed on the sun protection factor and UV-A protection factor exhibited by the incorporation of the inorganic sunscreen, titanium-dioxide in NLC of carnauba wax and decyloleate [144]. Fig. 6 shows the structures of some lipid nanodispersed vehicle systems.

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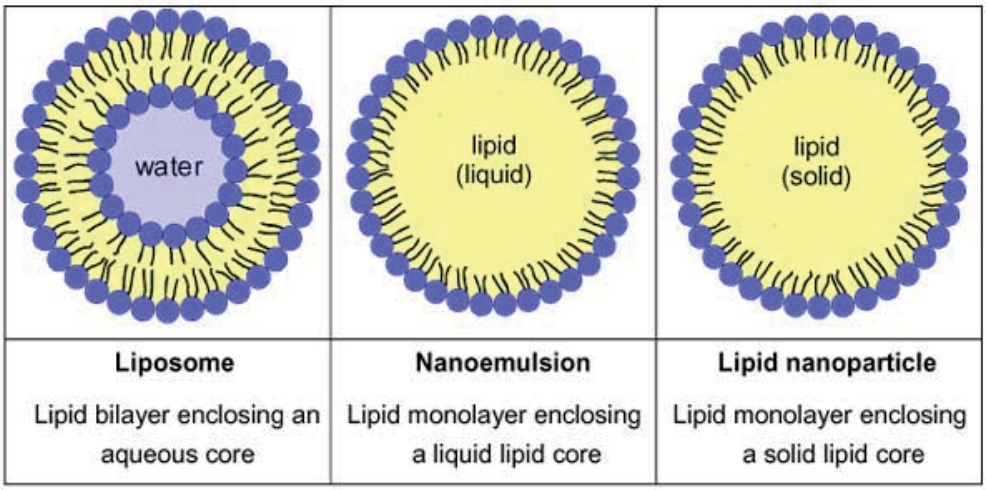


(http://www.skin-care-forum.basf.com/en/author-articles/strategies-for-skin-penetration-enhancement/2004/08/12?id=5b9a9164-6148-4d66-bd84-6df76bd6d111&mode=Detail. Downloaded April 26, 2014).

Figure 6. Structure of lipid nanodispersed vehicle systems

10.6. Polymeric nanoparticles

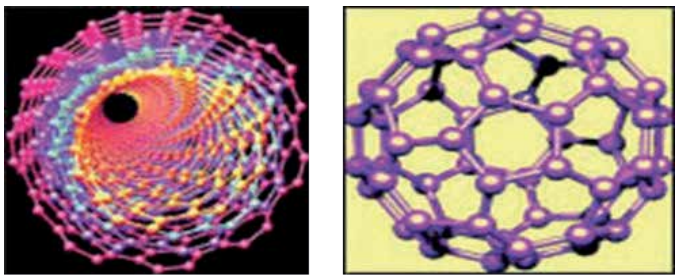
Polymeric nanoparticles are prepared from biocompatible and biodegradable polymers with size between 10-1000 nm, where the drug is dissolved, entrapped, encapsulated or attached to a polymer nanoparticle matrix. The penetration and transport extent of these systems through the skin depends on the ingredients' chemical composition, the encapsulation mechanism influencing the drug release, the size of nanoparticles and on the viscosity of the formulations. The polymeric nanoparticles are able to modify the activity of drugs, delay and control the drug release, and increase the drug adhesivity or its time of permanence in the skin. Briefly, the nanoparticles can be useful as reservoirs of lipophilic drugs to deliver them in the stratum corneum becoming an important strategy to control their permeation into the skin [145].

10.7. Carbon nanotubes (CNT) and fullerenes

Carbon nanotubes are stable carbon nanoparticles with potential anti-oxidant ability and cytoprotective effect. Carbon nanotubes (Fig. 7) have extremely small mean diameters (<100 nm). Their large inner volume allows the loading of small biomolecules while their outer surface can be chemically modified to render themselves various novel features that can be used to load proteins and genes for effective drug delivery [146], even through the skin.

Fullerenes (Fig. 7) are 1-nm scale carbon spheres of 60 carbon atoms. Although fullerenes are hydrophobic, they can be organically functionalized by attaching hydrophilic moiety and become water-soluble and capable of carrying genes, proteins and other biomolecules for delivery purposes [146]. Their small size, spherical shape and hollow interior all provide

therapeutic opportunities and have been proposed for use in cosmetic products like sunscreens, moisturizers, long lasting makeup, etc.



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3853888/figure/F4/#108;93 A; http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3853888/figure/F5/#117.75;90.75 B

Figure 7. Carbon nanotube (A) and fullerene (B)

11. Influence of nanoparticulate formulations on biochemical processes of the skin

Apart from environmental protection against radiation, functions of the skin include heat regulation, immune response, biochemical synthesis, sensory detection, regulation of absorption/loss of water and electrolytes. The stratum corneum formed from nonviable corneocytes plays the major role.

A crucial question for the investigation of nanoparticulate drug delivery carriers is the site of drug release from the particles, i.e., does the release occur in suspension or on the skin surface leaving the carrier particles outside or do the particles penetrate the skin to release the drug within the tissue? Nanoparticles formulations for dermal delivery or transdermal delivery influenced some of the traditional functions of the skin. Once applied to the skin, enzymes activated by body heat led to the formation of an active ingredient (allyl isothiocynate). Transport of the active drug component took place by passive diffusion across the skin-the very basis of transdermal drug delivery [147,148]. The alcohol in ethosomes initiates the process of transdermal permeation and drug release by its permeation enhancing effect [149]. The major hindrance to TDDS is the stratum corneum layer that forms a strong barrier and limiting factor to skin penetration and permeation of many drugs.

The processes involved in drug delivery from ethosomes through the skin are illustrated in Fig. 8 [46]. The alcohol makes the vesicles to be packed loosely and the vesicle membranes to become softer and malleable [13]. It also causes reversible perturbations in the deeper layers of SC and penetrates intercellular lipid layers of skin cell membranes making them more fluid

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and less dense [150]. The vesicles then squeeze through the intercellular spaces into the deeper layers of skin. It has been shown that drug particles are concentrated more on the inside wall than in the core of vesicles [151]. In this position, release of the vesicular content is thermodynamically favored. Owing to the increased affinity, due to its lipid content, the vesicle fuses with the lipid contents of the skin layers and releases its content which then diffuses into deeper layers of the skin or membrane and into systemic circulation. Other mechanisms, such as the free drug diffusion, may be involved in penetration.

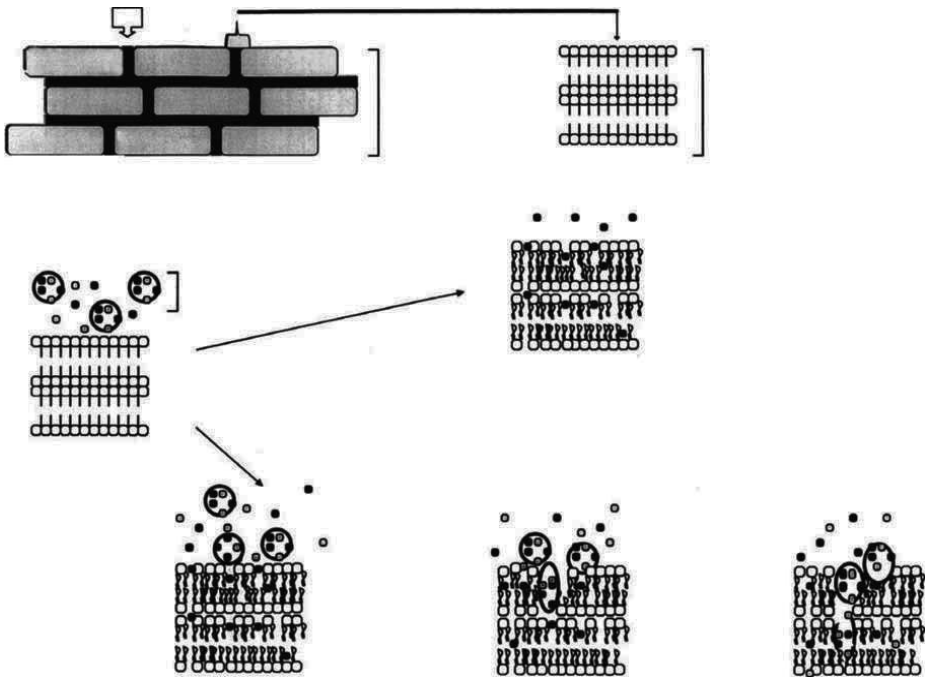

Figure 8. Mechanism of drug delivery from ethosomal vesicular carriers through the skin [46,115]. Note the initial fluidization of the skin architecture.

12. Regulations on dermal and transdermal delivery systems

Safety and toxicological issues are the most important issues for a drug delivery system. Safety is an obvious concern for the fast growth of nanoparticles mediated drug delivery [152]. Governmental regulatory agencies such as the United States Food and Drug Agency (USFDA) have established guidelines describing the kind of safety tests that should be conducted in animals in order to have a new drug approved for use in clinical trials and in order to get approval of a new drug application (NDA) for marketing. The rationale and circumstances for conducting reproductive, mutagenicity, carcinogenicity, irritation, and sensitization studies have already been mentioned. The requirements for acute, subacute, and chronic toxicity

studies for pharmaceutical products intended for use in humans as described according to are the requirements of the United States, Japan, and Europe because these areas represent the largest pharmaceutical markets in the world today. These requirements have been developed at the International Conference on Harmonization to provide uniformity among the three regions [153]. Phases I, II, and III refer to the different phases of human clinical trials. Phase I refers to the initial trials, limited to one or a few doses to determine absorption, pharmacokinetics, and an initial estimate of safety. Phase II refers to larger scale studies to establish safety and to get an initial estimate of clinical efficacy. Phase III refers to the final, large-scale, multicenter trials aimed at establishing efficacy.

The Food and drug agency (FDA) paradigm for regulation of new products is based on the concepts of risk management, which includes identification, analysis and control of risk [154]. The regulation and approval by the FDA is on a ''product by product'' basis, with the overall regulation process falling into three stages: premarket approval, premarket acceptance and post-market surveillance.

Premarket approval: Prior to market introduction of any new pharmaceuticals, high-risk medical devices, food additives, colors, and biologicals, FDA approval is required. The producer/sponsor of the product is responsible for identifying and assessing the risks presented by the product. This party will also be responsible for indicating means to minimize the risks in a product application.

Premarket acceptance: This category refers to products that are often copies of similar products that were approved previously or are products prepared according to approved specifications. For these products, the FDA receives and reviews some form of notice that the products will be marketed and the products undergo a more rapid review process than premarket approval.

Postmarket surveillance: In this category, FDA manages the risks of GRAS products like foods, cosmetics, radiation emitting electronic products and materials such as food additives and food packaging. For products in this category, market entry, and distribution are at the discretion of the manufacturer/producer. These products are generally regulated by the application of good manufacturing practices. FDA takes regulatory action if adverse events that threaten public or individual health occur.

The FDA coordinates policies within itself and with other government agencies. As and when new toxicological risks that derive from the new materials and/or new conformations of existing materials are identified, the FDA will require new tests.

The FDA regulations are for products, not technologies. In addition, the FDA regulates only the claims made by the product sponsor. If the manufacturer makes no nanotechnology claims regarding the manufacture or performance of the product, the FDA may be unaware at the time that the product under review employed nanotechnology. Finally, the FDA has only limited authority over some potentially high-risk products, such as cosmetics. Many products are regulated only if they cause adverse health-related events in use. To date there have been few resources available to assess the risks of these products.

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13. Dermal and transdermal formulations on the market

A lot of dermal and transdermal drug delivery systems have been licensed for manufacture after passing through the regulatory approval and trials as specified by different countries example FDA (United States of America). Some of the drugs currently available on the market are presented in Table 3.

Drug	Trade name	Type of transdermal patch	Manufacturer	Indication
Fentanyl	Duragesic	Reservoir	Alza/Janssen Pharmaceutica	Moderate/ Severe pain
Nitroglycerine	Deponit	Drug in adhesive	Schwarz Pharma	Angina Pectoris
	Minitran	Drug in adhesive	3M Pharmaceuticals	
	Nitrodisc	Micro reservoir	Searle, USA	
	Nitrodur	Matrix	Key Pharmaceuticals	
	TransdermNitro	Reservoir	Alza/Novartis	
	Nitroderm TTS	Face	Novartis	
	Diafusor	Matrix	Schering-Plough	
	Transdermal-NTG	Rim	Warner Chilcott Lab	
	Nitrocine	Rim	Kremer Urban	
	Nitro patch	Rim	Adria Lab	
	NTS patch	Rim	Bolar, Major, Qualitest, Bio-Line, Goldline, Geneva, Rugby WarnerChilcott Lab	
Isosorbide dinitrate	Frandol Tape	Matrix	Toaeiyo, Yamanouchi Pharm.	
Nicotine	Prostep	Reservoir	ElanCorp/Lederie Labs	Smoking Cessation
	Nicotrol	Drug in adhesive	Cygnus Inc./McNeil Consumer Products Ltd.	
	Nicotinell	Matrix	Novartis	
	Nikofrenon	Matrix	Novartis	
	Habitraol	Drug in adhesive	Novartis	
Testosterone	Androderm	Reservoir	Thera Tech/ GlaxoSmithKline	Hypogonadism in males
	Testoderm TTS	Reservoir	Alza	
Clonidine	Catapres-TTS	Membrane matrix hybrid type	Alza/Boehinger Ingelheim	Hypertension
Lidocaine	Lidoderm	Drug in adhesive	Cerner Multum, Inc.	Anesthetic

Drug	Trade name	Type of transdermal patch	Manufacturer	Indication
Scopolamine	Transderm Scop	Membrane matrix hybrid type	Alza/Novartis	Motion sickness
Hyoscine	Trasiderm-Scop	Matrix	Novartis	
	Kimite-patch	Matrix	Myun Moon Pharm. Co.	
Minoxidil 4%	Nanominox		Sinere, Germany	Hair growth promoter
Acyclovir	Supravir cream		Trima, Israel	herpes infection.
Many ingredients	Cellutight EF		Hampden Health, USA	Topical cellulite
Estradiol	Climara	Drug in adhesive	3M Pharmaceuticals/ Berlex Labs	Postmenstrual Syndrome
			Noven Pharma/Novartis	
	Vivelle	Drug in adhesive	Alza/Novartis.	
	Estraderm	Reservoir	Women First Healthcare, Inc	
	Esclim	Drug in adhesive	Johnson & Johnson	
Ethinyl Estradiol	Ortho Evra	Drug in adhesive		

Table 3. Currently available medications for transdermal delivery [155,156].

14. Dermal and transdermal delivery of phytopharmaceuticals

Novel drug delivery system is a novel approach to drug delivery that addresses the limitations of the traditional drug delivery systems. Phytopharmaceuticals are pharmaceuticals using traditional compounds derived from botanicals instead of chemicals. Because these natural ingredients are more easily and more readily metabolized by the body they produce fewer if any side effects and provide increased absorption in the bloodstream resulting in more thorough and effective treatments unlike pharmaceuticals produced from chemical compounds which are prone to adverse side effects [157]. The formulation of dermal and transdermal delivery of phytopharmaceuticals is gaining interest owing to the benefits accruable from it. One of the first few attempts to utilize TDDS containing phytopharmaceuticals was investigation aimed to formulate transdermal films incorporating herbal drug components such as boswellic acid (*Boswellia serrata*) and curcumin (*Curcuma longa*), which utilizes skin as a site for continuous drug administration into the systemic circulation [157]. TDDS avoids first pass metabolism of the drug without the pain associated with injection; moreover the system provides a sustained drug delivery with infrequent dosing via zero-order kinetics and the therapy can easily be terminated at any time. For the local action of the drug at the site of administration of TDDS, turmeric are used which is considered a new version of ayuverdic turmeric *poultic*e or *lepa* [158].

Application of vesicular encapsulation holds great promise in the development and use of phytomedicines considering the difficulties of their formulation into stable dosage forms. Certain physicochemical properties of many herbal extracts make their formulation difficult due to stability and processing challenges. By using appropriate techniques, vesicular products of herbal extracts with enhanced stability and efficacy have been produced. A new drug delivery device known as phytosome, composed of phosphatidylcholine, has been developed to overcome the poor absorption of flavonoids, a challenge due mainly to their large molecular sizes and poor miscibility with the lipid contents of cell membrane linings [159]. Phytosomes are well absorbed when taken orally.

Evaluations of phytosomes indicate that a bond is formed between a flavonoid and a phosphatidylcholine molecule to form a hybrid that is highly lipid-miscible. The development and applications of a variety of novel vesicular herbal formulations such as liposomes, phytosomes, transfersomes and ethosomes have been reported [160,161]. Ethosomes, by virtue of their special characteristics, may circumvent the hindrances to successful delivery of phytomedicines. Both soluble and insoluble phytomedines can be encapsulated in ethosomes. Ethosomes also offer protection from premature degradation and increased biodistribution, which would make for improved bioavailability and more beneficial therapeutic outcome for TDDS.

15. Conclusion

From the myriad published studies involving nanoparticles, it is clear that nanoparticles have the potential to effectively deliver drugs across the skin barrier. Conventional liposomes, flexible liposomes, ethosomes, niosomes and ultradeformable liposomes, etc offer potential value as dermal and transdermal drug delivery systems in addition to other lipid nanoparticles.

Author details

Okoro Uchechi, John D. N. Ogbonna and Anthony A. Attama*

*Address all correspondence to: anthony.attama@unn.edu.ng

Drug Delivery Research Unit, Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria

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Chapter 7

Nanoparticle Insulin Drug Delivery — Applications and New Aspects

Hatice Kübra Elçioğlu and Ali Demir Sezer

Additional information is available at the end of the chapter

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1. Introduction

Insulin is a hormone secreted from the β cells of the islets of Langerhans, specific groups of cells in the pancreas. Insulin is a protein consisting of two polypeptide chains, one of 21 amino acid residues and the other of 30, joined by two disulfide bridges. It was isolated in 1921 with its first clinical use in 1922 [1]. Insulin is prepared different techniques; One of these isolated from animals and the other is biotechnological preparation using with the recombinant DNA techniques [2, 3].

Insulin is a important player in the control of intermediary metabolism and profound effects on both carbohydrate and lipid metabolism. It has significant influence on protein and mineral metabolism [4, 5].

The traditional and most predictable method for the administration of insulin is by subcutaneous injections. This method is often painful and hence, deterrent to patient compliance especially for those requiring multiple dose injections of four times a day. Also, there have been reports of hypoglycemic episodes following multi dose injections of insulin [6, 7]. Several new approaches to the method have been adopted to decrease the suffering of the diabetic patients including the use of supersonic injector, infusion pump, sharp needles and pens. Some insulin delivery routs so problematic way for example oral administration; Oral delivery eliminates the pain caused by injection, psychological barriers associated with multiple daily injections. Oral delivery of insulin as a non-invasive therapy for Diabetes Mellitus is still a challenge to the drug delivery technology, because insulin is degraded by the enzymes in the acidic environment of stomach. Otherwise insulin delivery via transdermal delivery is so popular way of insulin administration but there are some disadvantages of this route, for example insulin molecular size and application problems etc. While some of them eased the pain encountered by the diabetic patients, they offer incomplete convenience. Even though

the ultimate goal would be to eliminate the need to deliver insulin exogenously and regaining the ability of patients to produce and use own insulin, new concepts are currently explored to deliver insulin using oral, pulmonary, nasal, ocular and rectal routes [8, 9].

The success of the route of administration is judged on the basis of its ability to elicit effective and predictable lowering of blood glucose level and therefore minimizing the risk of diabetic complications. It is clear that several difficulties have to overcome with the use of formulation and application devices technology [10, 11]. The various explored routes are reviewed in this chapter. On the other hand, the chapter is an attempt to illustrate the use of insulin drug delivery and their body route in diabetes management benefiting many diabetic patients with promising patient compliance.

Diabetes mellitus (DM) which is a metabolic disorder characterized by chronic hyperglycemia (increased blood and hepatic glucose levels) with disturbances in carbohydrate, fat and protein metabolism, resulted by diminished insulin secretion, impaired insulin action or both. It's expected to increase from 171 million in 2000 to 366 million by the year 2030 as predicted by the WHO so it continues to increase in prevalence and will become a serious threat of mankind health [12]. Insulin injections remain to be preferred approach for the treatment of insulin-dependent diabetes mellitus (T1DM) and for many patients non-insulin-dependent diabetes mellitus (T2DM) also. People with type 1 diabetes mellitus have an autoimmun mediated destruction of pancreatic islet beta-cells and insulin deficiency. T1DM usually occurs in children and young adults and require daily insulin administration by injection or an insulin pump for survival. On the other hand, insulin resistance (which is associated with excessive glucose production by the liver and impaired glucose utilization by peripheral tissue, especially muscle) is observed in T2DM. They have an impaired endogenous insulin secretion to deal with the increased blood glucose level and majority needs oral antidiabetic drugs. As the disease progresses, the pancreas looses its ability to produce insulin and necessity of insulin therapy increases [12, 13, 14].

Hyperglycemia, recurrence of ample fluctuation of blood glucose levels and insulin resistance can lead to long term complications such as micro and macrovascular. It is well known that improved metabolic control significantly reduces both microvascular (ie, retinopathy, nephropathy and neuropathy) or macrovascular [ie, cardiovascular disease (CVD), cerebrovascular accidents and peripheral vascular disease] complications in diabetes. The development of complications is a cause of considerable morbidity and increases disability and mortality for the individual with diabetes [15].

The conventional pharmacotherapies currently available for the treatment of type-2 diabetes include insulin sensitisers (metformin and thiazolidinediones), insulin secretagogues (sulphonylureas and glinides), alpha-glucosidase inhibitors, insulin and insulin analogues. Glucagon like peptide (GLP)-1 agonists and dipeptidyl peptidase (DPP)-4 inhibitors are the new therapies; that improve glycemic control have recently been developed [16, 17, 18, 19]. These therapies are proposed to treat the key metabolic abnormalities associated with T1DM and T2DM and minimize the side effects noted with conventional therapies. Also in development there are additional therapies that have effects on the kidney to promote glucose excretion [15]. SGLT-2 (proximal renal tubule) has high transport capacity for reabsorption of approximately

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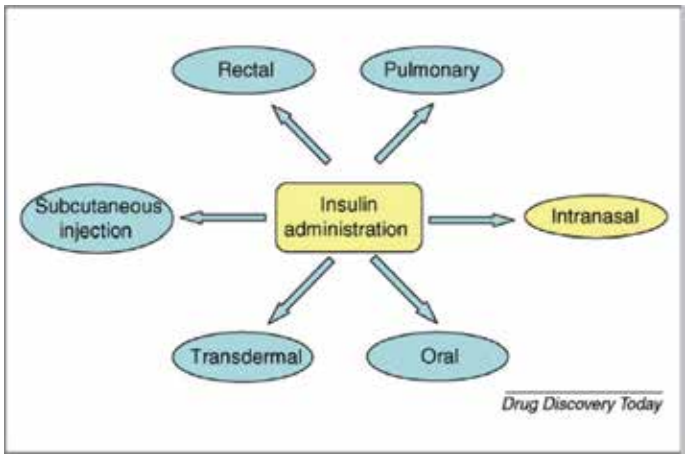

Figure 1. The main administration routes for insulin delivery (Reproduced with permission from Ref. [20], Copyright 2013 Elsevier)

90% of primarily filtered glucose. SGLT-2 inhibitors inhibit glucose reabsorption in proximal renal tubule. It results glycosuria leads to a decline in plasma glucose level. A wide variety of SGLT-2 inhibitors are currently under development with Dapagliflozin, Canagliflozin, Empagliflozin being the most advanced substances. Excretion of approximately 40% of primarily filtered glucose translates to a loss of 50–100 g glucose every day. The consequential decline in fasting and postprandial glucose leads to an HbA1c reduction of approximately 0.8%. The loss of energy substrate reduces body weight approximately 3 kg.

Current therapy for diabetes mellitus through oral anti-diabetic drugs and subcutaneous administration of insulin suffers from serious disadvantages, such as patient noncompliance and occasional hypoglycemia. Moreover, these approaches don't mimic the normal physiological fate of insulin release and doesn't provide better glucose homeostasis. In normal human physiology when the blood glucose level increases insulin releases from the pancreas, reaches to the hepatic portal vein and goes to liver which is its primary site of action. Subcutaneous administration of insulin moves firstly peripheral tissues and can produce peripheric hyperinsulinemia. In order to overcome the problems associated with parenteral administration of insulin, substantial progress has been made for insulin route such as ocular, vaginal, rectal, oral, pulmonary, transdermal, intranasal, and other routes (Figure 1) [20]. The barriers to reaching the bloodstream are either physical, such as poor absorption at barrier surfaces, or chemical, such as pH inactivation and enzymatic degradation. Delivery of insulin via the ocular route was tested in animal models in combination with different absorption enhancers, with particular attention given to toxicity as polymers were added to overcome low absorption. Vaginal and rectal routes of insulin have also been evaluated but the absorption rate and bioavailability are poor due to the thick mucosal layers in these tissues. Lots of absorption enhancers (bile salts, chelating agents, surfactants, cyclodextrins, and dihydrofusidate) used but they couldn't prevent local reactions with severe complications.

Nasal delivery has also been evaluated because of the easy access, high vascularity and large absorption area associated with this route. Unfortunately, highly active mucociliary clearance in the nose hindered prolonged drug action resulting in poor bioavailability. Buccal and sublingual insulin administration provide better results due to the low levels of proteolytic enzyme activity, the high vascularization of the tissue, the large surface area for absorption and the ease of administration. Unlike other delivery routes, the gut is the natural route of nutrient absorption into the circulation. The fact that the gut presents the largest absorption surface of all routes provides better efficacy. However, the multiple layers of oral epithelial cells represent a significant GI barrier to drug penetration, which, coupled with the continuous flow of saliva, leads to poor efficacy.

Taking all of this account oral administration is considered to be the most safest and convenient which delivers the drug directly into the liver through portal circulation, where it inhibits hepatic glucose production. Hence by oral delivery to a greater extent the natural physiological route of insulin can be mimicked (Figure 2) [21]. The highly acidic environment in the stomach and the presence of proteolytic enzymes cause structural instability of the oral delivery of protein and peptide drugs including in the harsh environment of the gastrointestinal system [22, 23, 24]. These drugs should overcome some various GI barriers such as chemical, enzymatic and absorption barriers to obtain adequate bioavailability [25]. Different formulation of polymers for insulin delivery such as liposomes, microspheres, microemulsion and nanoparticles (NPs) have been investigated to circumvent these GI barriers [26, 27].

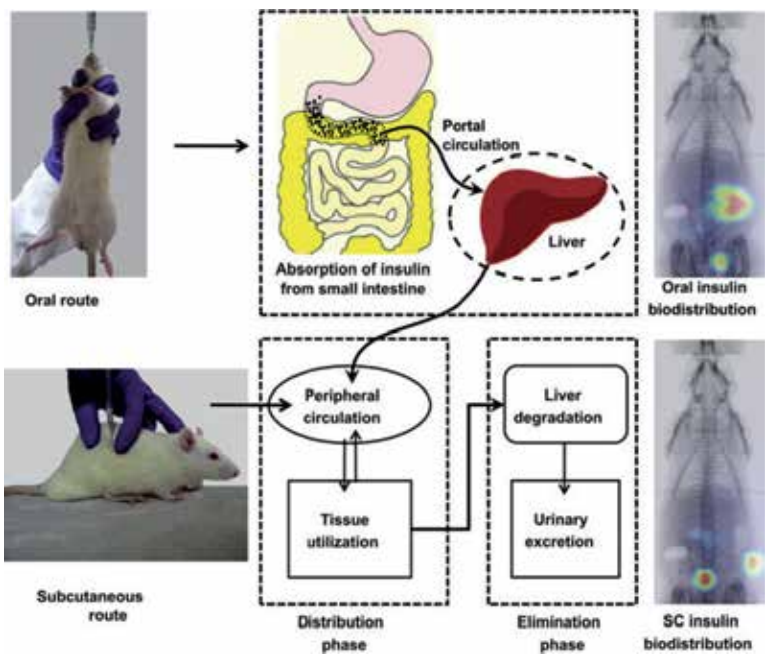

Figure 2. Schematic diagrams illustrating the absorption, distribution and elimination of aspart insulin following oral or subcutaneous (s.c.) administration to rats (Reproduced with permission from Ref. [21], Copyright 2013 Elsevier).

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Among these approaches nanoparticular sysytems have attracted special interest because of providing the protection to the highly acidic medium in the stomach (preventing enzymatic degradation), prolonging intestinal residence time, increasing the permeability of drugs to systemic circulation (increasing absorption) and providing controlled-release properties for encapsulated drug [12, 28]. For the conventional medicine it is well understood the nanosize along with other characteristics does play an important role as evident from the improved bioavailability/pharmacological availability [29, 30]. Owing to the high surface area to volume ratio of NPs the window of absorption is also high in comparison with microparticles, this is an added advantage in improving the bioavailability of the administered drug [31, 32].

2. Delivery route of insulin

2.1. Oral delivery

Insulin therapy is effectively used in treatment of diabetes mellitus. Insulin is a key player in lowering blood glucose levels for type 1 diabetes and also required at later stages in type 2 diabetes patients. The widely accepted route for delivery of insulin is by parenteral administration but this delivery of insulin usually requires at least three or four daily insulin injections for good glycemic control. Consequently more acceptable different routes of insulin delivery have been searched to decrease suffering from discomfort, local pain, irritation, infection, immune reactions and lipoatrophy at the injection site of insulin. Oral delivery of insulin would deliver the drug directly into the liver through portal circulation and could mimic the physiological fate of endogenously secreted insulin [33, 34, 35]. However polypeptides, like insulin are degraded in the stomach pH and undergo proteolysis by enzymes in the gastrointestinal tract [22, 36]. Moreover the gastrointestinal mucosa has low permeability for large hydrophilic peptides.

In order to overcome the problems associated with parenteral administration of insulin several strategies that are based on nanotechnology has been developed to enhance the intestinal absorption of different protein and peptides. NPs consist of naturally occurring biodegradable polymers are widely investigated in this regard. They have emerged as potential carriers of several therapeutic agents for controlled drug delivery as well as the oral route of insulin. Various natural hydrophilic and hydrophobic polymers used as carrier of oral insulin such as chitosan, alginate, dextran sulphate, etc. are commonly used to prepare NPs.

2.1.1. Polymers used as matrices for oral insulin delivery

Over the past few decades, enhancing attention has been paid to the use of polymeric NPs either hydrophilic or hydrophobic as carriers for insulin delivery. Hydrophilic polymers are of particular interest due to their non-toxic, biocompatible, biodegradable and natural polymers. Among them, chitosan is widely used because of its ease of chemical modification and promising biological properties.

2.1.1.1. Hydrophilic polymers

Chitosan (CS): CS is well known naturally occuring copolymer of beta [1-4] linked and N-acetyl glucosamine and have been generally found in crustacean (crabs, shrimps and lobsters) shell and in some fungi or yeast. It is a biodegradable, biocompatible, non toxic, non-allergic easily absorbable natural hydrophilic polymer properties that have resulted in a wide array of applications in biomedical and drug delivery research [29, 30, 37]. Moreover it prolongs the intestinal residence time that shows its mucoadhesive property [38] (Figure 3). It has also been shown as a paracellular permeability enhancer by interacting with the TJ proteins occluding ZO-1 and opens the tight junctions between epithelial cells [34, 39, 40]. In addition to these properties it increases the stability of nanospheres and faciliates effective encapsulation of proteins and drugs that make it as a suitable carrier material [38, 41, 42]. CS have been extensively used to develope new chitosan derivatized polymers. CS combined with poly(γ-glutamic acid) (γ-PGA) based insulin NPs are used as hydrophilic polymers for oral insulin delivery. *In vivo* preclinical studies of this formulations at a dose of 30 IU/kg in streptozotocin (STZ) induced diabetic rat models showed increased intestinal absorption of insulin from γ-PGA NPs. It has got long lasting hypoglycemic effect and 15% relative bioavailability compared to subcutaneous (sc) injection [43]. The same formulation filled in enteric coated capsules was even better at the same dose, showing 20% oral bioavailability. Also aspart insulin (=monomeric, 3 times faster than regular) is encapsulated in the same CS-γ-PGA; has got 15.7 % oral bioavailability [21, 44].

Moreover insulin loaded NPs with carboxylated chitosan and Poly-methyl methacrylate (PMMA) were developed to improve the insulin delivery via oral route. One of the most widely investigated polymer towards peptide delivery is acrylates which have high interest because of its pH sensitivity and carboxyl groups to enhance the bioadhesivity, alter the tight junction, chelate the Ca^{2+} there by inhibiting the proteolytic activity of proteases, etc. They evaluated their ability to reduce blood glucose levels in diabetic rats. *In vivo* experiments resulted in the reduction of blood glucose levels by 67% at a dose of 100 IU/kg and the pharmacological bioavailability of the 25 IU/kg at a dose of PMMA NPs was 9.7% [45, 46].

Chitosan with sodium alginate is being prepared another insulin loaded nanoparticle product which is used to improve the loading capacity and activity maintenance. It's observed that when insulin-loaded nanospheres (25, 50, 100 IU/kg) administered orally to diabetic rats they reduced glycaemia in a dose dependent manner. Their pharmacological availabilities are found 7.1, 6.8 and 3.4 %, respectively [33, 47, 48].

In addition hydroxypropyl methylcellulose phthalate (HPMCP) is a pH-sensitive polymer designed as an enteric coating material. It reduces the release of drug in acidic conditions and also to improve the colloidal stability of the particles. The release of insulin from CS/HPMCP NPs were significantly reduced at acidic pH and even after 6 h it was less about 25% only. Insulin was protected from enzymatic degradation in the case of CS/HPMCP in comparison with native chitosan particles. Insulin loaded chitosan and HPMCP NPs were orally administered to diabetic wistar rats. The pharmacological availability was 3.02% and 8.47%, respectively, for the chitosan and the modified NPs. In comparison to oral insulin solution the

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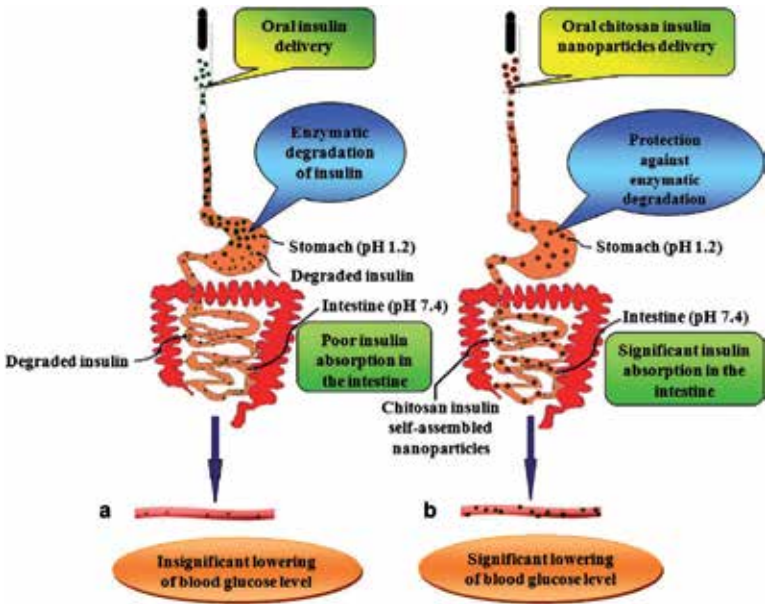

Figure 3. In-vivo efficiency of orally delivered insulin and chitosan/insulin self-assembled NPs (Reproduced with permission from Ref. [38], Copyright 2013 Elsevier).

hypoglycaemic effect was increased by 2.8 and 9.8-folds for the chitosan and the modified NPs, respectively [49, 50, 51].

Dextran sulphate-vitamin B12: Dextran sulphate is a non-toxic and highly water soluble different polymer used as matrices for oral delivery of insulin. Vitamin-B_{12} demonstrated as a ligand to enhance the uptake of the dextran NPs and their translocation across the gastrointestinal tract for high bioavailability. Insulin conjugated to dextran-vitamin B_{12} NPs to diabetic rats that had the least amount of cross linking were found to be most effective at lowering blood glucose levels (70-75%) in STZ induced diabetic rats. In addition the hypoglycemic effect lasted for 54 h. This modification showed the greatest hypoglycemic effect with a pharmacological availability of 29.4% (Table 1) [49, 52, 53].

2.1.1.2. Hydrophobic polymers

Poly (lactide-co-glycolide) (PLGA): Particles consisting of PLGA have been widely studied as therapeutic delivery vehicles owing to their biodegradabile and biocompatibile particles. The hydrophobic nature of PLGA matrices generally makes them incapable of entrapping water-soluble insulin. Intragastric administration of the insulin-loaded PLGA NPs (20 IU/kg) to diabetic rats reduced fasting plasma glucose levels to 57.4% within the first 8h of administration. The relative bioavailability of insulin following oral administration of NPs was 7.7% compared to subcutaneous injection of its solution. Star-branched PLGA (β-cyclodextrin-PLGA) NPs are highly promising for mitigating the burst effect and prolonging the release of insulin. Another study attempted to prevent the burst release of insulin in the stomach by

Particle	Size (nm)	Dose (IU/kg)	Pharmacological Activity (PA %)	Reference
CS-γPGA	218	30	15.1	
Entericcoated(EC)	233	30	20.1	21, 43, 44
Aspart Insulin	245	30	15.7	
CS-PMMA-PEG	1000	50	1.8	45
CS-PMMA	200-300	25	9.7	46
CS-NP	25-400	7, 14, 21	14, 15.6, 15.3	54
CS-dextran sulfate	500	50, 100	5.6, 3.4	55
CS/HPMCP	255	12.5	8.47	56
CS-Na alginate	748	25, 50, 100	7.1, 6.8, 3.4	57
B_{12}vitamin-dextran	192	20	29.4, 26.5	52, 53

Table 1. Hydrophilic Polymers.

using a cellulose derivative (hydroxypropyl methylcellulose phthalate, HPMCP) to prepare PLGA NPs. This modification reduced the initial release of PLGA NPs in simulated gastric fluid from 50% to 20%, and their relative bioavailability in diabetic rats was approximately 6.2% [49, 58, 59].

Poly lactide acide (PLA): PLA exhibit a strong affinity toward the small intestine due to their polyethylene oxide (PEO) blocks and a high permeation capability toward the cell membrane owing to their amphiphilic property. When orally treated with vesicular PLA NPs loaded with insulin to diabetic mice (50 IU/kg), the highest blood glucose reduction was achieved at 4.5 h. Although this effect lasted at least an additional 18.5 h, increasing the insulin concentration to 100 IU/kg did not enhance this hypoglycemic effect (hypoglycemic effect lasted for 23 hr) [60].

Poly-ε-caprolactone (PCL): NPs prepared with PCL and a monomeric form of insulin analog (aspart-insulin). Their results demonstrated that this formulation allows for preservation of biological activities of insulin, increase of serum insulin levels and improvement of the glycemic response. The maximum effect of reduction in hyperglycemia was found at 8 h after oral administration, which was more pronounced with aspart-insulin-loaded NPs (52%) at the dose of 50 IU/kg [61].

Lipidic polymers [*Solid lipid NPs (SLN)*]: Previous studies have demonstrated that nanoencapsulation of proteins in SLNs prolongs their blood residence time, modifies their biodistribution and improves their bioavailability [32]. Oral insulin delivery with SLNs administered to diabetic rats, their relative pharmacological bioavailability was 5.1% in comparison to SC injection of insulin; a considerable hypoglycemic effect was also observed during 24 h. To facilitate the transport of particles across the cellular barriers, in an another study the relative bioavailability increased to 7.1%. That study also suggested that increasing the drug entrap-

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ment efficiency and utilizing protease inhibitors in SLNs may further enhance the bioavailability of insulin (Table 2).

Particle	Size (nm)	Dose (IU/kg)	BA (relative bioavailability)%	Reference
PLGA	150	20	40 7.7	46
PLGA-HPMCP	169	20	60 6.27	59
PLA		50	Hypoglycemic effect lasted for 23 h	60
PCL, Aspart insulin loaded	700	50	52/ 12-24 hr	61

Table 2. Hydrophobic Polymers.

2.2. Nasal delivery

Nasal administration has attracted a lot of interest as a highly efficient route for the systemic delivery of insulin. It has been well known that the pharmacokinetic profile of intranasal insulin resembles the pulsatile pattern of endogenous insulin secretion in healthy volunteers during meal times [62]. In addition it's considered as a promising route for the following reasons: the nose has relatively large surface area [150 cm^2] of absorption because of numerous microvilli, high vascularized subepithelial layer that passes directly into the systemic circulation, thereby avoiding the loss of drug by first pass metabolism in the liver, high permeability of the nasal epithelial membrane and lower enzymatic activity than the gastrointestinal tract. Although nasal administration of insulin has many advantages, there are also some barriers that limit the intranasal absorption of insulin. Macociliary clearance of formulations from the nasal cavity, low permeability of nasal mucosa to large molecules and the low bioavailability of insulin act as barriers to intranasal absorption. To overcome the various barriers by the nasal route, researchers have studied many extensive range of enhancers such as bile salts and derivatives, sodium lauryl sulfate, laureth-9, phospholipids, cyclodextrins, chitosan and enzyme inhibitors.

At first CS NPs is seemed to be the safest and most effective as a carrier for the nasal delivery of insulin. It protected insulin from degradation in the nasal cavity and increased intranasal absorption of insulin with its positive charge [20, 63]. Also PEG-grafted(g) have been used to enhance the solubility and improve the biocompatibility of CS. Insulin PEG-g with CS NPs administered intranasally to transport insulin across the nasal mucosa in rabbits [64]. However recent studies showed that insulin-CS solution formulation was more effective than the intranasal NP complex (Bioavailability 17 %, 3.6 % respectively) [65]. Because of NPs couldn't enhance the uptake of insulin; PEGylated trimethyl CS NP results was also not found significantly different from the insulin-CS solution formulation. Besides chitosan reduced gold NPs could enhance insulin transport into cells effectively. After insulin loaded gold Nps adminis-

tered to diabetic rats by intranasally blood glucose concentration was decreased by 20.27 % [66]. Moreover intranasal route of insulin loaded starch NPs containing sodium glycocholate which's used as mucoadhesive carrier, caused 70 % reduction of plasma glucose levels and significant hypoglycemia untill 6h in the STZ induced diabetic rats [67].

2.3. Pulmonary delivery

Pulmonary administration is one of the most promising alternative route of insulin delivery. The lungs offer a large and highly vascularised surface area for drug absorption approximately 80-140 m^2. Alveoles are covered by a very thin (0.1-0.2 mm) monolayer epithelium, that permits rapid drug absorption. The alveoli can be effectively targeted for drug absorption by drug delivery as an aerosol with a mass median aerodynamic diameter of less than 5μm. First pass metabolism in this administration avoids gastrointestinal system metabolism. Although metabolic enzymes are found in the lungs, their activities and pathways may be different from those found in the GIT and this makes the pulmonary route of many therapeutic proteins and peptides very promising [68, 69, 70].

There are variety of inhalation devices such as metered-dose inhalers or drug powder inhalers. Such as AERx®. Insulin Diabetes Management system developed by Novo Nordisk which delivers aerosol of human insulin; Exubera® developed by Nektar/Pfizer which uses a dry powder formulation. [36].

Dry powder inhalers are currently the most commonly used devices because of their stability and sterility to develope pulmonary insulin. The surfactants, bile salts and fatty acids have been evaluated as absorption enhancers which increase the permeability of drugs through the epithelial membranes. However polyoxyethylene (PE) oleyl ether showed good enhancement sorbitan trioleate exhibited moderate enhancing ability. The enhancing effects of glycerol trioleate, ethyl oleate, oleyl alcohol, palmitic acid and stearic acid were very low. In contrast liposomes are very effective pulmonary absorption enhancers for peptide and protein drugs. They've biogenic phospholipids and biocompatible, biodegradable and non immunogenic natural properties.

Experimental studies investigated that insulin could be efficiently encapsulated in liposomes which has approximately 1μm particle size. Liposome mediated pulmonary drug causes enhancement in drug retention time in the lungs and decreases side effects which results increased therapeutic effects. When aerolized insulin liposomes delivered by the inhalation route in mice caused significantly reduction in plasma glucose concentrations [71]. Insulin-calcium phosphate (CAP) and polyethylene glycol (PEG) particles were administered to the lungs by a route of administration of respiratory tract and these particles positively affected the disposition of the insulin in the lungs of rats [72]. Poly-lactide-co-glycolide (PLGA) particles have used to improve insulin loaded particles which has a mean diameter of 400 nm. After the pulmonary administration of insulin with PLGA nanospheres, blood glucose levels were significantly decreased and has got prolonged hypoglycemic response over 48 h in guinea pigs [73]. In another related study poly butyl cyanoacrylate NPs have been used given by pulmonary inhalation of insulin in the lungs resulted stable and prolonged pharmacological effect. A significant reduction in glucose levels were found and the relative pharmacological

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bioavailability was 57.2 % [70]. Besides insulin/1, 2 dipalmitoyl phosphatidylcholine (DPPC) physical mixture used to enhance insulin absorption in pulmonary route of inhalation. This mixture caused higher blood glucose decrease because of their potentially effective, non-toxic and natural absorption enhancer property [74].

2.4. Buccal delivery

Insulin delivered by buccal route is through an aerosol spray into the oral cavity. It's absorbed through the inside of the cheeks and in the back of the mouth. The buccal mucosa is excellently accessible with surface area approximately 100-200 cm^2, lower risk to be traumatized and a relatively good permeability and perfusion [36, 75, 76, 77]. Several formulations and factors alone or in combination can influence release properties of buccal insulin delivery system. These formulations should contain absorption enhancers (such as surfactants, bile salts, chelators, sodium lauryl sulfate or fatty acids) to increase membrane permeability, enzyme inhibitors to protect the drug from degradation, protease inhibitors (aprotinin and sodium glycocholate) to function drug permeation across mucosa, lipophilicity modifications (conjugation with polymers) bioadhesive delivery systems (gels, films, patchs) and liposomal formulations [36, 76, 78]. Lysalbinic acid which is applied as an absorption enhancer was shown to enhance significantly buccal mucosa permeability for insulin. They investigated that it's a product of the alkaline hydrolysis of egg albumin and has no irritating or sensibilizing effect upon buccal use. Co-administration of lysalbinic acid and relatively small proteins such as insulin can increase insulin's permeability from the cheek mucosa of hamster [79].

In the last years a new innovative system has been developed by Generex Biotechnology Corporation (Toronto, Canada). It's based on a liquid formulation (Oral-Lyn®) of recombinant human insulin, absorption enhancers (which encapsulate and protect the Insulin molecules) and Rapid Mist® device (advanced buccal drug delivery technology). This device sends fastly small particles from an aqueous spray into the oral cavity. This allows rapid insulin absorption.

Oral-Lyn® has been evaluated in healthy persons and type I diabetes. It appears in the circulation within 10 min, the time to peak insulin concentration is is around 25 min. It has observed a more fast onset of action and less prolonged hypoglycemic action. Several studies in patients both type 1 and 2 diabetes demonstrated that this oral insulin can be efficient in controlling postprandial glucose levels. This new buccal insuin system needs further investigations in diabetic patients [35, 36, 76, 78].

2.5. Transdermal delivery

Transdermal insulin delivery is an appealing alternative to the invasive parenteral route of administration and other alternative routes of insulin such as pulmonary and nasal routes because the skin offers the advantages of an easy access and a very large surface area (1-2 m^2). It improves patient compliance and avoids both liver's first pass metabolism and degradation of drugs in gastrointestinal tract. The skin also represents an important painless interface for systemic drug administration. Despite these advantages the human skin limits permeation of foreign compounds especially large hydrophilic molecules like insulin. The

stratum corneum; which is the upper layer causes impermeability of the skin by its lipid-rich matrix. Several attemps have been made to overcome the skin barrier and to allow the transfer of large drugs such as insulin. They can be divided into chemical (liposome and chemical enhancers) and physical methods (mainly iontophoresis and sonophoresis).

2.5.1. Transdermal delivery methods

Chemical enhancers such as surfactants, fatty acids, fatty esters and azone-like compounds alter the lipid structure of the stratum corneum. They reduce its barrier properties and enhance its permeability for large molecule drugs that would not pass through the skin.

Iontophoresis is a non invasive technique used to increase transdermal insulin penetration through the skin by the application of a small electric current potential. Large drug molecules can be delivered in a shorter time with the help of this method and it increases drug's mobility.

Another non invasive technique *sonophoresis (ultrasound, phonophoresis)* which has been used to enhance (and or or delivery and activity of drugs) skin permeability to various low and high molecules weight drugs such as inulin. Low frequency ultrasound (20-160 kHz) decreases blood glucose levels both in animal and human studies [36, 72, 75].

Microneedles are minimally invasive painless and promising technology to deliver drugs into the skin without disruption of nerve endings. This technology create micronsized channels which interstitial fluid fills up the channels in the skin. It makes hydrophilic transport pathway, faciliates the stratum corneum barrier and increases skin permeability to large molecules [35, 36, 62, 75].

Also other methods have been investigated like microdermabrasion, pressure waves and electroporation but they're still in at a preliminary stage. Altogether chemical and physical methods they all need further investigations.

2.6. Ocular delivery

Ocular delivery is another the most promising and challenging delivery of ophthalmologically active peptides and proteins for the treatment of ocular diseases. The advantages of the ocular delivery are; less development of immunological reactions in eye tissues, less side effects, no tolerance and avoidance of hepatic first pass metabolism. While the enhancers such as saponin, dodecylmaltoside, tetradecylmaltoside, fusidic acid and glycocholate increases the systemic absorption of insulin in animals they may also increase the eye toxicity [36, 80, 81]. A series of alkylglycosides including tetradecyl-, tridecyl- and dodecylmaltoside and dodecylsucrose were potent stimulators of insulin absorption after topical ocular delivery in anesthetized rats when used at concentrations as low as 0.125 %. These are the most hydrophobic alkylglycoside reagents and were the most effective at enhancing systemic insulin absorption [82]. Moreover sucrose cocoate, a pharmacological excipient of cosmetic and dermatologic preparation was used to deter-

mine its possible absorption enhancer in ocular drug delivery. When insulin was delivered ocularly in the presence of 0.5 % sucrose cocoate, plasma insulin levels were significantly enhanced and blood glucose levels were reduced [83]. Because of this observation insulin-containing liposome was prepared to prolong the retention time of the formulation in the precorneal area [84]. This positively charged formulation decreased the blood glucose levels 65-70%.

More recently Gelfoam® an absorbable gelatin sponge ocular devices have been developed as insulin carriers for systemic administration of insulin. Although Gelfoam® containing 0.2 mg insulin has been showed prolonged systemic absorption of insulin within the desired therapeutic levels it may also cause long term toxicity such as slowing the tear production. Because of this toxicity sodium insulin and zinc insulin Gelfoam ocular devices have been developed and these devices were sufficient to control blood glucose levels (60 % of initial) for over 8 hours [62].

2.7. Vaginal delivery

In recent years numerous studies prove that vagina has got rich blood supply and large surface area that means good permeability and can be a potential route for systemic delivery to a wide range of compounds. The main advantages of vaginal drug route are avoidance of first pass metabolism, ease of administration and good permeability for low molecular weight drugs. For systemic delivery bile salts, dihydrofusidate, cyclodextrins, surfactants and chelating agents have been tested as enhancers to facilitate the rate of vaginal absorption but sometimes they induced several local reactions [36, 75].

2.8. Rectal delivery

Rectal route of delivery have been tested soon after the discovery of insulin but several investigators have met absorption problems through the mucosa. This administration's promising advantage is the possibility of avoiding, to some extent, the hepatic first-pass metabolism. Absorption promoters and surfactants were used to provide highest hypoglycemic effect in rectal insulin delivery. The most effective rectal absorption enhancer polyoxyethylene-9-lauryl ether (POELE) or sodium salicylate were used in insulin suppositories on diabetic dogs. It was investigated that hypoglycemic effect can be achieved about 50-55 % [85].

3. Conclusion

Over the last years numerous studies summerised polymeric NPs focused on different routes of insulin delivery. The association of insulin with NP formulations designed to protect insulin from degradation and enhance its uptake in the ileum. However, more research in this area is needed to achieve the goal that has plagued researchers for many

decades. At any rate, polymeric NPs for routes of insulin delivery seems to be the better alternative compared to others.

Author details

Hatice Kübra Elçioğlu[1*] and Ali Demir Sezer[2]

*Address all correspondence to: kubra.elcioglu@marmara.edu.tr

1 Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

2 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

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Chapter 8

Polymer Nanoparticles for Smart Drug Delivery

Devasier Bennet and Sanghyo Kim

Additional information is available at the end of the chapter

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1. Introduction

In the recent decades, polymers are widely used as biomaterials due to their favorable properties such as good biocompatibility, easy design and preparation, a variety of structures and interesting bio-mimetic character. Especially in the field of smart drug delivery, polymer played a significant role because it can deliver therapeutic agents directly into the intended site of action, with superior efficacy. The ideal requirements for designing nano-particulate delivery system are to effectively be controlled particle size, surface character; enhance permeation, flexibility, solubility and release of therapeutically active agents in order to attain the target and specific activity at a predetermined rate and time. The smart drug delivery systems have been successfully made by the advances in polymer science in the bio-nano-technology field. Recently, these advances have been found in various medical applications for nano-scale structures in smart drug delivery. The smart drug delivery systems should possess some important feature such as pre-scheduled rate, self controlled, targeted, pre-determined time and monitor the delivery. The smart drug delivery system enhances the polymer nanoparticle better stage to their therapy regimen. They are drug carriers of natural, semi-synthetic, and synthetic polymeric nature at the nano-scale to micro-scale range. The polymeric particles are collectively named as spheres and capsules. The most of the polymeric nanoparticles with surfactants offer stability of various forms of active drugs and have useful to smart release properties. There are numerous biological applications have been reported for the nano-scale to micro-scale sized particles, such as site-targeted, controlled, and enhanced bioavailability of hydrophobic drugs [1-4]. Due to the nanoparticles size the drugs have been targeting into various applications, such as, various cancers targeting has been shown to be promising [5]. Moreover, polymeric particles proved their effectiveness in stabilizing and protecting the drug molecules such as proteins, peptides, or DNA molecules from various environmental hazards degradation [2-4, 6, 7]. So these polymers are affording the potential for various protein and gene delivery. Numerous methods had been available to fabricate

nanoparticles; it depends on the physical and chemical properties of polymer and active ingredients. Most of the formulation techniques involve different mechanisms such as using organic solvents, temperature, ultra-sonication and mechanical agitation which can degrade the pharmaceutical active ingredients. So the nano-particulate system can be developed to consider the formulation methodology should not damage the active pharmaceutical ingredients. There are numerous biodegradable and biocompatible polymers with different physicochemical characters are offered to prepare smart nanoparticles, those polymeric nanocarriers can be natural or semi-synthetic or synthetic. Those nanoparticles can enhance the systemic circulation half-life and minimize unwanted internalization and prevents the denaturation of the therapeutically active moiety and could use to deliver the target agents. Several polymer systems are approved by the U.S. Food and Drug Administration (FDA) for human use. It is the belief that when inventions in fabrication can catch up with those in materials, design and development of drug delivery system can enter a new generation of enhancing clinical healthcare.

The most recent advances in the uses of carriers for sustained and targeted delivery, micro and nano fabricated self-regulated devices [8], bio-recognizable systems; micro-needles for transdermal drug delivery have shown the flexibility and enhanced permeability of these polymeric materials. Ultimately the goal in smart drug delivery is the emergence of a micro and nano-fabricated therapeutic drug release device with the capacity to enough hold and release of various active agents on demand. In modern system the micro-electro-mechanical systems give a distinctive possibility to produce micro-fabricated biomedical devices for different intentions, from implantable systems to lab-on-a-chip systems. The constant and prolonged drug release micro-fabricated systems have the several benefits, such as many active ingredients could be stored in an nano form within the system and sustainably released, the drug release is initiated by the dissolution and disintegration of outer membrane barrier by an mechanical/electric stimuli, the most potential drugs could be released more specifically with this technique, the complex drug release system such as simultaneous stable and periodically could be attained for local therapy by the micro-fabricated system; it can be achieved in high or low dose of drugs at the targeted site and increase the stability of drugs by the membrane barrier for preventing water diffusion into the reservoirs [9]. Owing to the advanced scientific sophistication of the controlled drug release system that has been achieved till now, or that are in dynamic progress, this delivery model can be categorized into various classes. The controlled drug delivery systems can be categorize four main mode of drug delivery, such as (1) rate-programmed drug delivery, where drug diffusion from the system has follow a specific release rate profile, (ii) activation-modulated drug delivery, where the drug release is induced by various factors such as physical, chemical electrical or biochemical modules, (iii) feedback-regulated drug delivery, where the rate of release is determined by biochemical substance (triggering agent) concentrations, it is dependent on the concentration exhibit in the target and (iv) site-targeting drug delivery systems, this is a complex process that consists of multiple steps of diffusion rate and partitioning for the rate of drug release is regulated by the specific targeting moiety, solubilizer and drug moiety. This chapter will brief discussion on recent innovative nano-fabrication methods for novel drug delivery system. Also, highlights some of these new technologies and consider their possibility ongoing clinical

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transformation of nanoparticles, which the particles are well-controlled formulated. This chapter will be followed by a more detailed novel drug delivery system development from a polymeric material viewpoint and their various bio-applications will be covered without attempting to all the work that has been done in this field.

Over a decade, investigators have appreciated the enrichment of potential uses of bio-nanotechnology in offering huge advancements in novel drug delivery and targeting. The novel drug delivery platform that provides diminishes toxicity and enhances therapeutic efficacy gives most possible benefits to clinical levels. In approaches to drug delivery systems the route of administration is one of the crucial roles of drug targeting. These nanoparticles can be used for various routes, including oral, nasal, transdermal, parenterals, pulmonary, ocular, etc. Nonetheless, the oral route is most convenient, preferred, and in several cases, also its cost-effective, but it does not cross easily some biological barrier; also easily degraded by various body fluids, then rapid hepatic clearance and other organs. So the drug delivery systems focus on overcoming the various membrane barriers, such as the blood brain barrier, tight junction barrier, to achieve the effective drug target and enhance the efficacy. To find an alternative and satisfiable route of administration for the effective drug delivery system should overcome the digestive tract problems, where the degradation could take place via acid-hydrolysis, enzymatic degradation and bacterial fermentation in the alimentary canal. This chapter will cover the more detailed novel route of administration and development from a polymeric material viewpoint and their brief discussion will be covered without attempting to all the work that has been done in this field.

2. General methods for polymeric nanoparticles preparation

Recently, various kinds of polymers are used to prepare the polymeric nanoparticles, among this all polymer biodegradable polymers and their co-polymers such as di-block, tri-block, multi-block or radial block copolymer structures have been generally used to prepare polymeric nanoparticles and to encapsulate the active ingredients. These multi-functionalized polymeric nano-carriers include micelles, capsules, platelets, fibers, spheroids colloids, dendrimers, core-shells, nanoparticle incorporated polymer matrixes, etc. The first polymeric nanoparticles were developed between the year of 1960 to 1970 for the therapeutic application, and this were Micelles[10-12].The micelles are formed by polymerisation methods, commonly the formation of polymer nano-carriers during the polymerization of monomers [13-16]. Then the various advanced polymerization techniques have been developed for the preparation polymeric based nanoparticles, and the nanoparticles were stabilised using various surfactants [1, 9]. The stabilised drug loaded nanoparticles consist of drug and non-toxic biocompatible polymer with stabilizing agents, the biocompatible polymer is either biodegradable or non-biodegradable. Numerous techniques are available for the preparation of the polymeric nanoparticles and mainly top-down and bottom up processes. The polymer nanoparticle drug carriers can be further categorized into nano/micro-capsules and nano/micro-spheres depends on the size and structure [1, 9, 17-19]. The fine particles are 100 - 2,500 nm and ultrafine particles are 1 to100 nm in size, and are collectively known as nanoparticles. 50 to 300 nm sized

nanoparticle have been prepared by emulsion polymerization method [20]. Drawbacks in polymerization techniques are evolving noxious factors such as toxic, reactive residues, unreacted monomers, the risk of a chemical reaction and the formation of unwanted oligomers [1], and these drawbacks are overcome by using preformed polymers for the polymerization process [1]. Generally the drug loaded nanoparticles were prepared by dissolving the drug and polymer into the water-immiscible organic solvents and producing a nano-emulsion, as an example by probe-sonication method. The organic solvent is removed by using elevated temperature or reduced pressure [21-23], as an example of rotary evaporation method, and the nanoparticle is washed and collected by certification. Followed by various changes and improvements of the emulsification techniques have been reported [24-29]. For example, the sonication process is a crucial step in the preparation of the sensitive drug loaded nanoemulsion, and the sonication process can increase the temperature, that leads to inactivate the active ingredients. In order to avoid the problems researchers utilized an on/off cycle to maintain a low temperature. Other examples of general methods to prepare the drug polymer nanoparticle are described in the Figure 1. The biodegradable polymeric nanoparticles are commonly prepared by five different techniques such as emulsification-solvent evaporation, solvent displacement, salting-out, emulsification-solvent diffusion and double emulsion solvent evaporation. The synthesizing methods include salting-out method [1, 30, 31]; it is based on the separation of a water miscible solvent from aqueous solution through the salting out effect, solvent displacement method [1, 32-34], phase separation method [35], evaporation precipitation [36, 37], antisolvent precipitation and electrospray methods [38].

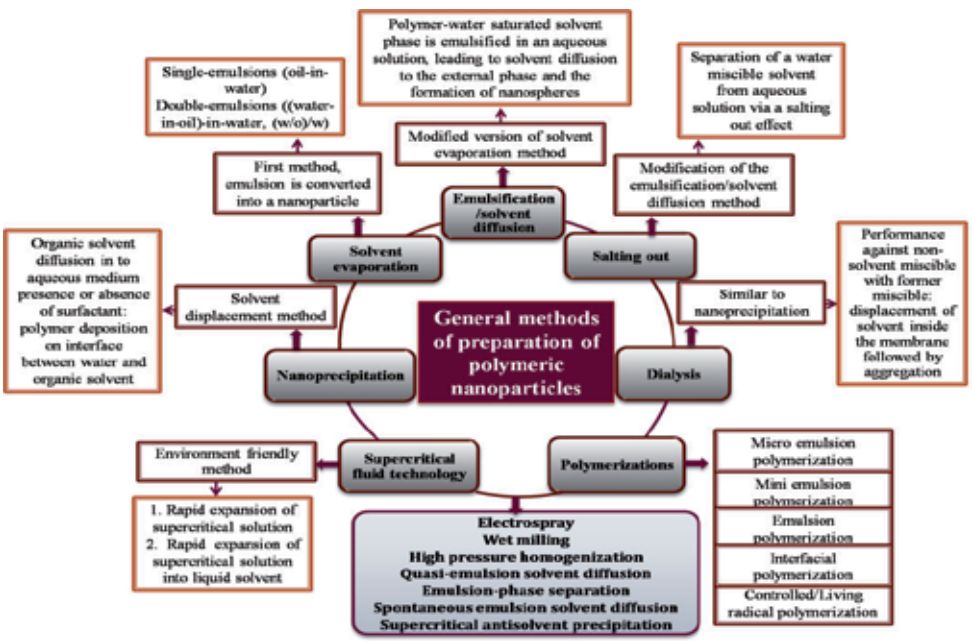

Figure 1. General methods of preparation of polymeric nanoparticles and their principle involved in the mechanisms

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Also, many approaches have been developed for the drug particle size reduction (increase in the surface) to the nanometer size range. For size-reduction, high pressure homogenization or wet bead milling is frequently used technique to produce reduced size nanoparticle [39-43]. Among these the high-pressure homogenization has been shown to be effective methods to produce size reduction particle. Moreover, its need sophisticated equipment to resist increasing pressures and temperature. Then, in order to obtain dried polymeric nanoparticle formulations researchers used various drying techniques such as atmospheric freeze drying, spray freeze drying, vacuum freeze drying, and lyophilisation. The uniformity of spray-dried nanoparticle is better than a freeze-dried nanoparticle. Moreover the lyophilisation and spray-drying are used to prepare the nanoparticle [44, 45], these nanoparticles easily tends to aggregates. Also the polymeric nanoparticles have also been synthesized by supercritical fluid techniques [46-52]. This method can get a dry product without any solution, also no need additional drying stages, but the supercritical fluid can swell some of the polymers and act as a softener, extender, and lubricant, which lead to aggregation. Moreover, this method is not easy to get the mono-dispersed multi-component particles because of different kinetics [52]. Nanoparticles prepared by spray-drying technique are one-step based on the conversion of a droplet to a dry particle by evaporation [53-55]. These one-step techniques have been revealed that the nanoparticle could be prepared without any problems [56-58], and the drug content in the particles is almost high [59], but produce an amorphous residual structure. In all above technique induce some unwanted noxious factors, as well as the organic solvents used in the preparations are increasing the risk of pharmaceutical application, also the increased processing time leads to microbial contamination [60, 61, 62]. Understanding the all risk factors, recently the modern instrument provides a promising and viable platform for the preparation polymeric nanoparticles.

3. Modern methods for preparation of polymeric nanoparticles

Recently, the polymeric nanoparticles have emerged as a most promising and viable technology platform for recognizing the targeted, environment-responsive and, multi-functional with navigated controlled drug delivery system. Polymer in smart drug delivery is a rapid-emerging new technological discipline in which various therapeutic applications of nano products are expected to overcome the patient complaints in healthcare. Smart delivery will give new solutions for therapeutic interventions. There is great interest from the beginning in smart medicine of advanced and well-characterized bionanotechnological products that will be especially effective in fighting diseases like cardiovascular diseases [63], diabetes [64], cancer [65, 66], aging [67, 68], some chronic metabolic syndrome and various degenerative diseases and disorders [69, 70]. For example, the innovative smart polymers with nanoparticulate drug-delivery systems can obviously advances in therapeutics by guiding the drugs to target cells and reducing the adverse-effect/side-effect on well being. At present, some of the smart polymer with multi-functioned nanoparticle system approaches in clinical trials, and it shows promising outcome. Certainly the morbidity and mortality rate of disease affected

patients could improve their lifestyle by the early course of smart therapeutic intervention. This smart intervention can be attained by developing high sensitivity and reliable smart drug delivery.

The rapid advancement in the above direction has been made with the initiation and development of more advanced alternative nanofabrication techniques to produce structures in various nano-scales level of controlled manners. Drug loaded polymeric nano-systems can provide controlled release of both hydrophilic and hydrophobic drugs over a long period of time while minimizing unwanted side effects in the body. This involves the synthesis of various novel biocompatible polymers with well-defined nanometers to a few micro-meters structures using several modern techniques such as microelectromechanical systems [71] microfluidic systems [72-76], electrodropping system [77], microneedle based system [78-81], advanced high pressure homogenization, interfacial emulsion polymerization and combined systems. Figure 2 described the few modern techniques for polymeric nanoparticles preparation with various concepts. The physiochemical characters of polymeric nanoparticles have to be optimized based on the specific application. Various methods can be used to produce various nano-particulate systems with various polymers. The multifunctional polymeric nanoparticles developments such as environment-responsive micelles, colloids, nano hydrogel, core-shell nanoparticles, nano-spheres and core-shell nano-spheres with layer-by-layer assembly for single/dual or multi drug release have been achieved so far. In order to get the desired properties, the mechanism of formulation method plays a vital role. Thus, it is extremely beneficial to have synthesis mechanism at hand to approach multi-functional polymeric nanoparticles with exact physiochemical properties for a specific application.

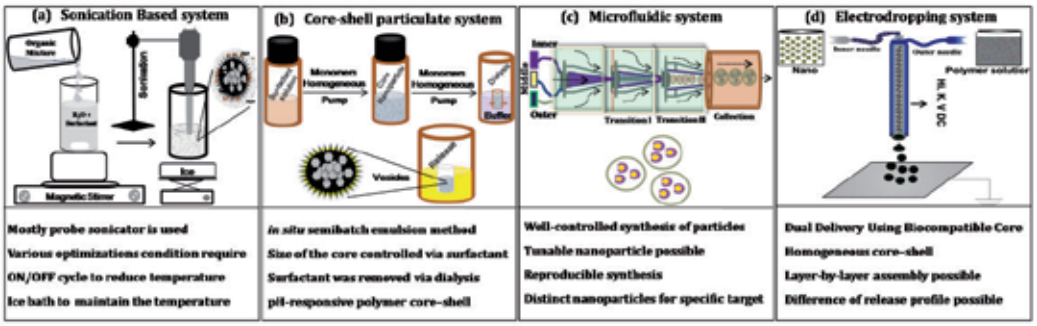

Figure 2. Schematic diagrams represent the advanced techniques of preparation of polymeric nanoparticles

The smart delivery systems of target bio-molecules have been concentrated of recent researches for various interventions. Particularly, various proteins, peptide, growth factors and cytokine therapy for various diseases play a vital role in regulating cellular responses, and thus the design of multi-functional polymeric particles delivery vehicles are closely associated with the regulation of multiple cellular events, likewise a wide variety of target bio-molecules have been investigated in numerous literature reports [82, 83]. Also numer-

ous of delivery vehicles have been studied and reported recently, this chapter will cover same viewpoint and their brief discussion will be covered without attempting to all the work that has been done in this field. Various concepts are utilized in the design of delivery vehicles that are capable of ferrying multiple active ingredients in a self-controlled manner, with different release profile kinetics. The distinctive self-assembly of multifaceted nanostructures from an easy colloidal system has been of interest to design a material with distinctive characters for the use of drug delivery vehicles. The inter-and intra-molecular linkage via van der Waals interaction leads to dense-packed self-assembly periodic nanostructures. These structures could be colloidal particle or clusters, based on the assembly [84, 85]. The natural or semi-synthetic polymer-based self-assembled nanostructures have inherent capacity of the nano-carrier for delivering many kinds of active ingredients, because of good biocompatibility and degradation/resorption properties [86]. In the sonication methods (Figure 2a), the self-assembled nanoparticle was achieved by probe sonication, the process has been done by cavitation, nucleation and reversible locking concept, the formed nanostructure have more flexibility in the nature [87]. In this self-assembled and core-shell particulate delivery systems, including water-soluble polymeric drug compounds conjugates [88], block polymeric micelles [89-93], long-circulating polymeric micelles [94, 95], nano encapsulations [96, 97], and core-shell nano-spheres [98, 99] have been synthesized by in situ two-step semi-batch emulsion polymerization technique (Figure 2b), as vehicle to target suitable dose of drugs in an accurate and controlled manner. Also the core-shell nano-spheres have been achieved for pH-responsive controlled release, and delivery of hydrophobic anticancer agents for acidic tumor tissues [100]. Recently Choi DH, et al have optimized electrodropping system to produce a homogeneous biocompatible core shell capsules for angiogenesis in dual delivery system [77], and they particularly focused on regenerative medicine. This electro-dropping system can overcome from the particle aggregation and drug encapsulation efficiency (Figure 2d). Coming to the micro-fluidics, the recent science and advanced technology of manipulating micro/nano-scale volumes in micro-fluidic channels have significant impact on the various applications. Advances and inventions in micro-fluidics are awaited to enhance the preparation of polymer nanoparticles and shifting to clinical evaluation [101] most of the micro-fluidic systems for synthesis, polymer nanoparticles are still under development and they have the widest possible to develop because they are highly reproducible, easily modifiable and can be incorporated with other techniques [102]. Recently, various micro-fluidic systems provide rapid mixing without any stimulator, such as stirring or electric force; have been originated [103]. Among these various systems the flow-focusing [104], droplet mixers [105] are widely utilized and it enables micro-mixing within the micro channel [106]. The flow focusing squeezes the solvent stream between two anti-solvent streams, resulting in a rapid solvent exchange via diffusion take place (Figure 2c). The effectuation of these rapid mixing methods for the development of nanoparticles in continuous flow; the micro-fluidic system has been achieved the continuous flow, narrow sized, mono dispersed with high drug entrapment and better batch-to-batch uniformity in compared with conventional methods [107].

4. Controlled drug delivery systems

4.1. Rate-programmed drug delivery systems

The recent advances in smart drug delivery systems with rate-programmed drug delivery systems have been achieved by functionalization of rate-controlling surface. The transdermal drug delivery have been achieved a new rate pre-programmed drug delivery system, transdermal patch which delivers a particular concentration of drugs to the blood circulation via the skin, it provides the therapeutic advantage to clinical levels. The rate-programmed drug delivery systems, the release of drug molecules from the rate controlling membrane system has been pre-programmed at particular rate kinetics. The rate controlling membranes made from natural and semi-synthetic polymeric material and proves their ability to use as a rate controlling membranes in any dosage form even nano to microscale level particle embedded matrixes or implantable or transdermal patches. It must be simple, cost-effective, and flexible enough not to split or crack on bending or stretching. Recently, some of novel rate-controlling composite membranes have been developed as rate controlling barriers for transdermal application, with flexible and smooth surface nanoparticles embedded scaffold which could reduce the risk of wounding or being rubbed off during dressing, and thereby improves upon traditional dressings and its can provides better patient compliance [108, 109]. This is achieved by optimized system design, which determines the diffusivity of active agents across the membrane. This rate-programmed drug delivery system can be categorized by various controlling dependencies, such as (1): membrane permeation-controlled, (2): diffusion-controlled, (3): membrane/matrix hybrid-type and (4): reservoir partition-controlled systems. The recent advance in the smart rate-programmed drug delivery systems the polymer and their scaffolds play vital roles, such as greater drug-loaded nano/micro-particle encapsulation ability, overcome pre-systemic metabolism, enhanced bioavailability and environmental responsive properties for various applications. For selecting the polymers, need to consider some important key factors for pharmaceutical application such as reduced tensile strength [110], water vapor permeability rate, biocompatibility, non-toxic [111], anti-infective, controlled release [112, 113], flexibility, emollient, adhesion, spreadability and retention properties of the drug-loaded nano/micro-particle encapsulation scaffold or film preparation [114-116]. So it can prevent the immunogenesis, secondary damage to cells, disease recurrence and finally enhance patient compliance [117]. In this type of rate-programmed controlled drug delivery systems, a drug-loaded nano formulation or rate-controlled nano formulation can be either totally or partially loaded in the reservoir space whose surface is covered by the rate pre-programmed polymeric membrane. The pre-programmed polymeric membrane can be optimized and achieved by multi-functionalization with block copolymers. The scaffold or membrane can be produced by the homogeneous or heterogeneous non-porous polymeric compounds or a micro/nano-porous or semi-permeable material. The drug release profile should be at a constant pre-fixed rate. The release profile is controlled by a pre-programmed rate-controlling membrane; it's based on the molecules, diffusivity, partition coefficient, and dimension of the outer membrane. Also the rate of release is determined by

the cross-linking ratio of the polymer network. The rate controlled release profile exists in many kind therapeutic formulations such as intrauterine devices [118], ocular insert [119, 120], some transdermal therapeutic system [109], polymer matrix, sub-dermal [121] and subcutaneous implantation [122-125].

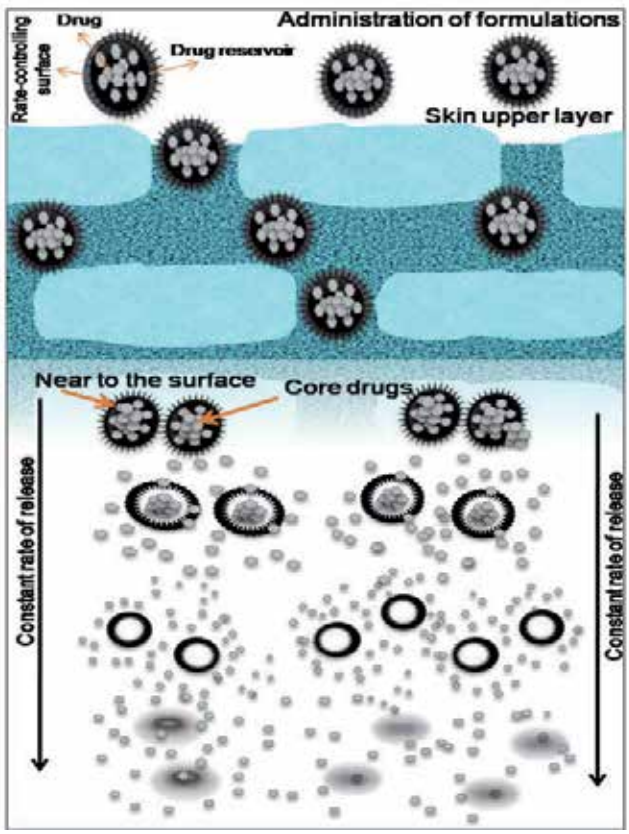

Figure 3. Schematic diagrams represent the rate controlled drug delivery systems of topical applications

4.2. Activation-modulated drug delivery

4.2.1. Environmental activation/stimuli responsive smart delivery system

The smart drug delivery with activation-modulated system has been achieved by external or environmental stimuli, these environmental responsive smart delivery systems achieved a lot more with double and multiple-responsive delivery system. The various activation/stimuli responsive drug delivery vehicles have been synthesized and tested, in various particle sizes, ranges from nanometers to a few micro-meters sized carriers for different routes of administration. The transdermal electro-activated or electro-modulated drug delivery has been established as an efficient model. In this group of activation-modulated controlled drug

delivery system, the release of active agents from the systems is activated by some physical, chemical, electrical, environmental condition or biochemical processes and/or facilitated by an energy supplied externally. The release profile has been controlled by the input energy. Based on the activation/stimulation process applied or energy type used, this activation-modulated controlled drug delivery system can be categorized into the various classes which are given in the Table 1. These stimuli-responsive materials show changes in the physicochemical character during the environmental condition changes. These changing properties can be fully utilized in smart delivery system, which certainly similar to the biological response behavior. Different types of body organs, different tissues and various types of cellular compartments might have great differences in every stimulus with great response. So that all the important cases considered in this chapter, deal with various environmental responsive smart delivery systems. Any specific behavioral changes in the system lead to a phase transition, these transitions will be key factors for the stimuli-responsive drug delivery system and some selected examples of applications are described in the Figure 4. The preclinical and clinical studies have demonstrated that drug-loaded polymeric nanoparticles has been well tolerated, extended systemic circulation, higher accumulation in the tumor sites through enhanced permeability and retention effect, minimized side effects and adverse effect, and/or higher bioavailability [153-155]. And most of the drug delivery systems are based on biodegradable polymer [156, 157]. Most of the environment-sensitive polymeric nano-particulate systems are leading to degradation and or disintegration by the internal or external local environmental stimulus such as pH, glucose, low oxygen content, ions, redox potential, and lysosomal enzymes; and then temperature, magnetic field, electric, ultrasound, and light respectively (Table 1).

These activations grew to achieve smart, targeted drug release in a particular time (spatial and temporal control release) [158-160]. At this place we describe a few examples. Particularly, the acidic pH levels in the body vary according to the different body environments (site and the organ) such as tumor cells and tissues (pH 6.5-7.2), endosomes (pH 5.0-6.5), lysosomes (pH 4.5-5.0) and entire GI tract with different pH value as comparatively varied with normal physiological (pH of 7.4) conditions in blood and tissues. So, the pH-responsive nano system have been considered and formulated to release the active agents in pH sensitive targets such as cancer site or endo/lysosomal regions [161,162]. The cytosol and cell nuclei have surrounded with elevated redox potential (in reducing glutathione) it higher than normal body fluids and it have been developed for intracellular release of various active bio-molecules [163-165]. Additionally, the cancerous tissues are extremely low in oxygen content (hypoxia) with higher glutathione levels compared to normal tissues [166]. This has been targeted with hypoxia-responsive polymeric nanoparticles. These internal stimuli-responsive nanoparticles have their own benefit of self-regulated drug delivery and effective target in clinical therapeutics. Also the external activated nanoparticles provide their own advantages such as high reproducible nature, also remote controlled delivery possible, then the release profile can be pulsatile delivered (means that switched on and off) possible [167]. On the other hand, the various light-responsive polymeric nanoparticles system has been developed for activating antitumor drug release [168]. Also numerous of temperature-sensitive multi-functionalized polymeric and copolymers nanoparticles have been formulated based on thermally-respon-

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sive release [169, 170]. Magnetically guided nano-carriers have been developed for the remote controlled cancer therapy and diagnosis [171, 172]; also the core-shell nanoparticles have demonstrated for improved tumor accumulation and antitumor therapeutic efficacy in various models.

Based on	Stimulus	Mode	Ref.
Physical stimuli	Osmotic pressure	Controlled through the permeability of water Controlled through a gradient of osmotic pressure	[126]
	Hydrodynamic pressure	Generate hydrodynamic pressure gradient Forces the drug to release through the orifice	[127]
	Vapor pressure	Pumping system contains vaporizable fluid Creates vapor pressure, vaporizes at body temperature	[128] [129]
	A mechanical force	Equipped with a mechanically activated pump First-pass elimination and pressure-sensitive delivery	[130]
	Magnetics	Electromagnetism-triggering vibration mechanism Magnetically activated, vibrate by an electromagnetic field	[131]
	Sonophoresis	Utilizes ultrasonic energy to activate the delivery	[132]
	Iontophoresis	Electrical current to activate and diffuse the charged drug	[133]
	Hydration	Utilized swellable polymer matrix Activated by hydration-induced swelling delivery	[128] [126]
	Electricity	Electric-sensitive capsule Electrically erodible matrix for delivery	[134] [135]
Chemical stimuli	pH	Deliver the drug in the intestinal tract not in the stomach Deliver the drug in the ulcer stomach by floating delivery	[136] [137]
	Salt concentration	Prepared by ionizable drug with ion-exchange resin Controlling the delivery of an ionic or an ionisable drug	[128] [138]
	Hydrolysis	Hydrolysis-induced degradation of polymer chains Hydrolysis activate the release of drug molecules	[139]
Biochemical stimuli	Enzyme	Polymer chains fabricated with biopolymers Deliver the drug by enzymatic hydrolysis of polymers	[140] [141]
	Biochemical	Enzymatic-activated, biodegradation Feedback-regulated delivery concept has been applied	[142] [143]
Environmental stimuli	Temperature	Depends on the transition temperature Shifting the hydrophilic/hydrophobic balance	[144] [145]
	Light	Polymers undergo isothermal phase transitions by photon Reversible phase separations through photo-irradiation	[146] [147]

Based on	Stimulus	Mode	Ref.
	Hypoxia	Hydrophobically modified imidazole derivative was conjugated to the carboxymethyl dextran, it can release the hydrophobic agents under hypoxic conditions	[148]
Dual-stimuli	Two different responses	Based on the polymer architecture Micelles are reported pH and thermo-responsive	[149] [150]
Multi-stimuli	More than two responses	Functionalization of pyrene-quaternized segments form a light-responsive shell and the unquaternized segments form a temperature/pH-responsive core	[151] [152]

Table 1. Overview of various stimuli responsive nano-carriers for smart drug delivery systems with mode of drug release applications

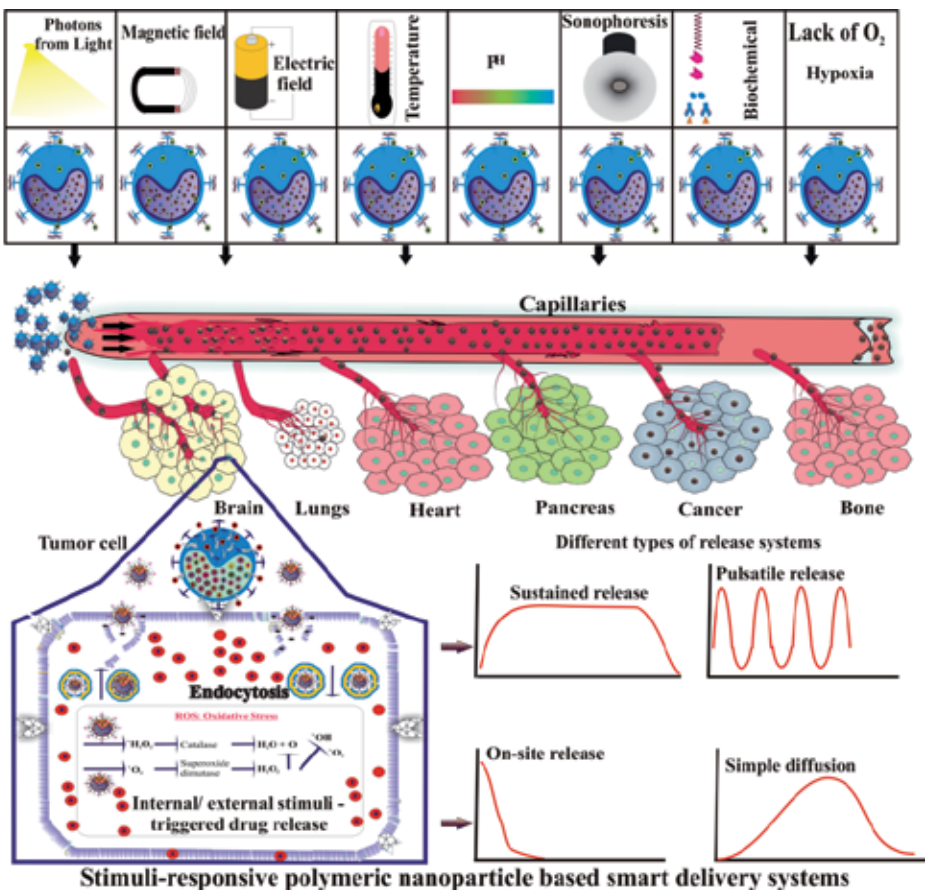

Figure 4. Schematic diagrams represent the activation-modulated drug delivery systems, which the polymeric nanoparticle activated by various stimuli such as physical, chemical, biochemical, environment, and/or a combination of two or more.

4.2.2. Dual and multi-stimuli responsive smart delivery system

In this chapter, provide the recent proposes and formulations of dual and multiple-stimuli responsive multi-functionalized polymeric nanoparticles and their promising targets in smart drug delivery in specific to the cancer therapy. With the booster development of the smart drug release and increase therapeutic efficiency of intelligent drug loaded nano-particulate system, polymeric nanoparticles that respond to dual and multi-stimuli, which have been aggressively reported. The double-response and multiple-responsive nano-particulate systems were described in the Table 2. It must be mentioned that the stimuli and responses happened at the same time at the same site or different mode. These dual and multi-stimuli responsive polymeric nanoparticles can provide control over the drug release profile, which leads to greater anti-tumor efficiency in vitro and in vivo models, and on the other side the nanoparticle formulation and drug loading under moderate conditions. In this section we describe a few examples. Especially, redox-responsive drug release multi-functionalized nano-particulate system have been formulated based on temperature and reduction, dual responsive tri-block copolymers functionalized by increasing temperature above the lower critical solution temperature after that cross-linking [173, 174]. These multi-functionalized nano-particulate systems were targeted to cancer cells and triggered by reduction oxidation mechanism, which leads to dissociate to release the active agents by de-crosslinking followed by disruption and degradation of nano-particulate system. pH/redox dual-stimuli multi-functionalized disulfide cross-linked micelles have been developed for increased drug release and accumulation in the cancer target, due to endo/lysosomal pH and intracellular redox environment the drug release was taken place [175].

4.2.3. Considerations for stimuli responsive targeted molecular systems

These multi-functional polymeric nanoparticles are capable to face the current problems of nanoparticle drug formulations including formulation and drug encapsulation, prolong stability, cellular internalization, site-targetability, enhanced cellular uptake, and inside cell target and drug release. These dual and multiple-activation responsive characteristics have provided novel and enthusiastic power over drug release kinetics and greater efficiency. All the described studies in dual and multiple-stimuli responsive drug delivery systems are mostly trial and error models, because most them non-biodegradable carriers, low encapsulation, and nonviable to clinical therapeutics. To overcome all the unfavorable conditions, immediate efforts could be focussed to improvement of dual and multiple-stimuli responsive biocompatible, biodegradable, non-toxic, and non-immunogenic smart polymeric nanoparticles that could effectively entrap and sustain the drug release in the systemic circulation, enhanced accumulation in the cancer target, and efficient release kinetics in response to more efficient external or internal stimuli. Moreover the smart polymeric nanoparticle system does not produce any secondary damage and any harmful to the healthy cells. In the case of clinical studies on dual and multiple stimuli responsive system shall be performed to obtain a real mechanism of action in anti-cancer target. In addition, the multi-functionalized smart polymeric nanoparticles system construct with targeting ligands and shall be incorporated into dual/multiple stimuli responsive nanoparticles to be achieved multidrug resistant cancers by

site targeting, site-specific, and rapid/sustained release, and we sure that dual and multiple stimuli responsive smart nano-particulate system going to be a good future in cancer therapy.

Responses	Stimulus	Nanoparticles	Ref.
Dual-stimuli	pH & Thermo	P(NIPAAm-co-DMAAm-co-UA) nanoparticles	[176]
		P(NIPAAm-co-AA)-b-PCL nanoparticles	[177]
		PLA-g-P(NIPAAm-co-MAA) nanoparticles	[178]
		P(NIPAAm-co-DMAAm)-b-PCL/PLA micelles	[179]
		PNIPAAm and PAA hollow nanogels	[180]
	pH & redox	PEG-SS-PDEA polymersomes	[181]
		DS-g-PEG/cRGD nanoparticles	[182]
		Poly(b-amino ester)s-PEG micelles	[183]
		PMAA-based nanogels	[184]
		mPEG-PAsp(MEA)-PAsp(DIP) micelles	[185]
	pH & magnetic	Fe_3O_4 nanocarrier with peptide mimic polymers	[186]
		DOX-tethered Fe3O4 conjugates nanoparticles	[187]
		mPEG-b-PMAA-b-PGMA-Fe_3O_4 nanoparticles	[188]
		Fe_3O_4-capped MSNs	[189]
		MCM-TAA-Fe_3O_4-capped MSNs	
	T & redox	EO-PAA-PNIPAAm polymersomes	[190] [191]
	Double pH	PPC-Hyd-DOX-DA nanoparticles	[192]
		Poly-b-amino ester ketal nanoparticles	[193]
	pH & diols	PEG-b-dendritic cholic acid telodendrimers nano-carriers containing boronic acid	[194]
	T & magnetic	Pluronic with Fe_3O_4 nanoparticles	[195]
	T & enzyme	DNA-capped MSNs	[196]
Multi- stimuli	T/pH/redox	PNIPAAm-SS-P(THP-protected HEMA) micelles	[197]
	T/pH/magnetic	P(NIPAAm-co-MAA) coated magnetic MSNs	[198]
	pH/redox/ magnetic	Fe(II) loaded PMAA crosslinked by N,N-methylene-bisacrylamide and N,N-bis(acryloyl)- cystamine	[199]
	T/redox/guest molecule	Vesicles based on hosteguest complex formation between C4AS and MVC12	[200]
	T/pH/guest molecule	Cucurbit(8)uril micelles, methylviologene-functionalize PNIPAAm and naphthalene-terminated PDMAEMA	[201]
	Light/pH/T	Pyrene-functionalized poly (dimethylaminoethyl methacrylate)	

Table 2. Overview of dual and multi-stimuli responsive materials for nano-carriers of various smart drug delivery systems

4.3. Feedback-regulated drug delivery

The recent advances in smart delivery systems with feedback-regulation of drug release. This self-regulated or feedback-controlled drug delivery comes under closed-loop systems. The self-regulated system drug release rate is controlled by feedback information, without any external stimulation, and utilized several approaches to control the release rate [202-205]. The feedback-regulated drug delivery concepts were schematically depicted in Figure 5. The feedback-regulated drug delivery concept has been applied to the development of various controlled delivery systems such as bio-erosion regulated, bio-responsive regulated and self-regulating drug delivery systems. Among this one of the concepts has been involved in the smart controlled delivery systems. For that various research efforts are also in progress to develop such nanoparticles that contain drugs capable of a feedback-modulated drug release. The drug release is activated by a triggering agent, such as a biochemical substance, in the body via some feedback mechanisms. The release rate has been determined by triggering agent concentration. When the triggering agent is above a certain level, the release is activated. This can induce and stop the drug release. It would be a high potential benefits if they were delivered by a system that recognized the particular warning signal caused by disease affected part, then they estimated the magnitude ratio of the signal, and then acted to release the exact quantity of active drugs in response. This kind of drug delivery system required to fulfil the physiological need by means of some feedback mechanism. The self-regulated drug delivery systems utilize several approaches for the rate-control release: pH-responsive polymers, temperature-responsive polymers, enzyme-substrate reactions, antibody interactions, enzyme-mediated, pH-dependent drug solubility nature, competitive binding mechanism and metal concentration-dependent hydrolysis. A hydrogel can swell in aqueous medium and retain their structure. The multi-functionalized polymer nanoparticle can be incorporated into hydrogel, such hydrogel used for the feedback-regulated drug delivery system. This hydrogels can protect the drug from dangerous environments such as enzymes and low pH in the stomach. This can control drug release through changing the network structure in response to particular stimuli, which can enable the sensor leads to reversible volume phase transitions upon small changes in the environment condition. For example, the polymers characterized by lower critical solution temperatures generally shrink, as the temperature is increased via lower critical solution temperature. Decreasing the temperature below lower critical solution temperature, the polymer can swell. Biomolecules can be encapsulated on or within the heat responsive polymers.

The sensor grafted in the delivery system can enable to mimic the recognition function of various bio-chemicals such as enzymes, cell mediated receptors and various proteins in human beings for maintaining the regulation and equilibrium. This approach is utilized for drug incorporated polymeric feedback controlled delivery systems, and this system approach is based on the observation that changes in control mechanisms, e.g.: pH or ionic strength or temperatures can affect large changes in drug solubility; this can be the main factor for control release rate. The external trigger molecule and polymer-bound enzyme can alter the pH inside the polymeric system. If the pH alteration happened inside the polymer system that can lead to changes in drug solubility, which is induces the diffusion or dissolution or disintegration,

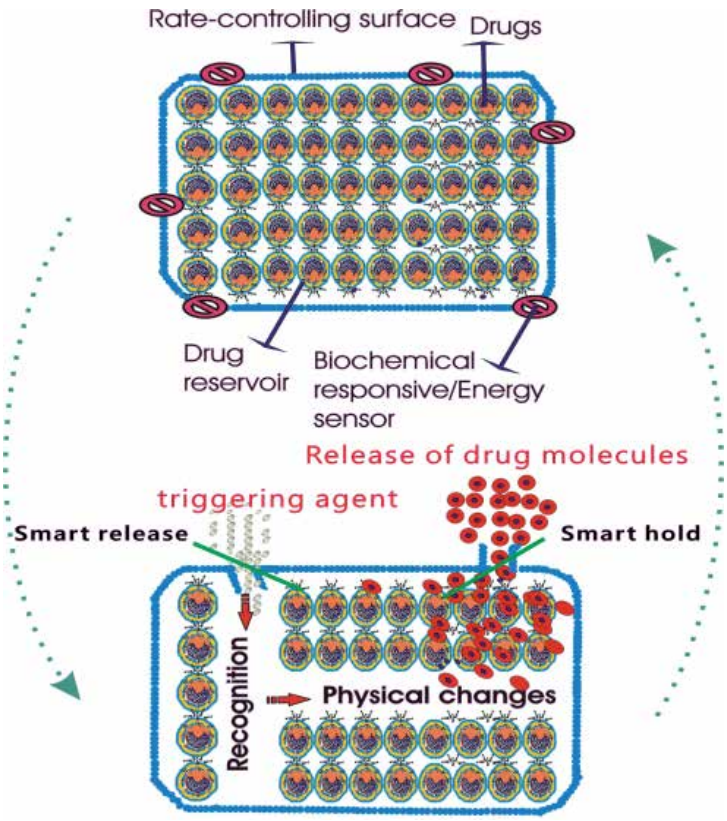

Figure 5. Schematic diagrams represent the feedback-regulated drug delivery systems

and rate of release has been changed accordingly. Many researchers have been developed a membrane to bypass the rumen but it allows the polymeric system to release the drug in the stomach via gastric retention mechanism [206]. Because of the polymer membrane it is impermeable to the rumen pH 7, but the swells and release at pH 4, which is the fourth stomach. Several studies have been performed on various polymers holding weakly acidic or basic functional groups in the polymeric backbone [207-112]. This polymeric system can swell or de-swell by changing the pH of the environments. By this way the drug will release from a matrix or device, which is developed by pH dependent polymers and this system can provides controlled release rates.

The bio-erosion controlled drug delivery system comprises of a drug-encapsulated bio-erodible scaffolds developed from biocompatible polymers (poly (vinyl methyl ether)), and were layered using immobilized urease. In a neutral pH the polymer erodes gradually, but in existing with urea, urea is metabolized by the system containing urea to form ammonia, it leads to increase the pH in the surrounding area, this increased pH degrade the polymer scaffolds then the drugs has been released [213], and some polymers require high pH to degrade.

The bio-responsive controlled drug delivery system, glucose-triggered insulin delivery has developed [214], the insulin is encapsulated within biocompatible polymer hydrogel scaffold comprising abundant NR2 functional groups present in the normal state. So in this state scaffolds are un-swollen and thus impermeable to insulin molecules. Enzymatically oxidized glucose is to form gluconic acid, this triggers the NR2 groups to form NR2 H+, it leads to swollen and insulin molecules deliver through the polymer membrane, and the amount of delivery has been controlled by glucose penetrating concentration.

The reversible and competitive binding mechanism also has been reported to insulin delivery. This mechanism role is to activate and to regulate the release of drug in the target; also it depends upon the glucose level present in the systemic circulation. Insulin-sugar-lectin complex has been prepared and entrapped into the semi-permeable polymeric membrane to achieve controlled release. The diffused blood glucose has competitively bound to particular binding sites, then activates the complex to release insulin derivatives, and the release acted based on the concentration of glucose presented in the systemic circulation. By this way the self-controlled drug delivery has been achieved. A further improvement on insulin delivery, they used glycosylated insulin-concanavalin A complex and entrapped inside polymeric membrane and the release has been achieved by self-regulated mechanism, depends on the glucose concentration permeate into the system [215]. Again in the development of self-regulating insulin delivery has achieved by enzymatically controlled implantable glucose-dependent insulin delivery systems [216]. Followed by various researches developed the different kinds of glucose-responsive insulin delivery [217-223]. Also the molecular imprinting technology developed system able to identify the specific compounds on the cell surface, and this can be appropriate for further developing and targeting the delivery system to specific tissues or cells. Recently, the pH-Sensitive polymer multi-functionalized with block co-polymeric nanoparticles have been developed for the triggered release of paclitaxel within a tumor microenvironment which the polymer acted as a feedback-regulated drug delivery carrier [224], and this carrier have a reversed swelling behavior. Most recently, the feedback controlled drug delivery system has been developed for cerebral cortical disorders with a feedback controlled mechanism. Drugs have been delivered via subdural/subarachnoid space, then diffuse into neocortical tissue and this diffusion can be controlled by electrophysiological feedback, the cerebral cortical area is exposed to the drug, and they were optimized for the drug concentration, delivery, frequency of delivery [225]. Moreover, the molecular imprinting technology has a huge possibility for producing acceptable dosage forms in the feedback-regulated drug delivery systems. The application of molecular imprinting enables the design of new systems and also in polymer based device fabrications. The advances in the preparation of molecular imprinting as spherical uniform particles [226] and scaffolds [227] can increase the field application potentiality of several polymers in drug delivery system. Moreover, these imprinted delivery systems have not yet touched in clinical therapeutics.

4.4. Site-targeting drug delivery systems

The recent advances in the smart delivery systems with site-targeting drug release. A site targeted drug delivery systems are complex of multiple steps of diffusion and partitioning.

Nowadays the site targeted drug delivery systems involve deep investigation as they are very eager to overcome the modern medical application [228]. A well-designed multi-functionalized polymeric carrier for site-targeted drug delivery in the interventions of various diseases such as colon disease, kidney/renal disease, nasal disease and genitourinary disease has been reported recently [229-234]. A variety of both natural and synthetic water-soluble polymers have been used for biomedical applications. These polymers have been used routinely in biopharmaceutics because of the effectiveness in controlled drug release. The traditional formulations are not significantly efficient at targeting molecules, thus the new and smart drug delivery systems are being studied to overcome the problem. The goal of the smart drug delivery systems is to allow a localized drug delivery, at the same time; it does not affect the healthy tissues and no unwanted effects. The drugs composed of micro-or nano-sized particulate system, which is able to spread through the systemic circulation, and transport through various body organs and body areas such as arteries, veins, and capillaries and even cross membrane barriers. The nanoparticle transport and targeting tissue are the complex process, so the transportation and communication have been viewed by the molecular communication paradigm. This transport of drug-loaded particles in the human body has been viewed, where the nanoparticle has transported this information is conveyed by signaling molecule. This communication system provides a clear reading of particle diffusion, distribution, disintegration over time throughout the biological system, which provides the importance to the invention of a smart particulate delivery system. Initially, the kinetic Monte Carlo method [235, 236], have been used computer simulation to solve the communication system. Lately, researchers developed an analytical approach based on the abstraction of targeted particulate delivery systems as a communication mechanism. This information is passed between sender and receiver by intracellular and intercellular signalling [237]. Different kinds of molecular communication have been analyzed so far, which involve passive or active transport of molecules [238, 239]). The smart site targeted delivery system takes an advantage of the systemic circulation for the distribution of active drug particle from where it's ingested to the systemic circulation to a targeted site. Basically, the delivery systems have been made with purpose and intention to control the rate of release from the systems, but the transport of nanoparticle to the target site still needs more control. Preferably, the route of administration and nanoparticle transport should also be strong enough controlled.

In this section also provides a few examples of site-targeted drug delivery systems, The ideal example is that the kidney site-targeted drug delivery systems, it acted as a smart delivery to enhance drug efficacy and safety in the therapeutics of kidney diseases. By this smart drug delivery treatment provides that reduces inflammation and reduce the formation of excess fibrous to proximal tubular cells, it can protect systemic infection and renal tubular inflammations. So targeting the renal proximal tubular cells is the novel and efficient routes to cure kidney disease [240-244]. Kidney-targeted drug delivery system can overcome from the various obstacles such as kidney transplantation, ureteral obstruction, diabetes, and other some important kidney disease. Figure 6 shows the kidney drug delivery of nano-particulate systems. Among all drug carriers the macromolecular carriers are extremely powerful targeting the kidney, because of the selective accumulation in the kidneys. Macromolecular carriers with prodrugs play crucial roles in targeting drugs to particular target cells in the

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kidney. The molecular weight and electric charge of polymers is one of the crucial role for effective renal clearance [245, 246], thus the active polymeric system can uptake and exists in the renal cells [247]. Especially the multi-functionalized polymeric nanoparticles showed higher uptake in glomerular mesangial cells [248, 249]. For nasal site-targeting specificity, the multi-functional particulate system design is the main role for site-targeting. So, design and preparation method has to be controlled according to the needs, the materials should be with quality of properties such as biocompatible, biodegradable, modifiable, mucoadhesive, antimicrobial, tumor or particular cell recognition, and maintain the drug release. In the example, N,N,N-Trimethyl chitosan nanoparticles achieved controlled intra nasal delivery to treat various diseases including hepatitis B and allergic rhinitis [250]. Also the amine functionalized chitosan has been shown their eminent characters such as biocompatible, enhanced solubility, strength, porosity, absorption efficacy, chemical tolerance, non-immunogenic and non-antigenic properties, and it has been used for various nasal delivery.

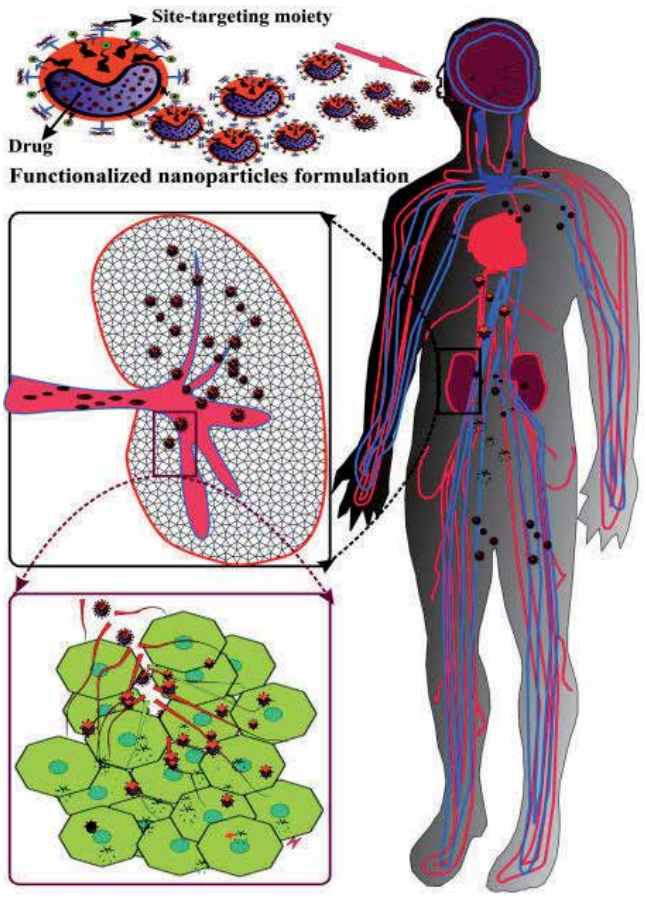

Figure 6. Schematic diagrams represent the site-targeting specificity particulate drug delivery systems

4.5. Targeting strategies for kidney diseases

Macromolecule is a very large molecule, which can accumulate in the kidneys. Generally, the molecular weight of the macromolecular vehicle is bigger than that of the prodrugs, so this kind of system can achieve the goal. Pro-drugs have the ability to select the target in the kidney because it can release the active drug by the action of renal enzymes. The various strategies of kidney-targeted drug delivery systems has to be considered such as biodynamical strategy of renal artery perfusion, macromolecular carriers which includes enzymes, immune proteins and peptide hormones, pro-drugs which includes folate, sugars, and amino acids, and other strategies including various nano-particulate systems. The molecular weight and charge [245, 246] of polymers is the main factor, it can influence their distribution in various organs including kidney. In general, increasing the molecular weight of polymers leads to decreases urinary clearance. Some of the polymers have been eliminated rapidly from the systemic circulation but it does not excrete from the kidney, and its accumulated in the renal systems. So it clearly proposed that the selection of effective and active multi-functionalized polymeric nanoparticles can uptake by the particular kidney cell types. So the selection of polymers is one of the prime strategies for consideration to achieve the efficient kidney targeting. These new possibilities to develop kidney targeting conjugates and other nano-particulate drug delivery systems. Including various polymers based nanoparticles give excellence strategies to achieve the goal of targeting drugs to the various renal diseases.

4.6. Common strategies for smart polymeric particulate targeted delivery

The ideal proposed model for site-targeting delivery is fabricated from a biocompatible, non-immunogenic and biodegradable polymer and acts as the central of support to three main characteristics of attachments such as site-specific targeting moiety, solubilizer and drug moiety, which should have drug delivery capacity, capable of transport and active molecule should bonded to the polymer via spacer, and the linkage is cleaved by particular enzyme(s) at the final targeted site respectively. In order to develop a new polymeric vehicle for a particular drug, the polymer distribution in the systemic circulation has to be analyzed since it's right away affects on activity of drugs. For controlling the systemic distribution of drugs, we need to consider minimum two strategies which are active or passive targeting. Previously, the drug is delivered to target site using some specific antibodies, which are specific to target cell-surface [251-254]. This method gives efficient targeting to tumor site; however, the antibodies can produce immunogenic activity. But, the passive targeting with bio-polymers vehicles cannot produce immunogenicity or toxicity, this might enhance the active molecule efficacy, such as increased half-life by increased size of the nano-particulate complex, increased permeability at the targeted area and polymer vehicle interacts to the body organs. Those elements must be increase the absorption of the drug molecule; which minimize the dosage and low unwanted effects [255, 256]. Moreover, in the advanced fabrication of molecular imprinting technology can provide efficient smart polymeric systems with the ability to recognize specific bio active molecules. This advanced fabrication technology has tremendous possibility to meet the requirements for satisfactory dosage forms developments. Depends upon the particular application the fabricated systems can decide the delivery, efficiency,

safety of the drugs, and when it should be reached. Described all above application strategies have a significant interest in targeting drugs into specific regions [257, 258].

5. Bioengineered materials: Ideal and recent advances for drug delivery systems

5.1. Nano-engines of drug delivery systems

Engineered materials have been utilized for developing smart drug delivery systems. Design and multi-functionalities fabricate of efficient smart drug delivery systems are vitally necessary for medicine and healthcare development. In the material science field provides biodegradable, biocompatible, environment-responsive, and highly effective novel polymeric system for targeted delivery. Nanotechnology provides bottom-up and top-down nanofabrication with size controlled and multi-functionality of particulate for targeted delivery. New materials invention and advanced technology have been synergistically achieved in drug delivery so far. The essential goals of medical pharmacology provide the right medicine, right dosage, and right route at the right time to the right patient, so more research need to optimize the therapeutic efficacy of the drug. This is the essential principles is behind the smart drug delivery. A smart, controlled delivery system needs synergistic consideration of several factors; these have been summarized in Figure. 7. It is difficult to get all consideration factors in a smart controlled delivery system due to other influencing factors. Also high quality, reliability, efficiency and reproducibility are the most significant issue while designing such a smart system. Also the smart systems have to induce the drug release and stop the release by their own manner. It would be highly benefited, if the system recognizes the disease affected part, estimated the disease affected ratio, and then acted to release the exact quantity of active drugs. This kind of drug delivery system can fulfil the medicine and healthcare requirements.

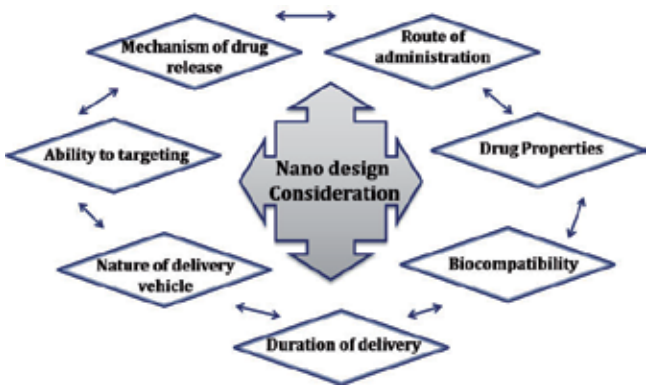

Figure 7. Requirements of several factors for simultaneous consideration to design a polymeric nanoparticle for the smart drug delivery system

6. Polymeric nanoparticles functionalization and considerations for smart application

In this section provides the recent research on the preparation and functionalization of various polymeric hybrid nano-materials including nanoparticles and microparticles by various techniques. Several techniques have been developed for the functionalization of polymeric nanoparticles with different therapeutic applications. The polymeric nanoparticles have been studied for their enriched properties in biological systems, with the nature of the materials and whether it has the specific properties for chemical modification and functionalization of the nanoparticle developed from various materials including bio-macromolecules. There are several researchers have been studied for functionalization and surface modification of nanoparticles and it would not cover all this in this section; so, this section covers some examples of nanoparticles functionalization and some important criteria to consider the fabrication process. In addition, the richness of surface chemistry and potential biomedical applications are described. The polymeric nanoparticles surface functionalization are mainly two types, one is functionalization with biological (macro)molecules such as peptides, carbohydrates, lipids, fatty acids, proteins, and nucleic acids (genes, oligomers, aptamers, and ribozymes/DNAzymes); another one is functionalization with specific ligands such as mono- or oligosaccharides (carbohydrates), folate receptor, antibodies and biotin are commonly used. This surface functionalization have been made by various modifications on preformed nanoparticles through adsorption, functional surfactants, emulsification, polymerization, covalently bounded functional molecules and various forms of bio-conjugation. There are few considerations for functionalization of polymeric nanoparticles properties such as: 1) the bio-molecule ratio should controlled by calculating the number of conjugate sites presents in the nanoparticles with different applications, 2) due to the environment and electrostatic interactions the alignment of functionalization has been varying, so the non specific attachment should be avoided in the performed nanoparticles, 3) depends on the applications requirements the nanoparticles bio-molecule distance should be maintained, 4) control the conjugation moiety attachment/linking affinity to the performed nanoparticles, 5) should maintain the optimal efficiency of physiochemical characters and 6) it should be high reproducible for all batches. The above all criteria can fulfil the requirement of design and functionalization of nanoparticles for a controllable release profile that satisfies the desired application. And better protection against environmental factors and maximum optimal control is achieved if drug loading is carried out by encapsulation instead of adsorption on to the particle surface. With the combinations of these above criteria in the fabrication of nanoparticles are potential to increase the clinical therapeutics by reducing unwanted effects.

With the field of bio-nanotechnology, enormous new research on the synthesis of polymeric nanoparticle based top-down or bottom-up approaches have been recently developed. Recent developed polymeric systems engrafted nanoparticles provide the optimal characteristic of the functionalized nanoparticles for various therapeutic approaches in harsh environments such as in the acidic and alkali environment [259]. Also polymer nanoparticles are broadly used in several therapeutic applications, mostly cancer targeting and therapeutics. And we

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provide some examples of various nanoparticles with different functionalization and different therapeutic uses based on the target, shown in Table 3. Therefore, the multi-functionalized nanoparticle over comes from the drawbacks of conventional therapy. In the latest study provided that more than 26 nanoparticle based therapeutic system have been approved for clinical treatment and several nanoparticles are under consideration [281]. In order to achieve the efficient nano-particulate system based therapeutics the nanoparticle synthesis and functionalization methods have to consider very carefully. Although several surface modified methods for various bio-applications have been reported previously, in this section highlight particular examples where this type of functionalization has been used.

Nanoparticles	Functionalization	Drug	Use	Refs.
Human serum albumin	Amino/acid group	Doxorubicin	Antineoplastic	[260]
Trimyristin	Sterically stabilized	Paclitaxel	Ovarian, lung, breast cancer	[261]
PLLA-b-PEG	Folate targeted	Doxorubicin	Solid tumors	[262]
PEG-PE	Lipid conjugated	Paclitaxel	Various cancers	[263]
PEG	Lipid conjugated	Tamoxifen	Lung carcinoma	[264]
Polymer-lipid hybrid	Lipid conjugated	Doxorubicin	Solid cancer	[265]
PCL-b-trimethylene carbonate-PEG	Serum protein	Ellipticin	Anticancer	[266]
PAMAM dendrimers	Folic acid	ethotrexate	Epithelial cancer	[267]
PEG	Albumin bound	Doxorubicin	Various cancers	[268]
Micelles	Biotin-antibody-conjugated	Daunomycin	Brain tumor	[269]
PLGA	Alendronate	Estrogen	Bone-osteoporosis	[270]
Poly(DEAP-Lys)-b-PEG -b-PLLA	Poly(lysine)	Doxorubicin	pH sensitive tumor	[271]
PLGA-b-PEG-COOH	PSMA	Anti cancer	Prostate- cancer	[272]
PEG or PE particles	Transferrin	Oligonucleotide	Brain- gene	[273]
PLLA-PEG	Biotin	Anti cancer	Cancer therapy	[274]
Polystyrol	Sc-TNF	Anti cancer	Cancer therapy	[275]
PLA	Aptamer	Anti cancer	Prostate cancer	[276]
PE	RGD peptides	siRNA	Vasculature cancer	[277]
mPEG/PLGA	Peptidomimetics	Anti cancer	Brain cells cancer	[278]
PLA	Galactose	Retinoic acid	Hepatocytes	[279]
PLGA	MP lipid A	Anti cancer	Dentritic cells	[280]

Table 3. Examples of various nanoparticles with different functionalization and therapeutic uses based on the target

Functionalization is defined as the improving performance of nanoparticle by a chemical functional group on their surface. Some basic components of functionalized nanoparticle are enabling to increasing the multifunctional applications in the field of biomedicine; the basic components are diagnostic agent, targeting ligand, spacer group, therapeutic agents, and polymer nano-carrier with proper functionalization. Here we introducing two strategies for surface functionalization, first one is direct functionalization, where the functional ligand is a bi-functional compound. In this method, one of the reactive groups is used to bind to the nanoparticle surface and the second group contains the required active functionality. Another one is post-functionalization, here the strategy is not changeable and the nature of the functionalizing group cannot be compatible with good control over the size and dispersion of the nanoparticles in the solvent used for the fabrication. Commonly, the nano-carriers have been functionalized with various chemical functional groups such as thiols, disulfides, amines, nitriles, carboxylic acids, phosphines and bio-macromolecules [282-287], based on their application. The functionalization of nanoparticle is to modify their outer surface with other specific chemical agents based on the desired application. After functionalization the particle physiochemical character has been changed. Also, it is a very important step for control because it can change their size and self-organization during the formation and should not promote aggregation. The prepared polymeric nanoparticles have emerged promising technology platform for recognizing the target with navigated controlled drug delivery system. Figure 8 shows the various functionalizations of the nano-engines for the development of smart drug delivery systems (Left side) and pre-regulated nanoparticle recognizes the tumor cells not the healthy (right side). This therapeutic drug concentration reaches the tumor site not in the normal cells or tissues. Polymer base smart drug delivery can overcome the patient complaints in healthcare.

In polymeric based nano-composites fabrication, the nanoparticles is used as backbone to enhance the physiochemical characters [288-290] such as flexibility, smoothness, enough strength and stiffness, which are much essential in the field of tissue engineering and bio-medical applications. The mechanical strength of polymer based nanocomposites is low due to the poor linkage between nanoparticles and the polymer, which leads to artificial defects in the composites [291-293]. It could be engineered with the appropriate interface to enhance the flexibility, smoothness, strength, stiffness and compatibility of the composite character [294]. The advanced functionalization of the nanocomposite have been prepared with suitable surface active agents, including anionic and non-ionic surfactants, it can lead to strong linkage between the nanoparticle and the polymer. The multi-functionalized nanocomposite enhances the physicochemical properties and no untoward effect on the biological system had been reported [295]. For the hydrophobic drug the phage display technique has been used for the functionalization [296], and the bioavailability have enhanced by post-polymerization. Additionally, the post-polymerization with copolymer produces efficient targeting in the extracellular compartment of the biological system [297-300]. With the nanoparticles the polymers like PEG establishes for prolonged systemic circulation [301, 302]. For the stimuli responsive targeted drug delivery has been achieved by the functionalization of suitable materials (light or magnetic or thermal or ionic responsive material). Particularly, the magnetic induction systems have been used with functionalized magnetic nanoparticles for cell or tissue

specific targeted delivery. For targeting brain delivery system the nanoparticles has been functionalized for specific or nonspecific binding mechanisms [303]. The fabrication and functionalization science has merged with software oriented technology for the development of controlled and targeted nanoparticle loaded micro-device system [304]. The recent trends in novel polymer and block co-polymer synthesis methods like radical polymerization and click chemistry has been provide well-desired multi functionality polymeric structures [305-312]. This is the potential method to fabricate the desired molecular weight polymer with well-defined characteristic features. This unique method of polymer synthesis gives the successful nano formulation for potential bio-application. The functionalized nanoparticles have been synthesized with potential biochemical moieties. Then these multi-functionalized nanoparticles have been examined for desired physicochemical property and biocompatibility.

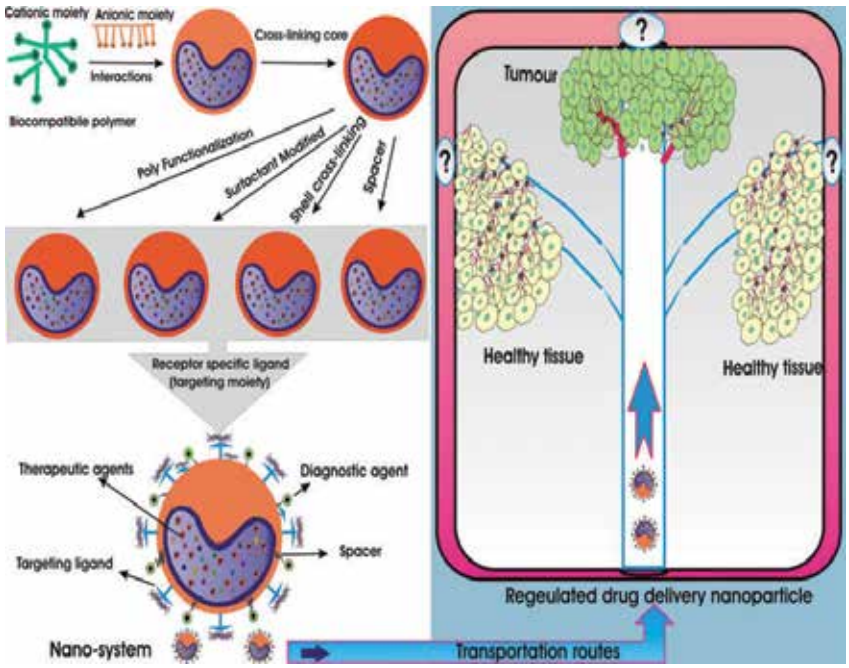

Figure 8. Schematic diagrams represent the various functionalizations of the nano-engines for smart drug delivery systems, which the pre-regulated nanoparticle recognizes the tumor cells not the healthy.

7. Recent developments, significant route of administration and targeting strategies

The route of administration of therapeutics is crucially important to cure the disease. Despite the invention of potential therapeutic moieties, the inefficient drug targeting by pills or injection on the appropriate site of the body limits therapeutics values to a larger extend. There

are multiple barriers involve in the anatomical and physiological system to lack the drug efficiency, including enzymatic degradation in the stomach, absorption across the intestinal epithelium, hepatic clearance, and accumulation in non-targeted tissues. These barriers also involve a range of complexities from the tissue to the organelle level along with the time that mismatch the drug potency in vivo. Collectively, these conditions challenge the active utilization of potent therapeutic molecules for disease treatment or prevention. Extensive research has been carried out in the field of drug delivery to overcome these challenges and thus to contribute a significant role in the overall drug-development process. After the evolution of nanotechnology and vast increment in knowledge about the human body, advances have been achieved in the drug delivery field as targeted delivery and sustained/controlled delivery system. By tuning the kinetic properties of therapeutics, the potentiality could be secured until it reaching the targeted organ and this factor is considered to be the most important in the field of pharmacology. Progresses in the nanomaterials development have been fruitful to fulfil the goals of drug delivery. Pharmacologically, the drug delivery is better explained based on the routes of drug administrations. Development of alternative drug delivery methods is crucially important to overcome the challenges experienced throughout the history of medicine. Scientists have been working on the creation of the smart drug delivery system and such approaches could provide an easy route of administration, ensuring patient compliance, decreasing toxicity, improving bioavailability and achieving precise therapeutic targeting. Creation of smart drug carrier as delivery systems and the discovery of new pharmacological compounds will potentially advance disease diagnosis and treatment beyond expectation. A variety of novel drug delivery systems have been developed using various nanomaterials during the last decade and several of them are already marketed. Nanotechnology manipulates the multiple properties including the size and other physical characteristics and thus achieves both controlled and targeted delivery of drugs. The bio-adaptability and multi-functional properties of smart delivery system minimize the undesirable properties of drugs in various routes of administration, including oral, rectal, nasal, ocular, topical route such as transdermal, and dermal, parenteral route such as intravenous/intravascular, intramuscular, subcutaneous, intradermal/intracutaneous, intraperitoneal and intrathecal. Figure 9 depicts the tremendous applications of new nanomaterials for the development of various routes of administration and targeting for therapeutics such as transdermal vaccine delivery, intranasal vaccine delivery and lung targeted delivery. Nasal mucosa offers numerous benefits as a target tissue for drug delivery, particularly for brain targeting because drug penetration through the BBB is favored by lipophilicity.

In particular, the non-invasive intranasal delivery offers large interests in the targeted route of administration. Nasal delivery helps drugs to bypass the blood-brain barrier and hence acts as an excellent platform for brain targeting. The intranasal drug delivery several approaches should be considered, attending, specifically, to the nature of pathological condition (acute or chronic) and intended effects of drug treatment (local, systemic or at CNS). Local delivery, nasal vaccines, systemic delivery and CNS delivery through nasal route is the prime route for drug administration to treat the various diseases. So the nasal vaccination is a promising alternative to the classic parenteral route because the nasal mucosa possesses abundant nasal associated lymphoid tissue (NALT), dentritic cells, large surface area, and low proteolytic

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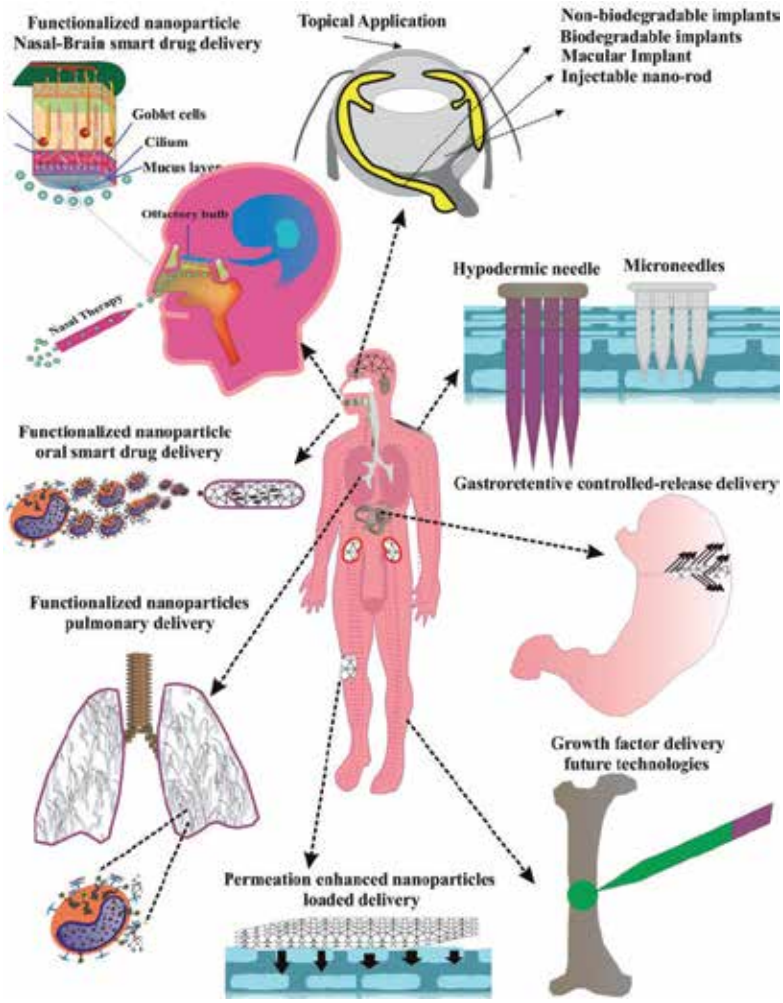

Figure 9. Schematic diagrams represent the recent developments of various significance routes of administration and targeting strategies

enzymes that serve as a primary defense system against pathogens. It can exhibit high drug concentration, permeation, no first-pass effect and compliance administration without enzymatic destruction. Moreover, antigens encapsulated nanoparticles ensure enhanced uptake and controlled release of antigens from the nasal vasculature membrane with strong immunogenicity and improved systemic therapeutic responses. Also, the bio-nanotechnology applied to the parenteral administrations techniques such as microneedles, jet-injections, ultrasound, iontophoresis, and electrophoresis. Theses systems extend painless, patient-friendly alternatives to injections for the delivery of molecule [313-317]. Drug administration using microneedles for the transdermal delivery routes have been reported elsewhere [318-320]. Microneedles are arrays of micrometer-sized shallow needles that penetrate only into the superficial layers of skin, thereby eliminating the pain associated with standard

hypodermic needles [321]. Microneedles have been made from a variety of materials and in particular the polymers have been shown to be effective. They have also been produced in solid and as well as in hollow forms. Solid microneedles are used to render skin permeable, whereas hollow microneedles actively deliver drugs into the skin at a controlled rate. In contrast, jet injectors deliver a high-velocity liquid jet stream into the skin, delivering drugs into various skin layers, depending on the jet parameters [322]. Jet injectors have a long history, particularly in the delivery of vaccines, insulin, and growth hormone. Ultrasound enhances skin permeability by cavitation, which temporarily disrupts skin structure [323]. Iontophoresis and electroporation use electric fields to alter the skin structure and/or provide additional driving force for drug penetration through the skin [324]. These new routes of administration of therapeutics with improved responses have been achieved by high drug concentration in target, permeation, no first-pass effect, high bioavailability and compliance administration without enzymatic destruction [325, 326].

8. Conclusion

The uses of bio-nanotechnology in therapeutics a number of unexpected inventions have been done recently on polymer based nanometers, which have great attention in the field of smart drug delivery applications. The biomaterials including protein based polymers, polysaccharide based polymers, natural or synthetic or semi-synthetic polymers, various biomaterials and combination of polymer have utilized to prepare various kinds of nano-formulations towards the smart drug delivery applications. Several polymeric nanoparticle-based therapeutic systems have been established for the treatment of various diseases. Several nanoparticle based drug delivery systems have been approved in clinical trials, some of them in under pre-clinical trial levels, this nanoparticle based system can provide the increased half-life, high biocompatibility, and minimum immunogenicity, site targeting and overcome the membrane barriers. Also the last era, major and new identifications have been drastically established in the smart material that alter its own structure and function in response to the environment. This performance has been used for the fabrication smart drug delivery systems, Smart polymer matrices release drugs by environment responses this system have been successfully achieved. In parallel the new method of bottom-up and top-down nanofabrication technologies provided precisely controlled size and shaped nano-particulate delivery system. Simultaneously, various advanced significant routes of targeting have developed and successfully achieved to the site of action. At present, the field of microfluidics for synthesis, micro-needle for transdermal and site targeted delivery is still in its infancy. So the pharmaceutical industry has to bring these products into industry-led investigation and the improvement in this would possibly to quicken their progress.

9. Future perspectives

Although there are considerable amount researches have been done in the field of drug delivery so far. In the polymeric nanoparticle based drug therapy has to be enhanced by incorporating by the combination therapies, Smart delivery has been achieved successfully in the case of cancer, but need to be concentrating more on other pathologies, also numerous challenges remain. From the material viewpoint, most of the smart delivery systems mechanism do well in vitro studies but flops the in vivo studies. So the research has to be re-considering to come up with simple, straightforward, efficient and reasonably accurate preparations with broadly applicable strategies, the pharmacologically active agent targeting to pathological sites, for the development of smart drug delivery systems. In technology vice the research has to focus into the fusion technologies. Although several specific specialized technologies have been shown to in polymer synthesis, functionalization, analysis, in vitro and in vivo study in the field of polymer science, the combinations of two or more techniques are often more effective than single technologies like a combination of controlled radical polymerization with click chemistry. The fusion technologies can fulfil the various existing drawbacks of some individual technologies, and this has the high potentiality, synergistic enhancement in safest nanoparticle based drug delivery. Consider merging and adopting two or more right technologies for getting a high-throughput technology by selecting the right combinations is a fruitful area for research that is still largely unexplored. This new understanding must be incorporated into the future of newer polymeric based nanoparticle synthesis development and evaluation of smart drug delivery. Also the next generation of polymeric nanoparticle based delivery systems with drugs like growth factors, hormones, antibodies, genes, peptides, etc.; should also enhance the efficiency and minimize the unwanted effects.

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Author details

Devasier Bennet[1] and Sanghyo Kim[1,2*]

*Address all correspondence to: samkim@gachon.ac.kr

1 Department of Bionanotechnology, Gachon University, Bokjeong-Dong, Sujeong-Gu, Seongnam-Si, Republic of Korea

2 Graduate Gachon Medical Research Institute, Gil Medical Center, Inchon, Republic of Korea

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Chapter 9

Mannan as a Promising Bioactive Material for Drug Nanocarrier Systems

Songul Yasar Yildiz and Ebru Toksoy Oner

Additional information is available at the end of the chapter

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1. Introduction

Polysaccharides are natural, non-toxic and biodegradable polymers that cover the surface of most cells and play important roles in various biological mechanisms such as immune response, adhesion, infection and signal transduction. Investigations on the alternative treatments applied by different cultures throughout the history revealed the fact that the utilized plants and fungi were rich in bioactive polysaccharides with proven immunomodulatory activity and health promoting effects in the treatment of inflammatory diseases and cancer. Hence considerable research has been directed on elucidating the biological activity mechanism of these polysaccharides by structure-function analysis [1].

Hemicelluloses are structural polysaccharides which are the second most abundant heteropolymers present in nature accounting for one third of total components available in the plants (Figure 1) [2]. Mannans and xylans are the two most important hemicelluloses and hence a lot of research is mainly focused on their value-added applications and hydrolysis [3]. Mannan is a biodegradable and bioactive polysaccharide that has been a focus of interest by various sectors due to its valuable properties. The film forming capacity and biodegradability of mannans make them an interesting alternative to the petroleum-based materials. Mannan-based films and coatings were shown to exhibit low oxygen and grease permeability and, in some cases, relatively high tensile strength [4]. There are also interesting reports on the successful use of mannan as a bioactive material in health related applications.

Mannans are linear polymers of 1,4-linked mannose residues and contain less than 5% of galactose [5]. In nature, mannan is present in four different forms, each having a β-1,4-linked backbone containing mannose (linear mannan) or a combination of glucose and mannose residues (glucomannan) and occasional side chains of α-1,6-linked galactose residues (galac-

tomannan / galactoglucomannan). The mannose and glucose residues in the backbone are sometimes acetylated at C-2 or C-3 (3,5).

Figure 1. Polysaccharide composition of plants

In plants, mannans have a structural role by binding cellulose, but also they serve a storage function as a reserve carbohydrate in endosperm walls and vacuoles of seeds and vacuoles in vegetative tissues [5]. Recently, mannan has also been proposed as a signaling molecule in plant growth and development [6].

Mannan is a biodegradable and bioactive polysaccharide that has been a focus of interest by various sectors due to its valuable properties. The film forming capacity and biodegradability of mannans make them an interesting alternative to the petroleum-based materials. Mannan-based films and coatings were shown to exhibit low oxygen and grease permeability and, in some cases, relatively high tensile strength [4]. There are also interesting reports on the successful use of mannan as a bioactive material in health related applications. Mannan conjugated to vaccine preparations are already in the clinic [7,8]. Tang et al. [9] utilized a mannan-based system to target DNA vaccines to antigen presenting cells and demonstrated that it could induce far stronger immune responses in mice compared to naked DNA immunization. By further studies, they could explain the molecular basis of the observed immune enhancing attributes of mannan-based DNA vaccination [9]. Successful use of carboxylic mannan-coated iron oxide nanoparticles in targeting immune cells for *in vivo* lymph node-specific Magnetic Resonance Imaging was also reported recently [10]. Moreover, to target mannose receptor expressed on the surface of antigen-presenting cells, biocompatible self-assembled mannan nanogels were designed to provide a therapeutic or vaccine delivery platform [11,12]. In a recent review on oral drug delivery research in Europe, mannan based nanogels were considered as a new approach for the oral delivery of labile molecules [13].

In this chapter, after a brief description of mannan, its production by algae, fungi, bacteria and other eukaryotic microorganisms will be mentioned with special focus on microbial resources. Then, use of mannan as a bioactive material in nanocarrier systems for drug delivery applications will be covered in detail by giving examples from literature and industry. The final

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section of the chapter will involve conclusions and future prospects on microbial mannan production and its potential uses in nanotechnology.

2. Structure of mannans

Mannan is one of the important member of the hemicellulose family and can be divided to four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomanan [14]. Mannan is present in different forms, each having a β-1,4-linked backbone containing mannose (linear mannan) or a combination of glucose and mannose residues (glucomannan) and occasional side chains of α-1,6-linked galactose residues (galactomannan / galactoglucomannan) (Figure 2). In the backbone, mannose and glucose units can also be acetylated at C-2 or C-3 (3,5) .

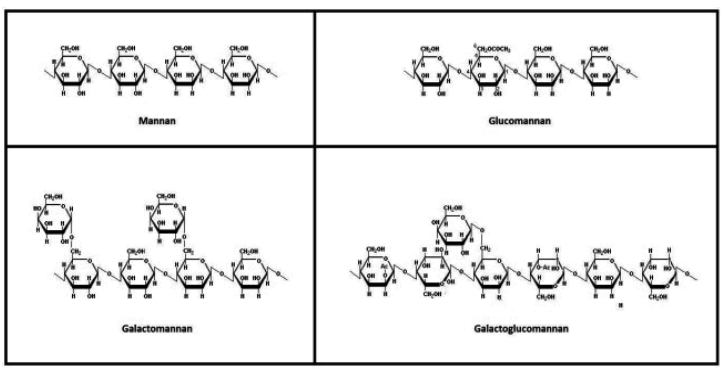

Figure 2. Four different forms of mannan

Glucomannan is mainly a straight-chain polymer, with a small amount of branching. The component sugars are β-(1,4)-linked D-mannose and D-glucose with a reported ratio of 1.6:1 [15], or 1.4:1 [16]. Softwoods and hardwoods consist of glucomannan with a glucose/mannose ratio of 1:3 and 1:1.5–2, respectively [17-20]. There is a significant similarity between conformation of glucomannan chains and those of cellulose A two-fold screw axis was observed because of the extended chains. Due to axial position of the hydroxyl group at C-2 of mannose, the interaction between C-6 and O-2 atoms of contiguous residues is prevented, and the chains are loosened, weakening the packing and organization [17]. Different structures were reported for glucomannans isolated from different sources. For example, (1 → 4)-linked structure, acetyl groups at C-2, C-3 positions and O-acetyl group at C-6 position were reported for glucomannan extracted from seeds of *Lupinus* [21]. Irregular distribution of acetyl groups was reported for pine glucomannan [22]. Studies on a nonionic glucomannan with a main chain of β-(1 → 4)-linked mannopyranosyl units to which D-glucopyranosyl units are linked by α-(1 → 6)

linkages, isolated from seeds of *Bryonia lacinosa* was also reported [23]. Galactomannans are polysaccharides consisting of 1,4-linked β-D-mannopyranose backbone with side chains of single 1,6-linked α-D-galactopyranose attached along the chain [24-26]. Galactose to mannose ratio show differences among different sources. More than 5% galactose residues can be considered as galactomannans [27].They are mainly found in the seeds of the family of *Leguminoseae* [28,29]. They are also present in the species of *Annonaceae*, *Convolvulaceae*, *Ebenaceae*, *Loganiaceae*, and *Palmae* [29]. Unusual backbone structure, containing (1 → 3)-linked residues together with a small proportion of (1 → 4)-linked β-D-mannopyranosyl residues with galactopyranosyl units attached at position 6, of galactomannan isolated from *Retama raetam* was reported in 2004 [30]. Presence of arabinosyl and glucosyl residues in the structure of galactomannans was observed in the studies of green and roasted coffee [31]. Several lichen species have been also reported as a source of galactomannan [32]. (1 → 6)-α-D-mannopyranosyl backbone with a different substitution pattern at O-2 and O-4 was observed in galactomannans isolated from lichens. The four major galactomannans of commercial importance in food and non-food industries are guar gum (GG, *Cyamopsis tetragonolobo*, M/G ratio: 2:1), tara gum (TG, *Caesalpinia spinosa*,M/G ratio: 3:1), locust bean gum (LBG, *Ceratonia siliqua*, M/G ratio: 3.5:1) and Fenugreek (*Trigonella foenum-graecum* L., M/G ratio: 1:1) [33].

Galactoglucomannan consists of a backbone of randomly distributed (1 → 4)-linked mannose and glucose units with (1 → 6)-linked galactose units attached to mannose units. The hydroxyl groups in locations C2 and C3 in mannose are partially substituted by acetyl groups [34,18]. Molar ratio of mannose, glucose and galactose was reported as 3:1:1 in the study of Puls and Schuseil [35]. Some of the mannosyl units are partially substituted by O-acetyl groups, equally distributed between C-2 and C-3 on the average one group per three to four hexose units [18, 36]. 5.9%-8.8% acetyl content was also observed [18].

The acetylated galactoglucomannan is mainly found in hemicellulose of softwoods. They can be either galactose rich or galactose poor with 10-15% and 5-8% of the dry woods respectively [36-38]. Acetylation at C-2 and C-3 positionsin the ratio of 2.2:1 was reported for galactoglucomannan backbone from native Norway spruce wood [36]. Formation of strong hydrogen bonds due to large content of D-galactose side-chains prevents the macromolecules from aligning themselves and hence galactoglucomannan is soluble in water [39].

3. Sources of mannans

Mannan is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms, but is the minor hemicellulose in hardwood from angiosperms [35]. Unsubstituted beta-1,4-mannan, composed of a main chain of beta mannopyranose residues, is an important structural component of some marine algae [40] and terrestrial plants such as ivory nut [41] and coffee bean [42].

A variety of plants store energy in the form of mannans in their endosperm tissue, including members of the Palmae, Liliaceae, Iridaceae, and Leguminosae families [43,44]. Glucomannans also are used for energy storage in corms of plants within the genus Amorphophallus. In

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addition to carbohydrate storage and structure, mannans serve a variety of other functions. In fern roots, mannans are deposited as constituents of cell wall appositions as a defense mechanism to limit microbial ingress [45]. Besides plants, algae are also a viable resource for mannan polysaccharides. In particular, the Dasycladalean alga Acetabularia acetabulum, also known as 'mannan weed', has long been known to contain mannan-rich walls [46]. Moreover, mannans are a common feature of fungal walls and a recent review points to the importance of cell surface mannans of pathogenic Candida species since they were found to participate in the adhesion to the epithelial cells, recognition by innate immune receptors and development of pathogenicity. Hence, clarification of the precise chemical structure of Candida mannan was reported as indispensable for understanding the mechanism of pathogenicity, and for development of new antifungal drugs and immunotherapeutic procedures [47]. Also, some yeast species stand out for their capability for excreting mannan to the fermentation medium. Yeast Rhodotorula acheniorum MC bioreactor cultures have been reported to produce 6.2 g/L mannan when grown for 96 hours in sucrose containing media [48]. Moreover, psychrophilic Antarctic yeast Sporobolomyces salmonicolor AL1 reached maximum glucomannan yield of 5.64 g/L in medium containing sucrose after a 5 days of fermentation [49]. Mannan synthesized by R. acheniorum MC, as well as the glucomannan, synthesized from strain *S. salmonicolor* AL_1 were both found to form stable emulsions making them suitable for various applications in pharmaceutical and cosmetic sectors [50]. On the other hand, studies also point to adverse toxic effects of fungal mannans when administered [51].

Although mannan production is established by numerious algal, fungi and other eukaryotic microorganisms, they are not normally products of bacteria [52]. There are only very few reported examples on extracellular mannan production by bacteria. Gram negative phytopathogenic bacterium *Pseudomonas syringae pv. ciccaronei* was reported to produce a highly branched phytotoxic mannopyranose polymer, which consisted of a backbone of α-(1,6)-linked mannopyranose units with 80% substituted at C-2 by mono-, di-and trisaccharide side chains [53]. Then, to understand the role of this mannan polymer in the activation of plant defence responses, various concentrations of the polymer was infiltrated in the abaxial side of tobacco leaves. Mannan polysaccharide was found to induce chlorotic and necrotic symptoms even at very low concentrations very effectively suggesting that it was identified by the plant cells as a signal of pathogen attack or environmental perturbation [54]. Two mannans at different chain lengths were reported to be produced by the marine bacterium *Edwardsiella tarda*, an opportunistic pathogen in human, and the polysaccharides were found to have good antioxidant and hydroxyl and DPPH radicals scavenging activities [55]. The lower molecular weight mannan was associated with higher antioxidant activity than the longer mannan and could be used as possible food supplement or ingredient in the pharmaceutical industry [55]. Recently, about 20-fold increase in mannan production has been reported in the pathogenic, constitutive biotin-producing *Pseudomonas mutabilis* bacteria [56]. The rheological properties of the highly branched mannan isolated from *P. mutabilis* T6 showed that its viscosity was over 30 times greater than that of the wild type *P. mutabilis* ATCC 31014.

Table 1. illustrates mannan producer organisms.

Source	Organism	Mannan type	Reference
Plant	*Ebenaceae* family	Galactomannan	[29]
Plant	*Arabidopsis thaliana*	Mannan	[57]
Plant	seeds of the family of *Leguminoseae*	Galactomannan	[28,29]
Plant	*Caesalpinia spinosa* Kuntze	Galactomannan	[33]
Plant	*Annonaceae* family	Galactomannan	[29]
Plant	*Amorphophallus konjac*	Glucomannan	[58]
Plant	*Ceratonia siliqua*	Galactomannan	[33]
Plant	*Convolvulaceae* family	Galactomannan	[29]
Plant	*Cyamopsis tetragonoloba*	Galactomannan	[33]
Plant	*Loganiaceae* family	Galactomannan	[29]
Plant	*Senna tora* seed	Galactomannan	[59]
Plant	*Trigonella foenum-graecum L.*	Galactomannan	[33]
Plant	*Palmae* family	Galactomannan	[29]
Plant	*Picea abies*	Galactoglucomannan	[60]
Plant	*Cercis siliquastrum*	Galactoglucomannan	[61]
Plant	*Nicotiana plumbaginifolia*	Galactoglucomannan	[62]
Yeast	*Hansenula holstii*	Phosphorylated mannan	[63]
Yeast	*Rhodotorula acheniorum*	Mannan	[48]
Yeast	*Sporobolomyces salmonicolor*	Glucomannan	[64]
Yeast	*Saccharomyces cerevisiae*	Mannan	[65]
Yeast	*Meyerozyma guilliermondii*	Mannan	[66]
Yeast	Brewers dried yeast	Mannan	[67]
Yeast	*Candida utilis*	Glucomannan	[68]
Algae	*Porphyra umbilicalis*	Mannan	[69]
Algae	*Acetabularia acetabulum*	Mannan	[46]
Algea	*Charophyceae*	Mannan	[70]
Fungus	*Dactylium dendroides*	Galactoglucomannan	[71]
Fungus	*Pseudocyphellaria clathrata*	Galactoglucomannan	[72]
Bacteria	*Pseudomonas mutabilis*	Mannan	[56]
Bacteria	*Pseudomonas syringae pv. ciccaronei*	Mannopyronose	[53]
Bacteria	*Edwardsiella tarda*	Mannan	[55]
Bacteria	*Pseudomonas aeruginosa*	Mannan	[73]
Bacteria	*Brevibacillus thermoruber*	Mannan	[74]

Table 1. Mannan producer organisms

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4. Biosynthesis of mannans

Mannans are synthesized from activated nucleotide sugars such as GDP-mannose, GDP-glucose, and UDP-galactose [75]. Enzymes necessary for the nucleotide sugar conversion from sucrose to GDP-mannose and UDP-galactose have been reported in planta. However, the enzyme for the formation of GDP-glucose has not been identified [76]. Golgi-localized glycosyltransferases (GTs) utilize the activated nucleotide sugars and synthesize the polymer by facilitating the formation of the specific linkage between the monomers [77,78].

The cellulose synthase-like family A (CSLA) genes are considered the best candidates to encode enzymes that polymerize the backbones of β-linked noncellulosic polysaccharides [79,80]. Experimental evidence to support this hypothesis for the CslA family came first from Dhugga et al. [81]. In this research, the first β-mannan synthase (ManS), a member of the cellulose synthase-like family A (CSLA) from GT family 2, was identified in guar seeds (CtManS in Cyamopsis tetragonoloba, a AtCSLA9 ortholog) including the demonstration of its in vitro ManS activity [82]. One year later, three Arabidopsis CSLA genes were expressed in Drosophila Schneider 2 (S2) cells and demonstrated that the resulting CSLA proteins were capable of producing mannans when supplied with GDP-Man and glucomannans when provided with a mixture of GDP-Man and GDP-Glc [75]. CSLA genes appear to be present in all land plants, and ancestral genes with characteristics similar to CSLA sequences have been identified in a number of green algal genomes, in which they are thought to represent a homolog of the progenitor gene from which CSLA genes evolved [76]. In developing Trigonella foenum-graecum (Fenugreek) endosperm, a deep sequencing approach was used to identify genes involved in galactomannan biosynthesis [83]. This research reported a CSLA family protein involved in mannan backbone synthesis and a preference towards GDP-mannose as a donor substrate was observed from the activity assays with the heterologously expressed protein. Heterologously expressed CSLA proteins from a variety of species show mannan or glucomannan synthase activity in vitro [6,75,81,83]. Analysis of Arabidopsis CSLA mutants and over-expressing plants further confirmed that CSLA proteins function as glucomannan synthases *in vivo* [84]. Despite this progress in identifying and characterizing the enzymes responsible for galactoglucomannan biosynthesis, it is likely that other important enzymes are required, and many aspects of this process need to be better understood.

In tissues of *Arabidopsis*, that take role in tip-growth such as root hairs CSLD, (*At*CSLD2, 3 and 5) proteins were found to mediate mannan biosynthesis [85-92]. In Fenugreek, it was found that additional genes were involved in mannan biosynthesis, such as a golgi-localized mannan synthesis-related (*MSR*) gene that was observed in the fenugreek endosperm [83,93]. *Tf*MSR protein in Fenugreek and its homologs *AtMSR1* and *AtMSR2* in *Arabidopsis* were highly co-expressed with the ManS of the CSLA family. Glucomannan and ManS activity were significantly decreased in stems of *AtMSR* knock-out mutants [93]. While the biochemical activity of MSR proteins remains unknown, hypotheses include a role in primer synthesis to initiate mannan biosynthesis, the synthesis of oligosaccharides linked to CSLA or promoting folding, stability or activity of a mannan synthase complex [93].

Edwards et al. identified a mannan:galactosyltransferase (GalT) in *Trigonella foenum-graecum* [94], an enzyme that facilitates mannan *O*-acetylation. However, discovery of the involvement of a large plant-specific family of Trichome birefringence-like (TBL) proteins in *O*-acetylation of wall polymer as specific *O*-acetyltransferases suggested that this gene family encompassed a mannan *O*-acetyltransferase [95]. A highly expressed (among the 10 most abundant ESTs) homolog of *At*TBL25 in the *Amorphophallus konjac* deep sequencing database [96] revealed that this protein or the closely related *At*TBL26 could represent mannan *O*-acetyltransferase(s) in *Arabidopsis* [95].

5. Polysaccharide-based materials in drug delivery

Many properties of polysaccharides such as biocompatibility, solubility, potential for modification, and innate bioactivity provide great potential for their use in drug delivery systems (Figure 3).

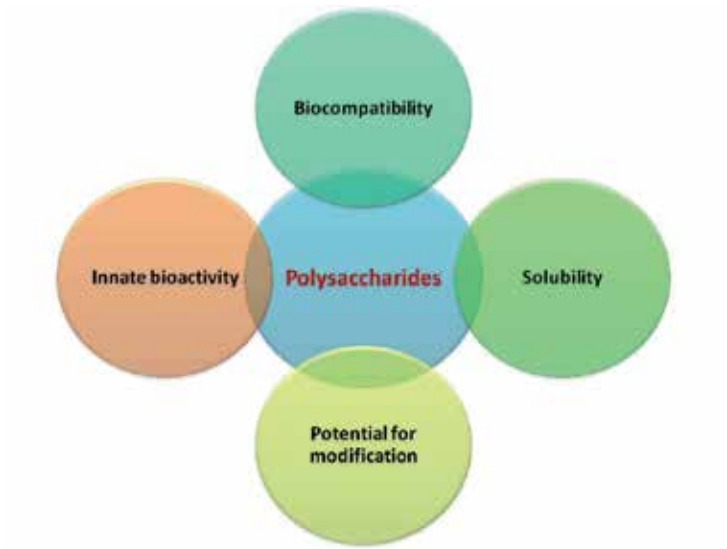

Figure 3. Properties of polysaccharides for potential use in drug delivery systems

Despite many synthetic polymers, polysaccharides have very low or no toxicity levels [97-100]. For example, dextrans are biopolymers composed of glucose with α-1,6 linkages, with possible branching from α-1,2, α-1,3, and α-1,4 linkages, that exhibit low toxicity and high biocompatibility, that makes them biocompatible hydrogels for controlled prolonged therapeutic release [101] and microspheres with no inflammatory response following subcutaneous injection into rats [102]. Since polysaccharides are naturally present in the body, most of them are degraded enzymatically. Through enzyme catalysis, polysaccharides can be broken down to their monomer or oligomer building blocks and recycled for use as storage,

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structural support, or even cell signaling applications [103]. As a result, mechanism of release for therapeutics associated with polysaccharide-based carrier systems is provided by enzymatic degradation [104].

The functional groups of polysaccharides such as hydroxyl and amine groups yield high aqueous solubility. However, this solubility can often be adjusted via monomer modification. For example, *O*-acetylation of glucomannan can be used to modulate the formation of intermolecular hydrogen bonds with water, thereby altering aqueous solubility [105].

Due to the presence of various derivable groups on molecular chains, polysaccharides can be easily modified chemically and biochemically, resulting in many kinds of polysaccharide derivatives. These modifications can change the character of the polysaccharides. For instance, hydroxyl group oxidation enhances biodegradability, while sulfonation generates a heparin-like polysaccharide with increased blood compatibility [106]. Quaternization of the primary amines with various alkyl groups can be used to enhance solubility and alter bioactivity [107-109].

Many polysaccharides possess innate bioactivity, particularly mucoadhesive, antimicrobial, and anti-inflammatory properties. Positively charged polysaccharides are capable of binding to the negatively charged mucosal layers through charge interactions [110-112]. For neutral or negatively charged polysaccharides, hydrogen bonding provides an alternative mechanism for mucoadhesion [113]. Nanoparticle carriers made of bioadhesive polysaccharides could prolong the residence time and therefore increase the absorbance of loaded drugs [114]. Several polysaccharides are also antimicrobial in nature, such as chitosan [115]. Other polysaccharides are known to reduce inflammation. Anti-inflammatory activity is thought to be due to binding with immune-related acute phase and complement proteins [111,116] and polysaccharides are known to interact with a variety of proteins.

Nanocarriers are nanoparticle drug delivery systems that are used to deliver drugs or biomolecules. Nanocarriers are sub-micro particle structures smaller than 100 nm in at least one dimension and cover nanospheres, nanocapsules, nanomicelles, nanoliposomes, and nanodrugs, etc. Nanoparticle drug delivery systems have noticeable advantages. Due to the ultratiny volume of nanoparticle they can pass through the smallest capillary vessels and avoid rapid clearance by phagocytes, that lead to greatly prolonged duration in blood stream. Due to small dimensions, nanocarriers are able to cross the blood-brain-barrier (BBB) and operate on cellular level. They can easily penetrate cells and tissue gap to arrive at target organs such as spleen, spinal cord, liver, lung, and lymph. Because of the biodegradability, pH, ion and/or temperature sensibility of materials, they could show controlled release properties. They can improve the utility of drugs and reduce toxic side effects; etc. As drug delivery system, nanocarriers can entrap drugs or biomolecules into their interior structures and/or absorb drugs or biomolecules onto their exterior surfaces. Presently, nanoparticles have been widely used to deliver drugs, polypeptides, proteins, vaccines, nucleic acids, genes and so on.

In recent years, a large number of studies have been conducted on polysaccharides and their derivatives for their potential application as nanoparticle drug delivery systems [114,117-120] and among them, mannan is a very promising bioactive material for drug nanocarrier systems

since an amphiphilic form of mannan can spontaneously incorporate proteins and other agents, potentially providing a new nanostructure drug delivery system.

6. Medical potential of mannans as a drug nanocarrier systems

Glucomannans have a variety of applications, including food industry used as an emulsifier and thickener and medicine as a preventative of chronic disease and weight control agent [21]. Likewise, galactomannans also found many applications in food industrial as a thickener and food additive due to their rheological properties [121]. Moreover, galactomannans are widely used as versatile materials in industries such as textiles, paper, pharmaceutics, cosmetics, petroleum, drilling and explosives [93,122].

Galactomannans have also significant potential in medical applications such as innate immune system stimulation. On the other hand, the mannooligosaccharides (MOS) derived from these polysaccharides have also prebiotic activity for selective growth of Bifidobacterium spp., and Lactobacillus spp. [123]. They have also been described to present anticoagulation and fibrinolytic activity [124] and the MOS may prevent adherence of toxic bacteria to the intestinal wall, mediated by lectins, thus presenting anti-infectious potential [123,125,126].

In the research of Apostolopoulos *et al.* oxidized mannan conjugated to MUC1 fusion protein (M-FP) was used as a target for tumour immunotherapy and M-FP appeared to confer the survival/disease-free interval advantage in patients with early stage breast cancer [8].

In another study, the factors important to gene delivery and DNA vaccination that could contribute to the improved immunogenicity of oxidized mannan poly-L-lysine (OMPLL)–DNA and reduced mannan poly-L-lysine (RMPLL)–DNA immunization were investigated [9]. It was shown that OMPLL and RMPLL were able to complex with DNA to form particles that were taken up by charge dependent binding and endocytic pathways. High possibility of delivery of DNA was observed since the particles formed were able to protect DNA from DNase I digestion. More significantly, direct effect of OMPLL and RMPLL was observed on the antigen presentation of dentritic cells (DCs).

In 2010, Guo et al. reported that marine bacterium *Edwardsiella tarda* produced two extracellular polysaccharides ETW1 and ETW2, mannans with different molecular mass, that exhibited strong antioxidant activities [55]. To investigate the antioxidant activities of the two polysaccharides, antioxidant properties based on hydroxyl, DPPH radical scavenging and lipid peroxidation inhibition assays were carried out. The scavenging abilities of ETW1 and ETW2 on DPPH radicals, hydroxyl radicals and lipid peroxidation inhibition were concentration-dependent.

In 2011, Ferreira et al. prepared nanogel made of mannan [11]. The properties of the resulting nanogel were characterized and cytocompatibility was tested by using two cell lines, namely, mouse embryo fibroblasts 3T3 and mouse macrophage-like J774. The results of study revealed that the mannose receptor binds ligands at the cell surface and these receptor-ligand complexes were internalized via the endocytic pathway. Internalization of the nanogel caused cytotoxicity

since the non-phagocytic cell line was not affected and internalization was confirmed with J774. The high nanogel toxicity observed with the macrophage cell line indicated that the cell line J774 was not suitable for studies with mannan-C16 nanogel and primary cultures of macrophages that do not exhibit cytotoxicity should be used instead.

In 2012, the mannan nanogel cytocompatibility was tested in mouse embryo fibroblast cell line 3T3 and mouse bone marrow-derived macrophages (BMDM). [12]. The essential focus of the study was to assess nanomaterial cytocompatibility and to analyze the internalization by macrophages. The results of this study indicated that the mannan nanogel was biocompatible to mouse embryo fibroblast 3T3 cells and mouse BMDM. Essentially, no cytotoxic effect was observed with mannan nanogel up to about 0.4 mg/mL in *in vitro* experiments. Cell survival rate only dropped significantly at higher tested concentration after 48 h of incubation. Comet assay, under tested conditions, revealed no DNA damage in mouse embryo fibroblast 3T3 cells but possible DNA damage in mouse BMDM. Upon internalization by mouse BMDM mannan nanogel was localized in vesicles, as judged by the non-even distribution over the cytoplasm, and concentration of the fluorescence in internalized structures. Exit of nanogel from the mouse BMDM was observed when cells were incubated in fresh medium. Confocal colocalization image analysis denoted that the entrance and exit of nanogel and FM 4-64 might occur by the same processes – endocytosis and exocytosis – in BMDM.

Sato et al. [127] examined the adhesion inhibitory effect of mannan coating on acrylic denture surfaces against *Candida albicans* and *Candida glabrata*. The outermost layer of the *Candida* cell wall is covered with hydrophilic polysaccharides, such as mannan or galactomannan [128]. These mannans on the fungal surface function as adhesins, which are involved not only in the adhesion to the host cell [129,130] but also in the adsorption to plastic plates. On the other hand, when the plastic surfaces of culture dishes were coated with mannan, the adherence of *C. albicans* to the dishes was significantly inhibited [131,132]. The results of this study indicated that mannan inhibited the adhesion of *Candida* in a concentration-dependent manner, but mannose was not able to inhibit *Candida* adhesion even at a high concentration. The application of 0.1 mg/mL of mannan coating overnight showed inhibitory effects on the adhesion of the hyphal form of *C. albicans.* In the case of *C. glabrata*, the inhibitory effect was also observed to occur in a concentration-dependent manner, and the 10 mg/mL of mannan led to significantly higher anti-adhesive effects. This indicated that mannan effectively prevented the adhesion of two major *Candida* species to the denture surface, indicating the possibility of applying such a coating for clinical dentistry.

Superparamagnetic iron oxide nanoparticles (SPIONs) have been used as a contrast agent in magnetic resonance imaging (MRI) or as a carrier platform in the applications of drug [133-135] and gene delivery [137,138]. It was previously reported that mannan-coated SPION (mannan-SPION) could be specifically targeted to macrophages by the interaction with mannose receptors on antigen-presenting cells (APCs) [139]. Vu-Quang et al., [10] investigated the physicochemical properties, the *in vitro* and *in vivo* uptakes of carboxylic mannan-coated SPION (CM-SPION) using MRI and assessment of systemic toxicity. Results of the study showed that CM-SPION achieve longer circulation than mannan-SPION without compromising specificity. The intracellular accumulation of CM-SPION in macrophages was higher than

those of either PVA-SPIONs or Dex-SPIONs. The intracellular localization of CM-SPIONs was pre-dominantly observed in the cytoplasm of APCs. In the light of these results, authors claimed that CM-SPION could be regarded as a safe and potential contrast agent in LN-targeted MR imaging.

The effective conjugation of iron oxide nanoparticles with various biomolecules has been used for novel therapeutic-and drug delivery purposes [139-142]. Ultrasmall superparamagnetic iron oxide (USPIO) targets biomarkers of atherosclerotic plaques and improvements of USPIO make possible to obtain better plaque images at lower doses. Mannose units of mannan polysaccharides are recognized by mannose receptors on immune macrophages and they lack of significant toxicity. As a result, in the study of Tsuchiya [143], MRI-and histologic analyses were performed to compare the uptake by the rabbit atherosclerotic wall of four types of SPIO particles, i.e. SPIO, mannan-coated SPIO (M-SPIO), ultrasmall SPIO (USPIO), and mannan-coated USPIO (M-USPIO). Resuts of study reveal that mannan-coated iron particles had a greater affinity for active atherosclerotic plaques than non mannan-coated iron particles. Intracellular iron uptake was also higher in cells treated with M-USPIO than USPIO.

Glucomannans have diverse applications in biomedical and pharmaceutical areas due to the advantages of the polysaccharide such as weigt loss in obesity, decreased carbohydrate absorption in diabetes type 2, antitumor activity against sarcoma in cancer, decreased LDL levels in cholesterol, recognition of mannose receptors in targeting, antiseptic coating and sustained release profiles, increase of stability, improvement of the interaction between polmers, enhancement protein association of pharmaceutical forms of glucomannan. Glucomannan has been investigated as a pharmaceutical excipient in tablets, films, beads and hydrogels, due to its gelling, solubility and biodegradable properties [143-146].

Electrostatic interaction between the negative carboxylic groups of carboxymethylated-GM and the positive amino groups of chitosan was used for the preparation of nanoparticles made of carboxymethylated-GM and chitosan [147]. These nanoparticles were within size range of 50–1200 nm and exhibited a positive charge. Additionally, these nanoparticles elicited an ability to entrap and release bovine serum albumin (BSA) [147,148] The objection of use of GM in these nanoparticles was to increase their stability and their controlled release properties. Sande et al. reported that the introduction of GM into the nanoparticles lead to a facilitated interaction with the intestinal epithelium both *in vitro* and *in vivo* [149, 150]. The results of studies revealed that GM–chitosan nanoparticles offer attractive features as carriers for transmucosal drug delivery applications.

In the report of Zhang et al., use of konjac glucomannan (KGM) in oral colon targeting drug delivery system (OCDDS) was reviewed [151]. Based on the previous studies of KGM, it could be considered as a significant natural polysaccharide in OCDDS. It was known that KGM gel systems were able to maintain integrity and control the release of theophylline and diltiazem for 8 hours [152]. KGM hydrolysate was reported to have prebiotic potential for beneficial gut microbiota [153,154]. KGM is a water soluble polysaccharide because of hydrogen bonding in its structure [155,156]. The stronger the hydrogen bonding between their molecules, the harder for it to dissolve in water. Water solubility can be either advantageous or disadvangeous according to its application. Due to the high water adsorption rate, deficiency of free water in

gastrointestinal tract occurs and leads to diarrhea when KGM was used in the applications such as pharmaceutical excipients or drugs. On the other hand, when prepared as styptic sponge, which used to stop bleeding, the higher the water adsorption rate of it, the better the hemostasis effect may be. Modifications of KGM lead to alteration in the water adsorption of it. Moreover, KGM have gel-forming and film-forming properties [157].

In the previous studies, it was reported that KGM can be specifically degraded by colon β-mannanase [158], an enzyme generated by human colon bacteria [159]. On the other hand, based on the toxicity tests Ancui et al. reported KGM as a stable and safety material for medicinal purposes [160].

Invention of a novel hydrogel systems designed for colon-targeting drug delivery was reported in 2004 [158]. This hydrogel was composed of KGM, copolymerized with acrylic acid, and crosslinked by the aromatic azo agent bis(methacryloylamino)-azobenzene. Chen, Liu and Zhuo, copolymerized KGM and acrylic acid (AA) with N, N-methylene-bis-(acrylamide) (MBAAm) to form a novel hydrogel system [161]. Studies on swelling behaviors and degradation showed that the gel is pH-sensitive and could be degraded by Cellulase E0240 containing β-mannanase. Further researches demonstrated that the IPN hydrogel composed of KGM and poly(acrylic acid) (PAA) and cross-linked by N, N-methylene-bis-(acrylamide) (MBAAm) was still pH-sensitive and a potential carrier for colon-targeting drug delivery. Xu et al., prepared oxidized konjac glucomannan (OKG) for OCDDS which was pH-sensitive and could be used without the destruction of drugs in gastric acid [162]. Furthermore, Korkiatithaweechai et al., prepared controlled release of diclofenac sodium (DFNa) film from CTS (chitosan)-OKG [163]. This study suggested that the proportion of OKG in the formulation may affect the release profile and the formulation may be used for further study as a long term intestine controlled release drug model (at least 3 days), including as colon targeting drug carrier.

Guar gum derived from the seeds of *Cyamopsis tetragonolobus* is a naturally occuring galactomannan polysaccharide that consists of 80% galactomannan, 12% water, 5% protein, 2% acid insoluble ash, 0.7% ash and 0.7% fat. Guar gum has been reported as an inexpensive and flexible carrier for oral extended release drug delivery [164]. Guar gum can be used for colon delivery since it can be degraded by specific enzymes in this region of the gastrointestinal tract. GG provides protection to the drug in the environment of the stomach and small intestine, and drug delivery to the colon where it is degraded by the enzymes excreted by specific microorganisms. Guar gum shows high potential as a carrier for oral controlled release matrix systems. Furthermore, excipients to GG can be used to modulate drug release from these matrix systems [165].

Locust bean gum also known as Carob bean gum consists mainly of a neutral galactomannan polymer made up of 1,4-linked D-mannopyranosyl units and every fourth or fifth chain unit is substituted on C6 with a D-galactopyranosyl unit. Locust bean gum is a neutral polymer and its viscosity and solubility are therefore little affected by pH changes within the range of 3-11 [166]. Locust bean gum was used to produce matrix tablets with and without the cross-linker, glutaraldehyde [101]. A commercially available tablet system (TIMERx®) developed

by Penwest Pharmaceuticals Company consisting of locust bean gum and xanthan gum showed both *in vitro* and *in vivo* controlled release potential [167].

Guar gum hydrates and swells in cold water [168]. This gelling cause to retardation of the drug release from the tablets [169,170]. Guar gum is being used to deliver drug to the colon due to its drug release retarding property and susceptibility to microbial degradation in the large intestine [171,172]. Guar gum based matrix tablets of dexamethasone and other antinflammatory agents were prepared and used in colon targeting [173]. Whereas negligible drug release was observed in simulated gastric and intestinal fluids, significant increase in drug release was reported in simulated colonic fluid.

Colonic drug delivery system based on pectin (polygalactronic acid) and galactomannan coating was reported by Lee et al. [174] and Pai et al. [175]. These two polysaccharides, pectin and galactomannan, were used as coating material of a conventional tablet or capsule. The coating of pectin/galactomannan mixture was shown to be strong, elastic and insoluble in simulated gastric and intestinal fluids such that it would protect drug from being released in the upper GI tract. Researches revealed that in the colon, bacterial degradability was preserved. Moreover, extended film resistance to hydration, subsequent solubilization, film degradation rate by enzymes and drug release rate were found to depend on the varying ratio of pectin to galactomannan. Higher galactomannan percentage caused to decreased bacterial degradation in the colon and prolonged duration of negligible drug release in the upper GI tract. Compared with the combination of pectin and ethyl cellulose [176] or amylose and ethyl cellulose [177], combination of pectin and galactomannan was advantageous due to faster *in vivo* degradation of both pectin and galactomannan by microflora in the colon.

Matrix tablet of indomethacin with guar gum was prepared and the suitability of guar gum as a carrier in colonic drug delivery was investigated in another study [178]. The results indicated the specificity of these matrices for enzymes triggered the drug release in the colon. In another *in vivo* study, matrix tablets containing around 77% guar gum were loaded with technetium-99m-DTPA as tracer and scintigraphs were taken at regular intervals in six healthy human male volunteers [179]. These tablets were found to remain intact releasing only small amount of tracer in the stomach and the small intestine. However, bulk of the tracer was released in the ascending colon thereby suggesting that the enzyme triggered degradation by colonic bacteria.

Rubinstein and Gliko-Kabir investigated a biodegradable property of guar gum cross-linked with borax [180]. The time required for degradation of these crosslinked guar gum and borax showed that release of drug would be in proximal colon. The same group analysed phosphated cross-linked guar gum hydrogels for their potential as colon drug carriers *in vitro* and *in vivo* in 2000 [181]. *In vitro* studies revealed that these hydrogels loaded with hydrocortisone were able to resist the release of 80% of the drug for 6 h in phosphate buffer. *In vivo* studies in rat showed that degradation of modified guar gum by enzymes was concentration dependent. Thus, the phosphated crosslinked guar gum could be considered suitable for colon drug delivery.

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Alginate is a non-toxic polysaccharide that have properties such as pH sensitivity. This pH sensitivity is favorable for intestinal delivery of protein drugs. However, drug leaching during hydrogel preparation and rapid dissolution of alginate at higher pH are major limitations since when it enters the intestine, these limitations cause to very low entrapment efficiency and burst release of entrapped protein drug. To overcome these limitations, George and Abraham used another natural polysaccharide, guar gum which is included in the alginate matrix along with a cross linking agent to ensure maximum encapsulation efficiency and controlled drug release [182].

In the study of Coviello et al. [101], two galactomannans, guar gum and Locust bean gum, have been investigated for their possible use as matrices for modified drug delivery. They were crosslinked with glutaraldehyde (Ga) and then used for the preparation of tablets. This preparations increased the rate of release of small guest molecules due to the fact that the chemical reaction with Ga introduced meshes with a size larger than those present in the simply entangled systems.

In the study of Voepel et al. [183], hydrogels based on acetylated galactoglucomannan (AcGGM) were synthesized and examined for their properties in drug-release systems using two model substances of different molecular weight, size, and polarity (caffeine and vitasyn blue). AcGGM was synthetically modified to yield a polysaccharide with either neutral or ionic pendant groups. These precursors were formulated to produce either a neutral, covalent hydrogel or a physically cross linked hydrogel. Neutral and ionic hydrogels based on HEMA-Im– modified AcGGM (M-AcGGM) and maleic anhydride modified M-AcGGM (CM-AcGGM) were studied in view of their chemical, physical and drug release properties. In the case of the neutral hydrogels, half of the total drug release (50 wt % release) was reported to occur between 13 and 35 min and 50 to 90 min for caffeine and vitasyn blue, respectively. The majority of the caffeine (80 wt %) was released between 40 and 120 min, on the other hand, the majority of vitasyn blue was released between 125 and 250 min. When maleic anhydride was added to the M-AcGGM, ionic poly(CM-AcGGMco-HEMA) hydrogels could be achieved. Slower release of caffeine was found in these hydrogels, especially at acidic conditions because of the pH responsitivity obtained through the introduced carboxylic functionalities.

Roos et al. synthesized hydrogels from *O*-acetyl-galactoglucomannan (AcGGM) with encapsulated bovine serum albumin (BSA), to investigate the influence of substitutions and the feasibility of BSA-release mediated by the addition of β-mannanase to hydrolyze the hydrogel [184]. Hydrogels were prepared from AcGGM substituted with various amounts of 2-hydroxyethylmethacrylate groups and loaded with BSA. The degree of substitution of HEMA and the presence of β-mannanase *An*Man5A were two parameters that influenced the release of BSA from the hydrogels in water. Increasing HEMA substitutions on the glucomannan backbone from 0.1 to 0.36 caused lesser spontaneous release of BSA. However, the addition of β-mannanase *An*Man5A increased the BSA release due to enzymatic hydrolysis of AcGGM. The hydrogel with DS_{HEMA} (degree of sustitution) 0.36 released almost all remaining BSA from the hydrogel within 8 h after addition. The results of the study provided significant insights into further developments of AcGGM-based hydrogels for the application of drug delivery

Bioadhesive poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles were reported as promising drug delivery systems [185], and surface modification of nanocarriers was provided by application of mannan-based PE-grafted ligands (MAN-PEs) [186]. Kong et al. investigated MAN-PE-modified bioadhesive PLGA nanoparticles as active targeting gene delivery system using plasmid enhanced green fluorescent protein (*pEGFP*) as the model gene [187]. In the reported study, in order to achieve active targeting to the liver, surface of of PLGA nanoparticles was modified by the application of MAN-PEs. *In vitro* and *in vivo* behavior of mannan-modified DNA-loaded PLGA nanoparticles were compared with nonmodified DNA-loaded PLGA nanoparticles. Spherical shapes were observed for nonmodified DNA-NPs while the mannan-modified MAN-DNA-NPs had a dark coat on the white balls, that indicated the successful coating of mannan-PE. The mean particle size of NPs was around 100–200 nm, which was ideal for the nanoparticulate system. MAN-PEs-modified *pEGFP*-loaded bioadhesive PLGA-NPs could be targeted to the liver and successfully transfected the Kupffer cells (KCs).

In the study of Wu et al., mannan-PEG-PE (MN-PEG-PE) modified bioadhesive PLGA nanoparticles were obtained as a targeted gene delivery system [188]. Mannan was the target part that bind to the mannose receptor (MR) in the macrophage, and PEG-PE was the spacer linked into the surface of NPs. The results of this study confirmed that mannose-mediated targeting could successfully deliver genes into MR expressing cells. Improved transfection efficiency was observed in the case of mannose containing targeting ligands, such as in DNA loaded PLGA NPs. The results supported the active targeting ability of mannan containing PEG-PE modified bioadhesive PLGA nanoparticles, and the resulting vectors would be very useful in gene delivery both *in vitro* and *in vivo.*

In the study of Kaur, sustained and targeted release nanoparticles of didanosine were formulated using gelatin as polymer and mannan-coating to further enhance its macrophage uptake and its distribution in organs that act as major reservoirs of HIV [189]. Coating of nanoparticles with mannan further retarded the drug release (42.5 ± 1.7% over 24 h) and increased the cellular uptake of nanoparticles (N-C3-M) as was evident by higher staining intensity and complete lysis within 2 h of incubation. The better cellular uptake of mannan-coated nanoparticles might be due to the presence of mannosyl receptor predominantly on the macrophage cell surface, which was used by the cells for endocytosis and phagocytosis [190,191]. The results showed higher accumulation of didanosine in brain when administered through mannan-coated nanoparticles. Didanosine is a hydrophilic drug and its ability to cross the blood brain barrier was very low; however, mannan-coated nanoparticles provided enhanced delivery of didanosine to brain. Hence, mannan-coated gelatin nanoparticles resulted in a significantly higher concentration of didanosine in spleen, lymph nodes and brain.

7. Future prospects

Overview of literature clearly shows the high potential of mannan-based biomaterials in health related applications. In these studies though, the monomer composition and structure of mannan polysaccharide plays the key role for a successful design. It is well known that the

composition of polysaccharides is highly influenced by the environmental conditions and strictly depends on the availability of the activated sugar monomers. Currently, main sources for mannan are plants, algae and fungi where production may take months and greatly depends on geographical or seasonal conditions. On the other hand, microbial sources could be a feasible alternative for the sustainable and economical production of mannan at industrial scale. Microbial fermentation would not only enable the use of low-cost resources for the economical production, but also provide control over the chemical structure, monomer composition and physicochemical and rheological properties of the final product. There are only few reports on microbial mannan production and from these, thermophiles stand out with their high production rates due to their high metabolic activity. Moreover, such simple systems enable the effective application of systems-based approaches to obtain tailor-made polymers.

Finally, mannan is a very promising bioactive material for drug nanocarrier systems since its amphiphilic structure can incorporate diverse biomolecules, potentially providing novel nanostructure drug delivery systems. Hence, development of high mannan producer cell factories would overcome the problems associated with the sustainable production of this important biomaterial.

Author details

Songul Yasar Yildiz and Ebru Toksoy Oner*

*Address all correspondence to: ebru.toksoy@marmara.edu.tr

Industrial Biotechnology and Systems Biology Research Group, Department of Bioengineering, Marmara University, Istanbul, Turkey

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Chapter 10

Nanoflora — How Nanotechnology Enhanced the Use of Active Phytochemicals

Fadwa Odeh, Hala Al-Jaber and Dima Khater

Additional information is available at the end of the chapter

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1. Introduction

Nanotechnology is the science of manipulating matter where at least one dimension is in the nano-scale. Nanotechnology is a very promising field to the medicinal and pharmaceutical sectors, hence, it plays an important role in enhancing humanity's quality of life. Nanotechnology has many applications in the medical sector, including diagnosis and therapeutic. However, therapeutic uses are considered the main application of nanotechnology in the health sector. Nanotechnology has an important impact on improving therapeutics by facilitating drug delivery, increasing the efficacy of the drug, improving its circulation and stability in addition to decreasing its toxic side effects [1]. Most active compounds that are proven to have certain effects to treat certain ailments or alleviate pain can be categorized as organic compounds. Most active organic compounds are insoluble in aqueous media, have poor bioavailability, instable and most of the time are toxic.

Herbal medicines, or active compounds derived from natural sources suffer from the same limitations of many other drugs [2]. However, in the case of synthesized drugs, many of these problems can be overcome by preparing derivatives of these drugs that retain some of their activities and at the same time enhancing their physical properties to a more suitable form for pharmaceutical formulations. However, herbal medicines are still the main source of drugs and their side effects are much lower than their synthetic counterparts, add to this a deep and strongly rooted trust in many societies in their efficacy to heal or prevent diseases, even though most of the time not proven in a scientific and systematic way. In order to overcome such limitations, many techniques have been employed such as solubilization in a non-polar solvent, preparing them as an injection and/or converting the active ingredients into their salt form in order to enhance their solubility in aqueous medium. However, these methodologies also suffer from various disadvantages. These include the toxicity of the solubilizing agent,

the actual or real activity of the salt form, the need for further studies to insure the presence of the bioactive form of the drug and the extent of its bioavailability. In this aspect, nanotechnology is a very promising tool for enhancing the use of herbal medicines, or in more accurate words, to re-discover their full potential in pharmaceutical formulation. Extensive libraries of nanoparticles-that can be used for the delivery of natural bioactive compounds to a certain target-have been studied. These different nanoparticles can be designed, prepared in different shapes, sizes, compositions, functionalized and modified chemically/physically to suite specific properties depending on the characteristics of both the drug and the targeted organ. These nanoparticles can be anything from emulsion and micro-emulsions, dendrimers, fullerenes, liquid crystals, quantum dots, nano rods, solid lipid nanoparticles (SLN), liposomes, gels and many other different types.

In this chapter, a brief focus on herbal medicines, nanotechnological approaches to enhance their promised action will be reviewed and discussed. At the end, successful stories of nanoparticle loaded active phytochemicals that reached the market will be presented as case studies in this field.

2. Herbal medicine

Natural products chemistry is one of the oldest sciences sought for medicine throughout the history of mankind. Its roots dates back in time, thousands of years ago, through the use of many herbal mixtures as remedies for many diseases. It was clear from the beginning that certain herbs, plants, etc… have certain positive influence when used as remedies for sickness, but it was unclear how the mechanism of remedy was attained. As a consequence of the accumulation of many years of knowledge, traditional medicine has come forth to group, organize and categorize these remedies according to their effects on diseases, whereby, biomedicine and chemistry came to shed more light on the active ingredients of these folk remedies and on the mechanism of their action.

Currently, natural product chemistry has evolved to be an interdisciplinary area of science, concerned with the isolation, characterization and determination of the biological activity of the pure phytochemicals. These active components, generally referred to as secondary metabolites, include phenolics, terpenoids, alkaloids and steroids. Even though it has been proved that many natural products have a strong therapeutic value, limitations related to their poor solubility and bioavailability in addition to toxicity and stability have severely hindered their use as drugs [3]. According to U.S. Food and Drug Administration (FDA), the definition of bioavailability is “the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action” [4].

3. Phenolic compounds

Natural phenolic compounds are secondary metabolites present in all vascular plants and embracing a vast range of aromatic organic compounds with one or more hydroxyl substitu-

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ent(s). The parent compound is phenol but most of these naturally occurring compounds are polyphenolics, which to date, exceeds 8000 structurally identified compounds. Plant polyphenolics are grouped into different classes depending on their chemical structure. Flavanoids are the largest and most important group of natural polyphenolics with more than 6000 molecules identified so far. Other classes include phenolic quinones, lignans, xanthones, coumarines, polymeric lignins and tanins [5]. Within each class of compounds, the variations around the basic chemical skeleton essentially concern the degrees of oxidation, hydroxylation, methylation, glycosylation and the possible connections to other molecules (primary metabolites such as carbohydrates, lipids, proteins, or phenolic secondary metabolites [6].

Polyphenols were the focus of special attention as a result of the so-called Mediterranean diet rich in fruits and vegetables. This diet appears to protect against cardiovascular diseases in addition to potential beneficial health properties, as evidenced by several epidemiological studies showing that diets rich in fruits and vegetables are generally associated with a lower cancer incidence and other diseases, such as inflammatory or cardiovascular pathologies [7].

The bioavailability of the orally administrated polyphenols is very low due to their low water solubility, poor absorption, extensive and rapid metabolism. To overcome these problems, several bioactive polyphenols were formulated into various pharmaceutical formulations that could improve their bioavailability.

Among the types of naturally occurring polyphenols, flavonoids (Figure 1) are a large and a highly diverse group of structurally related secondary metabolites produced by plants. The main flavonoids subclasses include flavones (Figure 1a), flavonols (Figure 1b), flavans (Figure 1c), flavanones (Figure 1d) in addition to dihidroflavanols (Figure 1e), isoflavons (Figure 1f) and biflavones. [8].

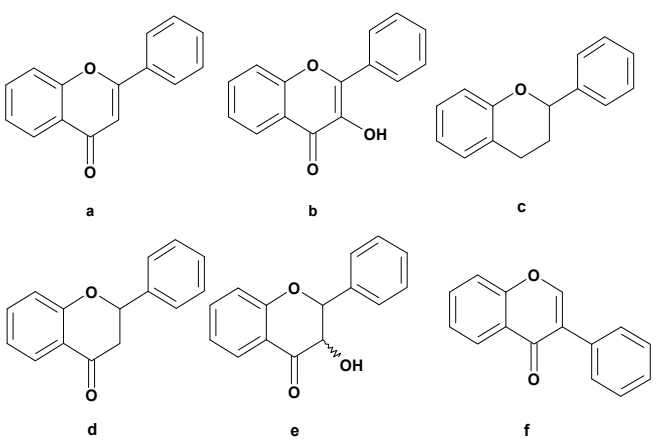

Figure 1. Basic structures of some flavonoids' backbone

Flavonoids received considerable attention due to their wide biological activities. Many of them are known to possess hepatotoxic, anti-inflammatory, antimicrobial, antiviral, anti-

allergic and anti-ulcer effects. Also, flavonoids are known to be potent antioxidants with free radical scavenging abilities [9, 10]. Some flavonoids are known to provide protection against cardiovascular mortality. Moreover, they have been shown to inhibit the growth of various cancer cell lines *in vitro*, and reduce tumor development in experimental animals [11]. Flavanoids, as natural compounds have several great advantages over therapeutic agents because many diets are rich in flavonoids and polyphenolic compounds [9]. The therapeutic potential of flavonoids makes them valuable targets for drug design [8].

Lignans (Figure 2) are among the important polyphenolic compounds that are recognized with a wide spectrum of biological activities. These compounds generally represent a group of dimeric phenylpropanoids where two C6-C3 are attached by its central carbon C-8.

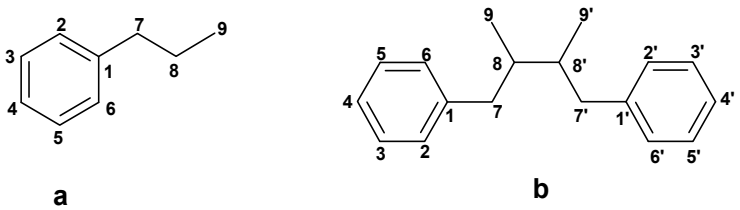

Figure 2. Basic structure of lignans: a) Phenylpropanoid unit; b) Lignan structure

Lignans are known for their antiviral, anticancer, cancer prevention, anti-inflammatory, antimicrobial and antioxidant activities. Also, lignans are known for their immunosuppressive, hepatoprotective and osterporosis prevention effects. Podophyllotoxin (Figure 3) has long been known to possess anti-mitotic activity with early clinical trials indicating its high efficacy. Unfortunately, the toxicity of this compound limits its direct application as a drug [12]. To solve the problem of toxicity, several modifications were made to the podophyllotoxin structure in hope of obtaining compounds with low toxicity but retain the desired activities of the parent compound. Thus, etoposide, teniposide, etopophos and GL-331 were synthesized; all are potent chemotherapeutic agents for a variety of tumors. Still, etoposide and its analogues suffer from poor solubility problems.

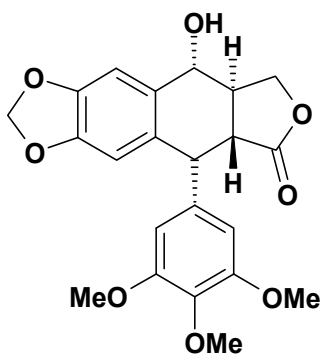

Figure 3. Structure of podophyllotoxin

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4. Terpenoids

Terpenoids, as represented by more than 40000 identified compounds, are among the most widespread group of natural products, with several new compounds being discovered every year. Terpenes can be generally defined as a group of molecules whose structure is based on various but definite number of isoprene (3-methyl-1,3-butadiene) units. Based on the number of isoprene building blocks, terpenoids can be classified into monoterpenes (such as thymoquinone), sesquiterpenes, diterpenes (such as retinol, *trans*-retonic acid, sclareol), sesterterpenes, triterpenes (such as oleanolic and usrolic acids) and tetraterpenes.

From a chemical point of view, terpenoids are usually cyclic unsaturated hydrocarbons, with different degrees of oxygen in the constituent groups attached to the basic isoprene skeleton. A wide range of terpenoids have been found to possess preventive and pharmacological activity against many human ailments including cancer and alzheimer [13, 14]. Several studies also indicated that terpenoids have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, anti-spasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory properties [15].

Monoterpenes are among the best known plant secondary metabolites and are one of the main classes of terpenoids detected in essential oils, floral scents and defensive resins (both constitutive and induced) of aromatic plants [16]. A number of monoterpenes have shown antitumor activity. Examples include thymoquinone (Figure 4), limonene and perilla alcohol. Thymoquinone, which is the main active constituent of the essential oil obtained from the medicinal plant *Nigella sativa* (commonly referred to as Black seeds in Arabic countries), has interesting anticancer, anti-oxidant and anti-inflammatory activities both *in vivo* and *in vitro* [14, 17, 18, 19] as well as chemo-preventive properties. The interesting *in vitro* anticancer activity of this monoterpene against different types of cancer cell lines including human colorectal cancer cells [13], myeloblastic leukemia cells [20], prostate cancer [17] pancreatic adenocarcinoma [14], ovarian and breast adenocarcinoma [21] were faced by several limitations that not only hindered its pharmaceutical applications, but also limited the available suitable approaches that can be used to enhance its bioavailability. Such limitations included the poor solubility, extreme lipophiliciy causing poor formulation characteristics in addition to light and heat instability.

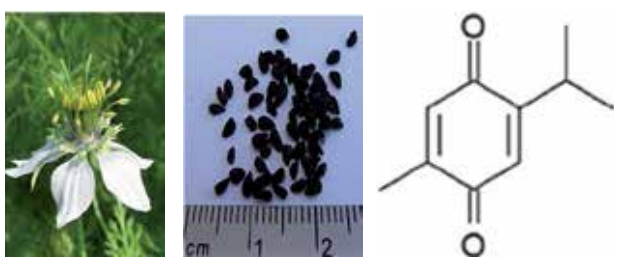

Figure 4. Flower and seeds of *N.sativa* and the structure of thymoquinone, the main component of *N. sativa* and many other plants

Triterpenoids are one of the most abundant natural products in plants. They exhibit huge structural diversity as more than 90 different triterpenoidal carbon skeletons are known. Further oxidative modifications and glycosidation of the skeleton generate even more diversity [22]. Oleanolic (Figure 5a) and ursolic acids (Figure 5b) are among the well-known natural occurring pentacyclic triterpenoids that widely exist in many food products and in more than 120 plant species [23]. Oleanolic acid (OA) is known to possess anti-inflammatory, antitumor, antiviral, hepatoprotective and antihyperlipidemic effects. Moreover, it has been used in Chinese traditional medicine to treat liver disorders for over twenty years. Ursolic acid (UA) is also known to exhibit a wide and interesting biological activities including anti-inflammatory, anti-ulcer, antihyperlipidameic, antihyperglycaemic, hepatoprotective, neuroprotective and anticacinogenic activities [23]. The oral bioavailability of these two natural triterpenoidal acids is greatly limited by their very poor solubility in water. In fact, this drawback limits their development as a medicine as well as their use in food, health and cosmetic products.

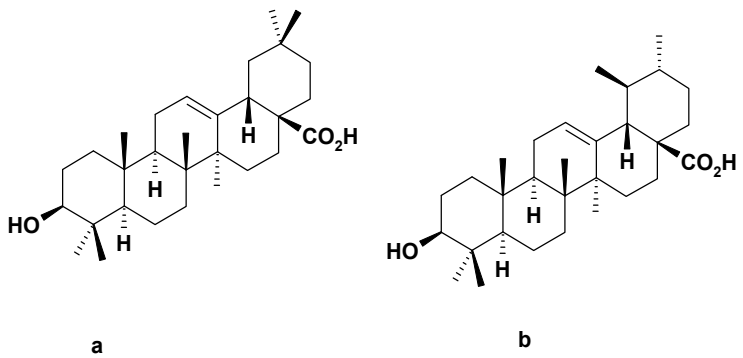

Figure 5. Structures of a) oleanolic acid and b) ursolic acid

Asiatic acid (Figure 6) is another natural derivative of oleanolic acid. This compound is known to be clinically effective on systemic scleroderma, abnormal scar formation and keloids [24]. Again, the poor solubility of this compound in water limits its bioavailability and hence hinders its usage as a drug.

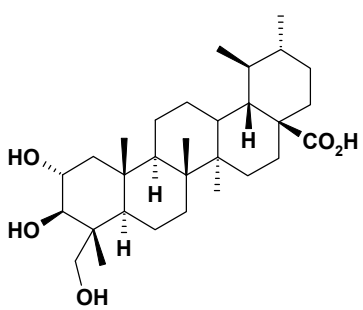

Figure 6. Structure of Asiatic acid

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Cucurbitacins (Figure 7) resemble another class of triterpenes with interesting pharmaceutical properties. Cucurbitacins are a group of highly oxidized tetracyclic triterpenoids that are widely distributed in the plant kingdom and well recognized for their bitterness and toxicity. Such compounds were initially isolated from plants belonging to the plant family *Cucurbitaceae,* but were later found to be present, either as non-glycosylated or glycosylated in many plant families including *Brassicaceae*, *Scrophulariaceae*, *Begoniaceae*, *Elaeocarpaceae*, *Datiscaceae*, *Desfontainiaceae*, *Polemoniaceae*, *Primulaceae*, *Rubiaceae*, *Sterculiaceae*, *Rosaceae* and *Thymelaeaceae*. In plants, cucurbitacins are known to act as heterologous chemical pheromones that protect plants from external biological insults [25]. Moreover, these compounds are known to possess a broad range of potent biological activity due to their cytotoxic properties. In traditional medicine, cucurbitacins-containing plants have been known for their antipyretic, analgesic, anti-inflammatory, antimicrobial, and antitumor activities [26].

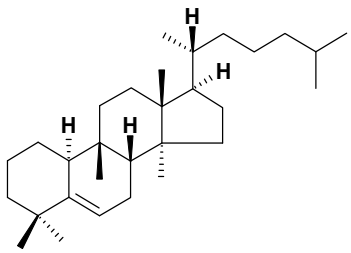

Figure 7. Basic structure of a cucurbitacin

There are 17 main molecules from cucurbitacin A to cucurbitacin T, and hundreds of compounds derived from them. Among them, cucurbitacins B, D, F, I and compounds derived from them have been extenively investigated for their anticancer activities.

5. Alkaloids

Alkaloids are a highly diverse class of secondary metabolites, with more than 5000 compounds being identified ranging from simple to highly complicated structures. These compounds contain a ring structure and a nitrogen atom, in most cases, the nitrogen is part of a heterocyclic ring structure. Alkaloids are known to exhibit significant biological activities. Examples include the relieving action of ephedrine for asthma, the analgesic action of morphine and the anticancer effects of vinblastine. Vinca alkaloids like vinblastine, vincristine, and vinorelbine are widely used cytotoxic drugs that elicit their effects through disruption of microtubules, resulting in metaphase arrest in dividing cells [27]. Thus, these compounds would benefit from a controlled release dosage form that would result in a prolonged duration of exposure over extended period of time. However, in spite of their significant bioactivities, these compounds suffer from side effects. The major adverse effect of vinblastine is hematologic toxicity which occurs much more frequently than with vincristine therapy. Other side effects include nausea, vomiting and constipation, dyspnea, chest or tumor pain, wheezing and fever. Many recent

publications dealt with loading vinca alkaloids in liposomal nanocarriers to lessen such side effects [27, 28].

6. Nanoflora

As discussed above, herbal medicines are accompanied with many problems that prevent them from reaching their full potential as pharmaceutical formulations. These problems include – but not limited to: low solubility in water, low bioavailability, high toxicity and instability. Nanotechnology has shown great promise for many medical applications such as cancer diagnosis, chemotherapeutic drug delivery, and diabetes treatments [29]. This technology is beneficial in overcoming some difficulties encountered with using bulk variable drug molecules in their synthetic and natural forms.

In the last decade, tremendous attentions have been paid on replacing synthetic drugs by natural bioactive phytochemicals to eliminate synthetic drugs side effects. In order to reach this goal, the above mentioned limitations need to be overcome. Nanotechnology can play an important role in reducing or even eliminating such drawbacks. Such possibility will open the door for a wide range of candidate compounds that were overlooked in the past due to these limitations, to be revisited again. In this chapter, a systematic overview of the various methods that can be applied to overcome one or more of these limitations and will lead finally to an acceptable formulation composed mainly of the active phytochemical attached to or encapsulated in a nanocarrier system forming what will be known throughout this chapter as a nanoflora. Such combination is capable of reaching the final phases of testing these active compounds and be helpful in improving health care systems.

7. Nanotechnology and nanoparticles

Several nanoparticle systems have been used to aid in the formulation, encapsulation and release of active compounds extracted or derived from natural resources. The main types of these particles are liposomes, solid lipid nanoparticles, inorganic nanoparticles, microemulsion, polymer nanoparticles, dendrimers and many other types.

7.1. Micelles and Liposomes

Micelles are spontaneous aggregates of amphiphiles (such as surfactants, Figure 8a) with usually spherical structures with a size range of 5-25 nm (Figure 8b). Their core is usually hydrophobic if they aggregate in polar media, however, they do form inverted micellar system in non polar media with a hydrophilic core. Micelles are perfect carriers for drugs and have been used more than any other system of nanoparticles [30]. Micelles come first in mind when tackling solubility issues in aqueous media [31, 32]. The solubilization power of micelles has been known and used for a long time especially as detergent. Their mechanism of action was discovered in early 1900s. Micelles can be used as drug carriers and as solubilizing agents.

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However, they cannot be used to tackle other problems accompanying natural active compounds such as toxicity and stability since micellar systems are very dynamic ones and suffer from fast clearance rate and stability issues. However, some of these disadvantages were to some extent reduced via the preparation of polymeric micelles [33]. Polymeric micelles are more stable with longer shelf life and stay longer in the body.

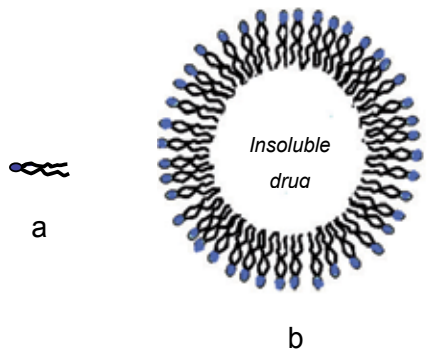

Figure 8. a) Sketch of a lipid or surfactant molecule capable of forming micelles and liposomes with a polar head and non-polar tail, b) Schematic presentation of a micelle with an insoluble drug encapsulated in the vicinity made by the non-polar tails

Liposomes are spherical vesicles that are composed of lipid bilayer (Figure 9a). Liposomes were discovered in 1961 by the British haematologist Alec Bangham and its resemblance to the cell membrane attracted immediate attention [34, 35, 36]. The name liposome was derived from the two Greek words *lipo* meaning fat and *soma* meaning body, which perfectly describes these spherical objects that are made mainly from lipids. In some cases other constituents are added to modify their chemical and physical properties (Figure 9b). Liposomes are easily prepared by disturbing the lipid film in aqueous medium. This disturbance may be a result from a large shear force produced via several techniques such as sonication. Liposomes are different from micelles (Figure 9) in that they are composed of bilayer lipid membrane whereas micelles are made from monolayer lipid vesicles.

There are different types of liposomes, including Small Unilamellar Vesicles (SUV, Figure 10b), Multilamellar Vesicles (MLV, Figure 10c), Large Unilamellar Vesicles (LUV, Figure 10d), Multivesicle Vesicles (MVV, Figure 10e) and cochleate vesicles (Figure 11). Each type of liposome is formed depending on experimental conditions. In addition, a dominant liposome type, size can be determined and/or made, after they are prepared via a series of extrusion process accompanied by shear or via several freeze-thaw processes since these structures are dynamic.

Liposomes are very important as drug carrier systems due to many factors including their suitability to encapsulate polar and non polar drugs, their stability and long shelf life, controllable properties such as size and charge, ability to functionalize and modify the surface due to the presence of many functional groups, and finally their biocompatibility and degradability. However, liposomes suffer from various disadvantages which include their short half-life

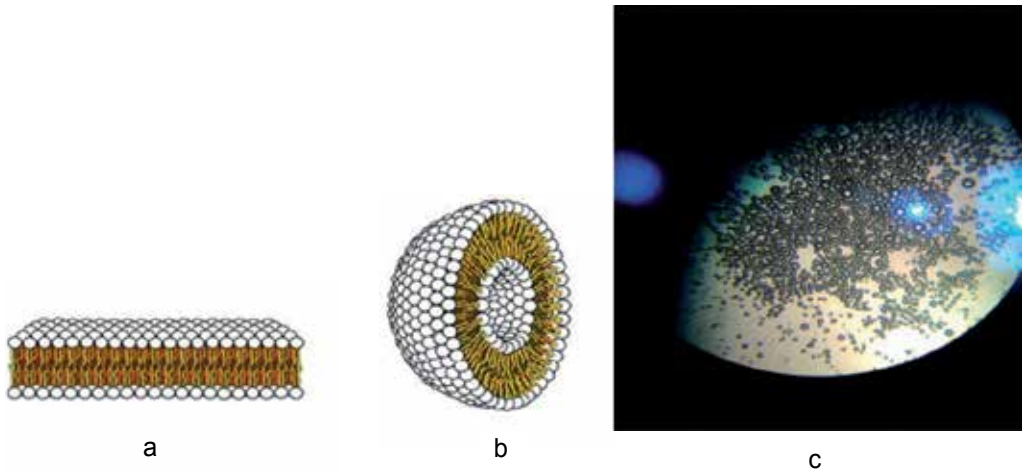

Figure 9. Basic structure of: a) bilayer lipid sheet, b) unilamellar liposome and c) optical micro-image of liposomes loaded with Thymoquinone [37]

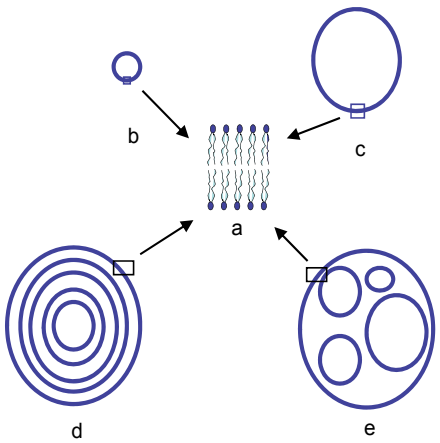

Figure 10. Schematic representation of the different types of liposomes a) the lipid bilayer, b) Small Unilamellar Vesicle (SUV), c) Large Unilamellar Vesicle (LUV), d) Multi Lamellar Vesicle (MLV) and e) Multi Vesicle Vesicle (MVV).

in the circulation system, although it can be enhanced by better controlling the size of the liposome vesicle and modifying its composition. Even though liposomes are suitable to encapsulate non polar drugs in the hydrophobic bilayer of the vesicle (Figure 9a and 10a), sometimes, such drugs affect the integrity of these vesicles rendering them unsuitable for non polar drugs.

Another promising type of nanoparticles is the phytosomes. The phospholipid in these types of nanoparticles (considered mainly as liposomes) is covalently attached to the phytochemical. Phytosomes are gaining increased interest and are the focus of more research to be used as drug delivery systems [38, 39, 40].

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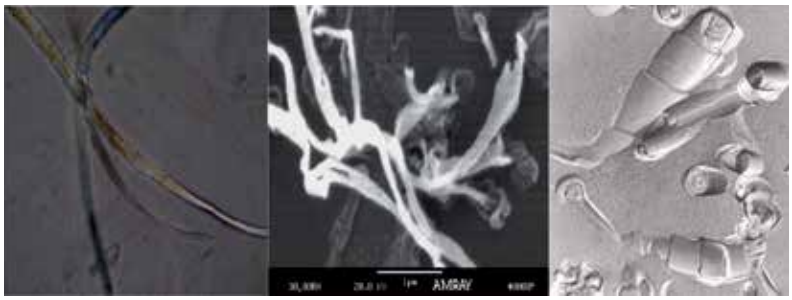

(a) (b) (c)

Figure 11. a) Optical micro image of cochleates [41], b) SEM image of cochleates [42], and c) freeze fracture electron micrographs of cochleates cylinders [42]

7.2. Solid Lipid Nanoparticles (SLN)

These are usually spherical structures composed of a lipid core, capable of solubilizing lipophilic drugs, surrounded with surfactants that stabilizes the lipid core and can be used for the hydrophilic drugs and other fictionalizations processes (Figure 12) [43]. SLN share other types of nano-carriers' their common advantages like their suitability to encapsulate non polar insoluble drugs in its polymeric core, shielding the drug the outside environment - which could be sometimes harsh - and as consequence increases the drug stability and reduces its toxicity to the body. Other advantages include not only the ability to functionalize the SLN surface with markers and targeting devices in order to enhance the targeting process, but also their ability to produce a sustained and slow release of the drug in the targeted site. However, SLN have advantages over other types of delivery systems in that they are easier to prepare, cheaper and much easier for scale up productions.

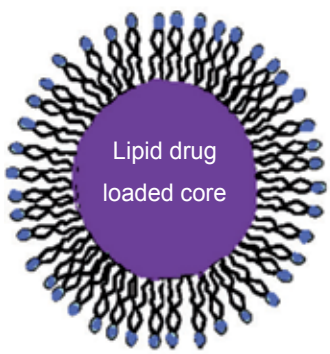

Figure 12. Structure of Solid Lipid Nanoparticle (SLN) stabilized with surfactant molecules

7.3. Polymer Nanoparticles

Another type of particles that draw large attention is polymer nanoparticles [44]. These types of nanoparticles (Figure 13) are easily made-mostly from biodegradable polymers and can increase the stability and time of circulation. Moreover, in addition of being non toxic, other advantages of these polymer nanoparticles include controlled drug release, biocompatability and their suitability for scale up methods [45]. The most used two polymers include both poly (lactide-co-glycolic acid) (PLGA) and poly (lactic acid) (PLA) [46]. Other polymers are also good candidates to form polymeric nanoparticles and be suitable to act as drug carriers. These include sugars [47], proteins [48] such as albumin [49], gelatin nanoparticles [50] and many other naturally occurring macromolecules.

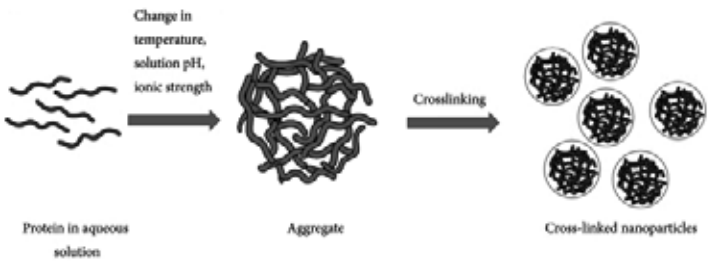

Figure 13. Schematic representation of polymer nanoparticles preparation [48]

Dendrimers are repetitive branched molecules attached to each other in a tree-like manner and typically are symmetric around their core. They can also be categorized as polymeric nanoparticles, and are characterized by their structural perfection, water solubility and monodispersity. Dendrimers are good encapsulating agents for hydrophobic drugs due to their non polar core.

7.4. Microemulsions (ME)

Microemulsions are usually made from oil, water, surfactant and a co-surfactant. They are thermodynamically stable (in contrast to emulsions), transparent, and form spontaneously. The particle size ranges from 10 to 100 nm, which enhances their penetration through cellular membranes making microemulsions suitable as drug carriers. Due to the presence of polar and non polar components in ME's, they are very good solubilizing agents. Their properties can be adjusted to suit the drug to be carried by optimizing compositions, types of both the surfactant and the co-surfactant in addition of course to the oil used in the composition [51].

7.5. Inorganic nanoparticles

Inorganic nanoparticles such as gold nanoparticles, silver nanoparticles, ceramics, carbon nanoparticles and nanotubes were the focus of very extensive research and in many fields. Inorganic nanoparticles can be mainly classified into three different main categories including

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the: i) transition metal nanoparticles, ii) ceramics nanoparticles and iii) carbon nanoparticles in addition to other types.

Transition metal nanoparticles (Figure 14) such as Au, Ti, Pt are gaining increasing interest in the medical field [52]. There are many methods were transition metals can be applied in medicine, drug delivery being one of them. For example, many transition metals can act as drugs themselves when excited by light radiation. Depending on the excitation process and the type of metal involved, when the absorbed energy is released, it can damage the DNA and/or modify the protein, promote lipid peroxidation and destroy the cell microenvironment, hence causing cell death. This method is promising in fighting cancer cells. Also, these nanoparticles can be very powerful in imaging, which is very important in both diagnosis and therapy monitoring [53]. One of the most promising nanoparticles in combating cancer is Au nanoparticle [54]. However, these metals can be used as drug carriers. For example, Au shuttles can be used for site specific delivery of toxic drugs [52]. However, these nanoparticles suffer from loaming safety and clearance concerns. Metals in the nanometer scale range have special properties and penetrations, making them very powerful catalysts that can trigger undesired reaction. Nowadays, there are large concerns regarding safety of specifically metal nanoparticles and nanoparticles in general.

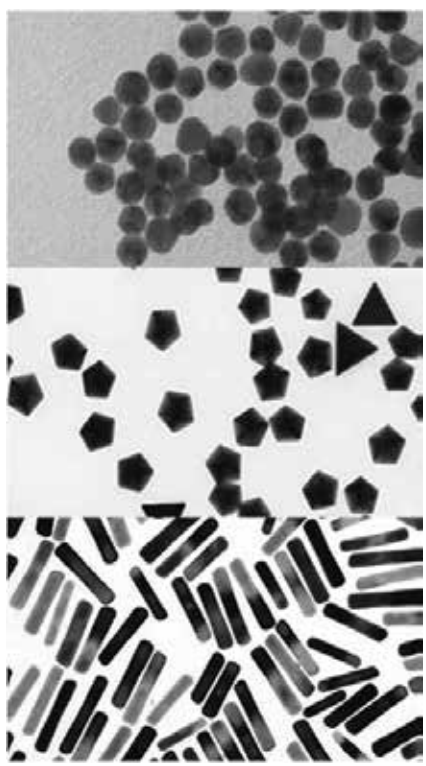

Figure 14. TEM images of metal nanoparticles with different geometries [55]

Ceramics nanoparticles are mostly composed from oxides, nitrides and carbides with silica (Figure 15) (SiO_2) being the most used. Mainly they are used as hollow shells or cores that are coated with biodegradable and biocompatible polymers. Such surface modifications improve the properties of these nanoparticles especially for targeted delivery.

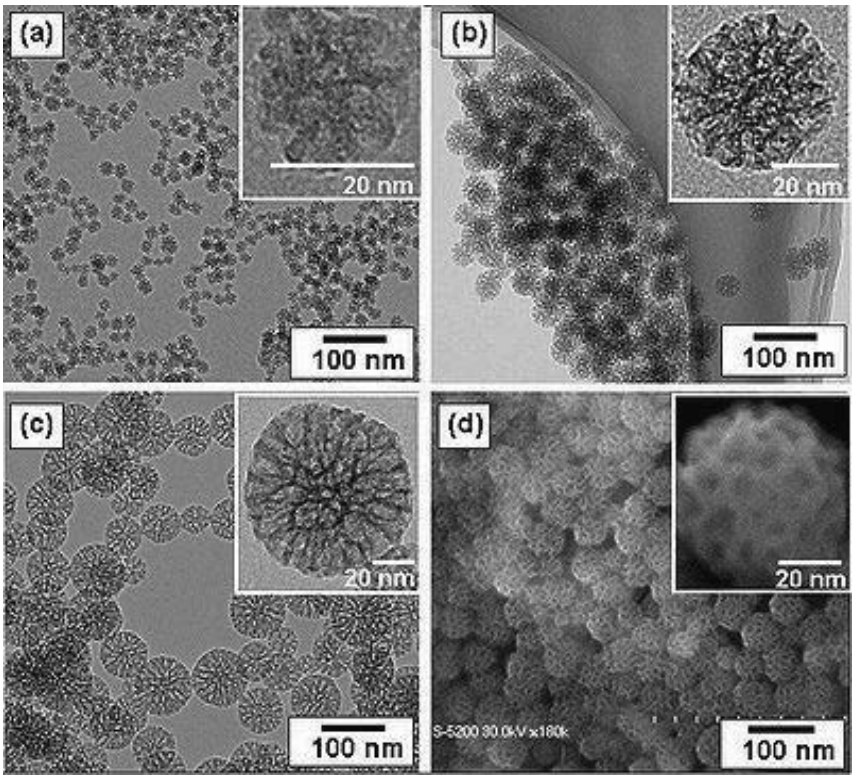

Figure 15. SEM images of silica nanoparticles with different sizes

8. Enhancing solubility and bioavailability

Low solubility of active phytochemicals is considered the main obstacle that hinders their usage in pharmaceutical formulations. This is due to two main reasons; the first is that the medium at which drugs work is mainly aqueous. Low solubility in aqueous media will drastically lower the concentration of the drug causing a poor bioavailability. Nanoparticles can provide an alternative medium for these drugs to be solubilized in and carried out through the body to the targeted tissue or organ. The extensively small sizes of nanoparticles give high surface area to volume ratio and as a result, more and more water molecules can surround the particles and the solubility of hydrophobic compound is enhanced [56].

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Several nano-vesicles can be used to enhance the solubility such as micelles, liposomes, solid lipid nanoparticles, polymer nanoparticles and many others [57]. Triptolide, is an example of a bioactive diterpenoid epoxide ingredient isolated form *Tripterygium wilfordii*, a plant used in traditional Chinese medicine. This compound was found to be active *in vivo* and *in vitro* mouse models against polycystic kidney disease and against pancreatic cancer. It can also be used in the treatment of autoimmune diseases especially rheumatoid arthritis, psoriasis, and leukemia. However, it suffers from low solubility and high toxicity. In order to overcome its solubility and toxicity issues, it was prepared as a biocompatible and biodegradable tripolide-loaded poly [DL-lactic acid] nanoparticles [58]. It was also studied as a micro-emulsion system for transdermal delivery.

The solubility of quercetin (a flavonoid that is naturally present in a wide range of fruits and vegetables especially in onion, apples and many edible fruits) was enhanced 100 times after encapsulation in polymeric nanoparticles suspensions [59].

Tetrandrine, bis-benzylisoquinoline alkaloid, exhibits antitumor activity and is known to acts as a nonselective calcium channel blocker. This compound has very limited clinical applications due to its poor water solubility. However, the solubility of this alkaloid was enhanced as a result of its incorporation into SLN [60].

Cryptotanshinone is an active quinoid diterpene isolated from the roots of the Asian medicinal plant, *Salvia miotiorrhiza* Bunge. This diterpene is known to exhibit variable interesting pharmacological activities including anti-inflammatory, cytotoxic, anti-bacterial, anti-parasitic, anti-angiogenic and anti-oxidative activities but suffers from very low bioavailability as a result of its extremely low water solubility. Cryptotanshinone oral bioavailability was highly enhanced by introducing solid lipid nano-formulations [61].

Hypericin is a natural photosensitizer with limited ability to be used in diagnostic applications because of its high hydrophobicity and limited solubility. Different nano-formulations like hypericin-loaded solid lipid nanoparticles (Hy-SLN) and suspension of Hypericin-polymeric nanoparticles have been developed in order to obtain better photo-detection and photodynamic therapy [57, 62].

Thymoquinone (Figure 4) is an active ingredient found in Black Seeds (*N. sativa*). It has anticancer activity in addition to other therapeutic effects [13, 18, 63]. However, this compound suffers from poor solubility and high hydrophobicity leading to poor formulations for pharmaceutical applications. This problem was solved by encapsulating thymoquinone in various carriers such as polymer nanoparticles [45], liposomes (Figure 16) [37] and in cyclodextrin (Figure 17) [64].

Bioavailability can also be enhanced due to encapsulation of drugs or active compounds in nanocarriers. For example, ampelopsin, a flavonoid extracted from *Ampelopsis grossedentata*, is known to possess many pharmacological activities including anti-inflammatory, antimicrobial, anticarcinogenic activities in addition to its antioxidation, antihypertension, hepatoprotective and cough relieving effects. However, not only ampelopsin suffers from poor solubility in water, it also has very low permeability. Ampelopsin was successfully encapsulated in a

microemulsion that enhanced its bioavailability by both enhancing its solubility and penetration through the intestinal mucosa [65].

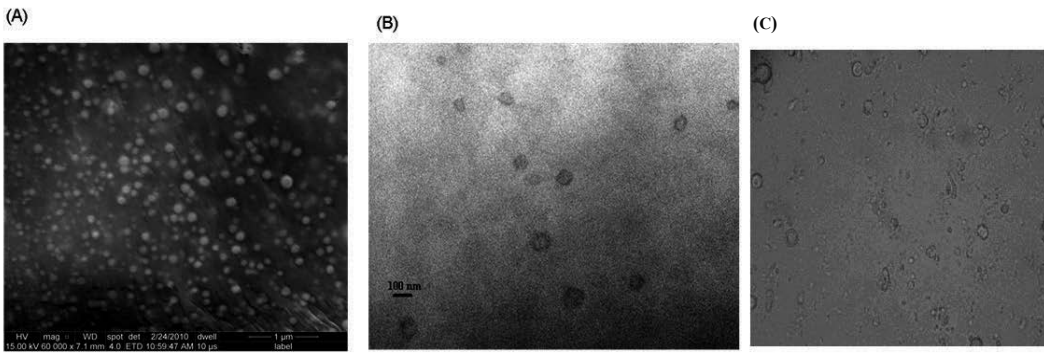

Figure 16. a) SEM, b) TEM and c) light microscope images of TQ-loaded liposomes [37]

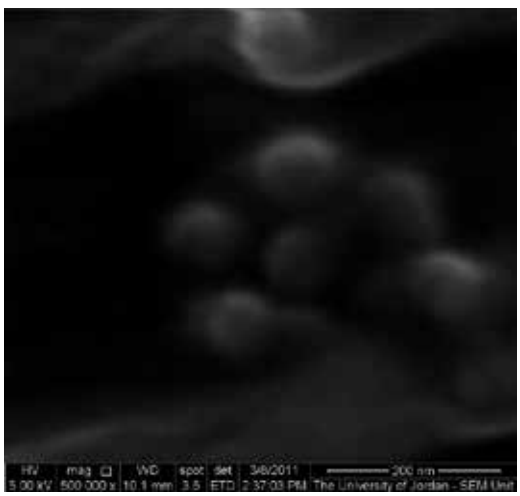

Figure 17. TEM image of a nanoparticle of thymoquinone with cyclodextrin β [64]

9. Reduced toxicity and side effects

Most research focuses on reducing the toxicity and side effects of drugs, especially for those used in chemotherapy. Many Nanoparticles systems can be used to encapsulate the toxic drug and deliver it to specific sites in the body. These Nanoparticles are mainly made from biodegradable, biocompatible materials such as natural polymers which include polysaccharides

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and proteins. For example, triptolide, which was mentioned previously to have anti-tumor activity, has a side effect of being irritating to the gastric systems. Such gastric irritation can be reduced by changing the nanoparticle carrier. When triptolide is encapsulated in Solid Lipid Nanoparticles (SLN), such irritations are minimized [32]. Nanoparticles in general shield these toxic drugs and isolate them until reaching their destination via targeted delivery mechanism. Such mechanism of delivery depends on the capability to functionalize the surface of the nanoparticle carriers.

Targeted drug delivery is deliberately increasing the concentration of the drug in specific part of the body relative to other parts in order to increase the efficacy of drug and decrease the side effects. The complex cellular network of organisms makes targeting a difficult mission. There are variable methods of targeting such as modification of surface charge, inserting ligands, and using biomarkers. The targeting mechanism can be divided into two mechanisms: passive targeting and active targeting.

Passive targeting refers to the preferable accumulation of chemotherapeutic agent in solid tumor as a result of enhanced vascular permeability of tumor tissue as compared to healthy tissue [66].

Tumors have unique features, which make them distinct from normal tissue. The intact tissue has non leaky microvasculars, while tumor tissue has leaky capillary beds. This situation promotes the delivery and retention of drug loaded nonparticles through tumor tissue. This phenomenon is recognized as the enhanced permeability and retention effect (EPR). The hydrophobic surfaces of nanomaterials are highly susceptible to osponization and clearance [66, 67]. When the surfaces were modified by becoming more hydrophilic, the rapid clearance problems can be solved and longer circulation can be obtained because the hydrophilic coating on the surfaces repels plasma and protein so drug loaded nanoparticles become invisible to mononuclear phagocytic system (MPS).

Active targeting refers to the use of drug carriers with ligands (antibody, peptide) that are selectively recognized by a receptor on the cell of interest. Since ligand-receptor interaction can be highly selective, a more precise targeting is achieved with improved target cell recognition and target cell uptake [67].

Particle size and size distribution are the most important characteristics of nanoparticle systems. Many studies have proved that nanometer particles are more effective and beneficial than micrometer particles in drug delivery system. In nano scale, the surface area to volume ratio is high; this situation makes the loaded drug less susceptible to reticular endothelial system clearances. The nano-sized particles have better ability to penetrate through cells and even small capillaries [68]. However, the ultimate small size of particles and large surface area lead sometimes to limited drug loading and burst release pattern [69]. Surface modification can be helpful in increasing residence time in the blood and reducing nonspecific distribution.Unsuccessful surface modification ultimately is the main limiting factor for long-circulating nanoparticle systems [70].

10. Enhanced activity

Encapsulation of active compounds into nanocarriers improves their activity. This might be attributed to several factors such as enhanced solubility, better stability for both *in vivo* and *in vitro*, and better formulation. However, the better penetration of the introduced nano systems through cellular membrane via unique interaction mechanism is an additional factor that participate in enhancing the bioactivity of drugs encapsulated in nanocarriers. For example, *Origanum dictamnus* extracts are known to have antimicrobial and antioxidant properties due to the presence of considerable concentrations of pehnolic compounds like flavones and cumarines. Research showed that encapsulating this extract into liposomes improved its activity [71].

11. Release profile

Obtaining constant drug release is considered an urgent necessity to obtain sustained drug level in tumor tissue which can lead to a lower dose requirement. For example, paclitaxel gelatin nanoparticles are highly effective in the treatment of bladder cancer because the rate of paclitaxel release from paclitaxel gelatin nanoparticles is limited by the drug solubility in aqueous medium. So the drug concentration remains constant and does not dilute when urine production is increased [58].

Sometimes, a fast release rate is desired, accompanied with enhanced solubility and penetration of non polar drugs. For example, *Hibiscus rosa-sinensis* and *Murraya koenigii* extracts are acclaimed to prevent hair loss and promoting its growth. However, proving such claims scientifically was hindered by many limitations, especially the poor solubility of the extracts in water. Since the desired formulation is intended to be used as a topical treatment with fast release rate to study its effectiveness, microemulsions were the ideal choice for improving the solubility and systemically study the extracts of these plants [72].

12. Enhanced stability

The instability of most phytochemicals in bio environments results in quick degradation and reduced activity. Encapsulation of such phytochemicals in carriers shields them from harsh conditions that lead to their decomposition. Quercetin has a life time of only five minutes in plasma whereas; quercetin loaded in liposomes modified with polyethylene glycol (PEG) has a life time of more than five hours in plasma [73]. Quercetin was also encapsulated in polymeric nanoparticles. Its antioxidant activity was maintained while its stability was highly enhanced when encapsulated in eudragit nanoparticles (polymeric nanoparticles). This polymeric nanoparticle protected quercetin and improved its stability in in acidic medium. However, the polymeric nanoparticles released the antioxidant under neutral to basic conditions, making

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such nanoparticles suitable for oral delivery of bioactive compounds and deliver them beyond the acidic stomach environment [74].

13. Success stories

Nanotechnology is one of the most powerful tools in the modern life, as it has made a revolutionary impact in every aspect of human life. The speed by which nanomedicine has advanced-particularly through the utilization of the various types of nanoparticles in the prevention, diagnosis and treatment of many complex diseases like cancer-is fascinating. The world is starting to witness the benefits of the application of nanotechnology in the field of herbal/natural products' drug delivery. One such benefit is Abraxane®. This drug is considered as a major success story of the nanomedicine approach to treat cancer [3]. Abraxane, an approved FDA drug, is a solvent free nano-version of the natural alkaloid, Taxol. Abraxane is both more effective and less toxic and has been successful in addressing the solubility problem associated with Paclitaxel (Taxol). Other examples of successful stories in the field of herbal drug delivery are the two companies Cosmetochem and Indena. Cosmetochem launched Herbasec® technology in the market. This product consists basically of liposomal encapsulated, standardized botanical extracts that are used in cosmetics for their antioxidant effects for the prevention of aging. Examples also include liposomal preparations of various herbal constituents like extracts of White tea, Green tea, white hibiscus, Gurana and *Aloe vera*. Indena commercializes the plant constituents/extracts of liquorice (18ß-glycyrrhetinic acid), *Ammi visnaga* (visnadin), *Centella asiatica* (triterpenes), *Ginkgo biloba* (ginkgoflavonglucosides, ginkgolides, bilobalide), Hawthorn flower (vitexin-2″-O-rhamnoside), milk thistle (silymarin and Silybin), horse chestnut (escin ß-sitosterol), *Terminalia sericea* (sericoside), *Panax ginseng* (ginsenosides), grape seed (polyphenols) and Green tea (polyphenols) [75]. Moreover, a long list of recent patents on controlled release novel herbal formulations is becoming even longer [76] proving that nanotechnology for drug delivery is becoming the future of phytochemicals and opening the era for re-exploring and investigation the full potential power of traditional herbal medicine represented either by the herbal extracts and/or their pure isolated phytochemicals.

14. Conclusions

The application of nanotechnology to drug delivery has already had a significant impact on many areas of medicine. Currently, more than 20 nanoparticle therapeutics are in clinical use, validating the ability of nanoparticles to improve the therapeutic index of drugs. In addition to the already approved nanoparticles, numerous other nanoparticle platforms are currently under various stages of preclinical and clinical development, including various liposomes, polymeric micelles, dendrimers, quantum dots, gold nanoparticles, and ceramic nanoparticles. More complex systems such as multifunctional nanoparticles that are concurrently capable of targeting, imaging, and therapy are the subjects of future researches.

Nanotechnology is offering several advantages to phytochemicals delivery and to drug delivery in general [77]. Of the many advantages, the most important ones are:

- Enhancing solubility and bioavailability.
- Reducing toxicity and side effects of these phytochemicals, especially that most bioactive phytochemicals are very toxic.
- Increasing the stability of active phytochemicals to stand harsh conditions along their way to their site of action.
- Enhancing the biocompatibility and reducing the toxicity of the formulation itself, since these nanocarriers limit the use of toxic non polar solvents that were traditionally used to increase solubility and improve the formulation characteristics.
- However, an important thing that we must pay attention to, is the safety and hazards of nanoparticles. In order to comprehend the safety of any formulation, long studies over a long period of time is needed. Nanoparticles are relatively new in the medical market and sector, and it is known that material in the nano scale usually has different properties compared with bulk scale, making it difficult to predict hazards of such small particles.

The currently approved nanoparticle systems have in some cases improved the therapeutic index of drugs by reducing drug toxicity or enhancing drug efficacy. Future research efforts need to be directed towards finding new methods for nanotoxicology, recognition of biological effects of nanoparticles in the environment, and creation of the bases of nanobiomonitoring.

Author details

Fadwa Odeh[1], Hala Al-Jaber[2] and Dima Khater[1]

*Address all correspondence to: f.odeh@ju.edu.jo

1 Department of Chemistry/The University of Jordan, Amman, Jordan

2 Department of Applied Sciences/Faculty of Engineering Technology/Al-Balqa Applied University/Marka, Amman, Jordan

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Chapter 11

Electroporation – Advantages and Drawbacks for Delivery of Drug, Gene and Vaccine

Azam Bolhassani, Afshin Khavari and Zahra Orafa

Additional information is available at the end of the chapter

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1. Introduction

Lack of potent drug and gene delivery is one of the major problems of cancer chemotherapy and biotherapy. Different non-viral approaches have been proposed for drug and gene delivery such as physical and chemical methods. Physical delivery systems are one of the efficient non-viral methods including electroporation, micro-injection, gene gun, tattooing, laser and ultrasound [Bolhassani and Rafati, 2011]. Electroporation (EP) is the formation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) high-voltage pulse to overcome the barrier of the cell membrane. This transient, permeabilized state can be used to load cells with a variety of different molecules including ions, drugs, dyes, tracers, antibodies, oligonucleotides, RNA and DNA [Faurie et al., 2005]. Electroporation has proven useful both *in vitro*, *in vivo* and in patients, where drug delivery to malignant tumors has been performed. In addition, the data show that electroporation of DNA vaccines *in vivo* is an effective method to increase cellular uptake of DNA and gene expression in tissue leading to marked improvement in immune responses. Electroporation represents a way of increasing the number of DNA-transfected cells and enhancing the magnitude of gene expression, while reducing intersubject variability and requiring less time to reach a maximal immune response compared to conventional intramuscular injection of the vaccine [Monie et al., 2010].

Delivery of DNA vaccines using electroporation has already been tested successfully in a wide range of disease models. Electroporation has been used to enhance immune responses using DNA vaccines directed against infectious diseases such as influenza, HIV, hepatitis C, malaria, anthrax or to treat or prevent the development of tumors including breast cancer, prostate cancer and melanoma [Daemi et al., 2012; Best et al., 2009]. The studies have shown that *in vivo* EP mediated vaccination is a safe and effective modality for the treatment of prostate cancer and has potential to be used as a neo-adjuvant or adjuvant therapy [Ahmad et al.,

2010]. Electroporation has been successfully used to administer HPV DNA vaccine to mice as well as rhesus macaques, which has prompted its use in an ongoing phase I clinical trial such as VGX-3100, a vaccine that includes plasmids targeting E6 and E7 proteins of both HPV subtypes 16 and 18, for treatment of patients with CIN 2 or 3. In addition, electroporation has been used as an effective vaccination technique for the treatment of HPV induced cancers using the pNGVL4a-CRT/E7 (detox) DNA vaccine [Monie et al., 2010]. The application of *in vivo* electroporation to the sites receiving injected plasmid DNA has allowed for dramatic increases in immune responses compared with plasmid DNA injection alone. Among the tissues targeted for *in vivo* electroporation have been skin, liver, tumors and muscle [Widera et al., 2000]. Regarding to *in vivo* EP is predominantly carried out intramuscularly (i.m.), currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ-producing T cells and protective efficacy to infection [Daemi et al., 2012; Brave et al., 2011]. Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+T-cell immune responses while limiting invasiveness of delivery [Brave et al., 2011].

However, this method sometimes leads to cell death, primarily when the electrical fields cause permanent permeabilization of the membrane and the consequent loss of cell homeostasis, in a process known as irreversible electroporation [Rubinsky, 2007]. This is an unusual mode of cell death that is not understood yet. The electroporation procedures used in many laboratories could be optimized with limited effort. Moreover, electroporation, used alone or in combination with other enhancement methods, expands the range of drugs (small to macromolecules, lipophilic or hydrophilic, charged or neutral molecules) that can be delivered transdermally [Escobar-Chávez et al., 2009; Denet et al., 2004]. The efficacy of transport depends on the electrical parameters and the physicochemical properties of drugs. The *in vivo* application of high-voltage pulses is well tolerated, but muscle contractions are usually induced. The electrode and patch design is an important issue to reduce the discomfort of the electrical treatment in humans [Denet et al., 2004]. It was shown that poloxamer 188, added before or immediately after an electrical pulse used for electroporation decreases the number of dead cells and at the same time does not reduce the number of reversible electropores through which small molecules (cisplatin, bleomycin, or propidium iodide) can diffuse. It was suggested that hydrophobic sections of poloxamer 188 molecules are incorporated into the edges of pores and that their hydrophilic parts act as brushy pore structures. The formation of brushy pores may reduce the expansion of pores and delay the irreversible electropermeability. These techniques show a potential for drug and gene delivery. However, site-specific and efficient delivery still remains a difficult problem [Tsoneva et al., 2010]. The voltages generally used for electroporation in animals range from 100 to 1200 V/cm. The investigators have shown that low-voltage electroporation can induce immunity and protect mice effectively [Daemi et al., 2012; Zhou et al., 2008]. In addition, intradermal DNA electroporation is one of the most efficient non-viral methods for the delivery of gene into the skin [Lin et al., 2012]. Previous studies have demonstrated that a combination of a short high voltage pulse (HV) and a long duration low-voltage pulse (LV) was efficient for DNA electroporation in the skin and that intradermal electroporation was suitable to deliver DNA vaccine when a Th1-oriented response is desired [Pavselj

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and Préat, 2005]. Various cell types of the skin are involved in the development of immune response. Langerhans cells (LC) due to their long dendritics and their horizontal orientation, create an almost continuous network that enables them to capture most antigens that enter through the skin. Delivery of DNA into the skin could induce direct-presentation of the encoded antigen by APC or cross-presentation after uptake by keratinocytes. Some studies have indicated that EP induces IgG and Th-cell responses higher than Intramuscular (IM) delivery [Lee et al., 2011]. This chapter is further focused on the use of electroporation-induced delivery of anti-cancer drugs, gene and vaccines in human cancer cells along with description of its advantages and disadvantages.

2. Non-viral delivery systems

Generally, the methods of delivering a gene, vaccine and drug are divided into: **a)** Physical/ non-viral approaches such as tattooing, gene gun, ultrasound, electroporation, laser; **b)** Chemical/ non-viral systems such as: cationic lipids/liposomes, polysaccharides, cationic polymers, cationic peptides, micro-/ nano-particles and **c)** Biological/ viral vectors [Bolhassani et al., 2011]. Non-viral vectors are safe in human body and easy for use. Among them, electroporation can be used to distribute nucleic acid fragments, oligonucleotides, siRNA and plasmids to cells. Studies using electroporation were performed *in vivo*; however electroporation is sometimes harmful to differentiated adult cells [Anwer, 2011; Wang et al., 2012]. Non-viral vectors are attractive tools in gene therapy and vaccine delivery [Draghia-Akli et al., 2005].

3. History and definition of electroporation

Electroporation was introduced in the 1960s and comprises the application of controlled electric fields to facilitate cell permeabilization. The success of *in vitro* delivery by electroporation has led to the development of *in vivo* applications [Takei et al., 2008]. The first *in vitro* and *in vivo* attempts to use electroporation in gene transfer were demonstrated in 1982 and 1991, respectively [Al-Dosari and Gao, 2009]. *In vivo* electroporation depends on electric pulses to drive gene transfer. These pulses generated transient pores in cell membranes followed by intracellular electrophoretic DNA movement. Typically, *in vivo* electroporation is performed by first injecting DNA to the target tissue followed by electric pulses, with varied voltage, pulse duration and number of cycles, from two applied electrodes [Al-Dosari and Gao, 2009, Hao et al., 2012]. This technique is generally safe, efficient and can produce good reproducibility compared to other non-viral methods. When its parameters are optimized, this method can generate transfection efficiency equal to that in viral vectors [Al-Dosari and Gao, 2009]. The initial study of *in vivo* EP was the delivery of chemotherapeutic agents to solid tumors. In the mid-to late 1990s, the efficacy of this approach for drug delivery was demonstrated in a variety of different animal and human tumors. This technique was then tested for enhanced plasmid DNA delivery and subsequently, the initiation of the first clinical trials [Heller and Heller, 2006]. Furthermore, the expression of reporter genes was used to optimize *in vivo* EP param-

eters, to explore the mechanism of EP and to show delivery in a new tissue. The use of *in vivo* EP for gene delivery including immune modulators, cell cycle regulators, suicide genes, anti-angiogenic genes and genes encoding toxins has established its potential for many therapeutic applications [Heller and Heller, 2006]. *In vivo* electroporation as compared to other gene transfer methods, such as viral vectors, has several advantages: **a)** various types of DNA constructs (or RNAi vectors) are readily introduced to the cells without limitation of DNA size; **b)** more than two different DNA constructs can be introduced into the same cells [Matsuda and Cepko, 2007]. Altogether, delivery by electroporation has been performed to a number of tissues including skin, muscle, liver, testes and tumors employing a wide range of electrical conditions and electrodes. While this preclinical research is promising, further optimization of electrical conditions and electrodes would be necessary for clinical use [Fioretti et al., 2013; Heller and Lucas, 2000].

4. Electroporation mechanisms

The development of theoretical models has developed our understanding of electroporation mechanism. Electropermeabilization of cells mainly involves the interaction of the electric field with the lipid domains of the cell membrane. Experimentally measured quantities consist of the membrane lifetimes, the current, the membrane conductance and transmembrane voltage. Regarding to the accumulated evidence, the pores are formed because of the electric field. The transient aqueous pore theory describes the main features of electropermeabilization, which is one major consequence of electroporation. Molecular transport of charged molecules appears to be predominantly due to electrical flow through pores, such that the elevated transmembrane voltage plays two roles: (a) creation of pores and (b) provision of a local driving force [Weaver and Chizmadzhev, 1996]. Electrochemotherapy (ECT) is a cancer therapy that conjugates the administration of a chemotherapy agent to the delivery of permeabilizing pulses released singularly or as bursts. This approach results in higher number of anticancer molecules delivered to their biological targets, but is also associated to undesirable side effects such as pain and muscular spasms. A new electroporator delivering eight biphasic pulses at the voltage of 1,300 V/cm lasting + 50 μsec each, with a frequency of 1 Hz, and with 10-μsec interpulse intervals (total treatment time: 870 μsec/cm^2 of treated area) was tested on the human lung cancer cell line (A549) and both in mice xenografts and rabbits with spontaneous tumors. The tumor cell line treated with electroporation showed efficient drug delivery suggesting further cell death. In addition, *in vivo* data demonstrated that the new permeabilizing protocol adopting biphasic electric pulses displays a significant higher efficacy compared to previous ECT treatments and consequently, substantial reduction of the morbidity [Spugnini et al., 2014].

5. Applications of electroporation

Skin electroporation could be particularly appropriate for topical drug delivery. Skin electroporation temporarily permeabilizes the barrier to drug permeation and therefore could

broaden topical delivery to drugs not suitable for delivery by passive diffusion (i.e., hydrophilic, charged, and/or large molecular drugs). The use of high-voltage pulses could also enhance the permeability of viable cells as demonstrated by the electrochemotherapy of tumors (e.g., bleomycin) or DNA transfection [Escobar-Chávez et al., 2009]. Indeed, the application of electrical pulses to a cell creates a transient permeability that allows entry of hydrophilic molecules such as drugs and plasmid DNA. The exact mechanism by which the plasmid enters the cell following electroporation is unclear. Although, small molecules such as drugs can enter cells via transient pores, it seems that macromolecules such as plasmid DNA enter by a more complex interaction with the cell membrane. This interaction is enhanced by the application of repeated pulses that brings the plasmid into closer contact with the cell membrane. The voltage required for electroporation varies considerably and is dependent on cell size and shape [Wells, 2010]. It ranges from values of approximately 100 V/cm in large cells up to 1-2 kV/cm in small cells such as bacteria. Plasmid electrotransfer is a multistep process from interaction with the cell membrane, movement into the cell, intracellular trafficking and passage across the nuclear membrane [Wells, 2010; Nakamura and Funahashi, 2013]. A variety of different electrodes could be used depending on the cells to be treated. For *in vitro* studies, electrode patterns vary from a cuvette figure for cells in suspension to complex electrode arrays for adherent cells. An equal variety of electrodes have been developed for *in vivo* use, based on the nature of the tissue being treated [Wells, 2010]. A wide range of pulse patterns have been used both *in vitro* and *in vivo*. Repeated pulses appear better than single pulses. Some authors suggest a combination of one high-voltage pulse with a series of low-voltage pulses. Pulse magnitude and duration also has an effect on the damage caused to the cells. Pretreatment of skeletal muscle *in vivo* with hyaluronidase allows the use of a decreased voltage and so reduces damage while maintaining efficiency. Plasmid size has a significant effect on the efficiency of electroporation with a decreasing efficiency observed with increasing plasmid size using the same expression cassette [Wells, 2010]. The *in vitro* and *in vivo* studies using electroporation have been further described as following:

a. *In vitro* electroporation: Electroporation can be used to transfer a range of genetic materials into cells including DNA, RNA and oligonucleotides. In addition, *in vitro* electroporation is useful for synthetic oligonucleotides which have an uncharged backbone such as the phosphorodiamidate morpholino oligomers [Wells, 2010]. The effects of electrical treatment with high field intensity (200-1000 V/cm) were evaluated on two breast cancer cells (MDA-MB-231 and MCF-7) and one fibroblast cell line 3T3. The degree of electropermeabilization of the adherent cells elevated steadily with the increasing of the field intensity. Furthermore, cell replication of both cancer cell lines was disturbed after electropermeabilization. Altogether, the use of suitable electric pulses could trigger changes in the cytoskeleton organization and cell adhesiveness, led to the enhancement of anti-tumor effects [Pehlivanova et al., 2012].

b. *In vivo* electroporation: *In vivo* electroporation has been shown to be effective for a wide range of tissues, including tumors, skin, liver, lung, kidney, thymus, bladder, adipose tissue, vasculature, retina, cornea, ciliary muscle, brain, spinal cord, skeletal muscle and testis, for delivering a range of genetic material such as DNA, RNA and oligonucleotides

(e.g. siRNA, antisense oligonucleotides). *In vivo* plasmid electroporation has also been used as either a primary or booster vaccination strategy that enhances cell-mediated immune responses. Most of the studies using electroporation have involved local delivery into the target organ but a few have studied local electroporation following systemic (intravenous) delivery of the plasmid. For example, local electroporation targeted plasmid delivery to the liver was effective for liver, kidney and spleen but was not successful for skeletal muscle or skin [Wells, 2010]. Taken together, electroporation has been applied to efficient delivery of drugs, genes and vaccines as described below. Figure 1 shows common application of electroporation.

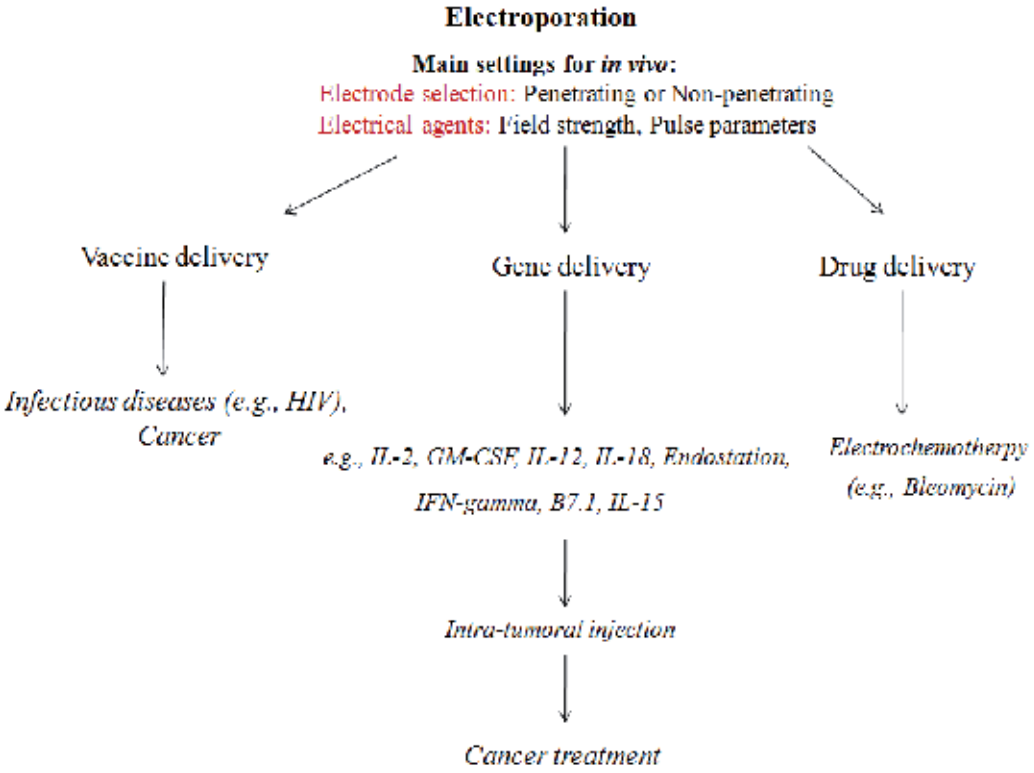

Figure 1. Common applications of electroporation

5.1. Drug delivery

Several studies have investigated the use of electroporation to enhance the efficacy of the drugs especially used for the treatment of various cancer types. Current electroporation protocols are based on preclinical studies. The authors reported the use of 1,000 V/cm (voltage/electrode distance ratio) up to approximately 1,300 V/ cm for electrochemotherapy. One simple way of lowering the applied voltage was to decrease the gap between electrodes, e.g., 0.4 cm [Gehl, 2008]. The threshold potential for transient electric breakdown of cell membranes is about 0.5

V. For a cell with a 10 μm diameter, the field strength needed to reach and exceed a potential of 0.5 V at each end is about 1,000 V/cm [Hui, 2013].

Transdermal drug delivery offers an attractive alternative to the conventional drug delivery methods of oral administration and injection [Escobar-Chávez et al., 2009]. The subcutaneous layer forms the major barrier to most water-soluble and many hydrophobic drugs and contributes the major portion of the electric resistance of the skin. Electroporation is one of the approaches to improve the transdermal delivery by transiently permeabilizing the skin to facilitate drug transport. Transdermal drug delivery has several potential advantages over other parenteral delivery methods. Apart from the convenience and non-invasiveness, the skin also provides a "reservoir" that sustains delivery over a period of days [Hui, 2013]. The authors have shown that if the voltage of the pulses exceeds a voltage threshold at 75–100 V (equivalent to the breakdown threshold of 8–10 lipid bilayers in the SC), microchannels or "local transport regions" are created through the breakdown sites of the SC [Hui, 2013]. Many small-molecule drugs have been successfully delivered through the skin by electroporation. Transport efficiency for small charged molecules (MW ≤ 1000, e.g., protoporphyrin IX), using the same polarity pulses, was higher than that for uncharged molecules (e.g., protoporphyrin IX methyl ester) or charged molecules with opposite polarity pulses. The results indicated that, besides passive diffusion through electropores, electrophoretic force of the pulses also contributes to the electroporation-enhanced transport of these charged molecules [Hui, 2013]. Therefore, the efficacy of transport depends on the electrical parameters and the physicochemical properties of drugs. Some studies indicated that the *in vivo* application of high-voltage pulses is well tolerated, but muscle contractions are generally induced. Furthermore, the electrode and patch design is an important issue to reduce the discomfort of the electrical treatment in humans [Escobar-Chávez et al., 2009]. The electroporation has been first used to enhance the delivery of chemotherapeutic drugs like cisplatin and bleomycin in cancer cells and solid tumors, respectively. This application has been termed electrochemotherapy [Tsoneva et al., 2007; Gehl, 2008].

5.1.1. Anti-cancer drugs

Electrochemotherapy, via cell membrane permeabilizing electric pulses, potentiates the cytotoxicity of non-permeant or poorly permeant anticancer drugs with high intrinsic cytotoxicity, such as bleomycin or cisplatin, at the site of electric pulse. Its advantages are high efficacy on tumors with different histologies, simple application, minimal side effects and the possibility of effective repetitive treatment. In clinical studies, electrochemotherapy has proved to be a highly efficient and safe approach for treating cutaneous and subcutaneous tumor nodules. The treatment response for various tumors (predominantly melanoma) was approximately 75% complete and 10% partial response of the treated nodules [Escobar-Chávez et al., 2009].

5.1.1.1. Bleomycin

A consistent finding is that lipo-or amphiphilic drugs traverse the cell membrane without electroporation, while an enhancement in cytotoxicity is found with drugs that, under normal

circumstances, do not pass the cell membrane easily. The most prominent example is bleomycin, which is a well-known drug. One bleomycin molecule can cause several DNA strand breaks and is highly toxic inside the cell [Gehl, 2008]. Drug doses used in bleomycin-based electrochemotherapy have been variable. Some groups have used intratumoral injection with relatively high doses, while others have applied its lower doses. Also, for *i.v.* administration, bleomycin is generally given in the doses used in standard treatment protocols. The results of the different regimens are comparable, but there may be more necrosis with the higher doses and a better chance to conserve normal tissue with the lower doses [Gehl, 2008]. In bleomycin chemotherapy, treatment was more than 1000 times more effective with electroporation than without electroporation. In comparison with bleomycin, other drugs such as daunorubicin, doxorubincin, 5-fluorouracil and paclitaxel had no electroporation benefits [Hui, 2008]. Bleomycin electrochemotherapy has been successfully applied to treat melanomas, head and neck squamous cell carcinomas, Kaposi's sarcomas, as well as lung, breast, kidney, and bladder cancers. Its cytotoxicity is higher in cancer tissues than in normal tissues, including arteries and nerves. In certain stage II and III clinical trials, 100% complete recovery has been reported. Bleomycin electrochemotherapy induces temporary vasoconstriction, which helps to retain the drug in the tumor tissue [Hui, 2008].

As described above, the bleomycin is used with electroporation (electrochemotherapy) for treatment of tumors in the clinical setting. Calcium electroporation offers several advantages over standard treatment options: calcium is inexpensive and may readily be applied without special precautions mentioned about cytostatic drugs. Therefore, details on the use of calcium electroporation are essential for carrying out clinical trials comparing electrochemotherapy [Frandsen et al., 2014]. Calcium electroporation can induce ATP depletion-associated cellular death. The effects of calcium and bleomycin electroporation (alone or in combination) were compared in three different cell lines (DC-3F, transformed Chinese hamster lung fibroblast; K-562, human leukemia; and murine Lewis Lung Carcinoma) [Frandsen et al., 2014]. Furthermore, the effects of electrical pulsing parameters and calcium compounds on treatment efficacy were determined. The results showed that electroporation with either calcium or bleomycin significantly reduced cell survival, without a synergistic effect at similar voltage parameters. At equimolar concentrations, calcium chloride and calcium glubionate resulted in comparable decreases in cell viability. Indeed, the effect of calcium electroporation is independent of calcium compound [Frandsen et al., 2014]. Briefly, the calcium electroporation can be suggested as a potential cancer therapy in future clinical trial.

5.1.1.2. Poloxamer 188

Poloxamer 188, added before or immediately after an electrical pulse, decreased the number of dead cells as well as it did not reduce the number of reversible electropores. It was suggested that hydrophobic sections of poloxamer 188 molecules are incorporated into the edges of pores and their hydrophilic parts act as brushy pore structures. The formation of brushy pores may reduce the expansion of pores and delay the irreversible electropermeability. Its advantage is the increased uptake and accumulation into reversibly electroporated tumor cells [Tsoneva et al., 2010].

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5.1.2. Analgesic and anti-inflammatory drugs

Electroporation increased the permeation of h-cyclodextrin (BCD) and hydroxy propyl h-cyclodextrin (HPCD), relative to passive transport. The presence of BCD and HPCD enhanced the total transport of the permeants piroxicam and carboxyfluorescein (CF), respectively, from both permeant solutions and suspensions. Another studies demonstrated that electroporation may enhance and control transdermal permeation of nalbuphine (NA) and its prodrugs including nalbuphine benzoate (NAB) and sebacoyl dinalbuphine ester (SDN). The results indicated that the use of iontophoresis or electroporation significantly enhanced the *in vitro* permeation of NA and its prodrugs. In addition, lipophilicity and molecular size had significant effects on skin permeation of NA, NAB, and SDN via passive diffusion or under the electric field. The permeation amounts of NA and its prodrugs may be increased by application of higher pulse voltage, pulse duration and pulse number [Escobar-Chávez et al., 2009].

5.1.3. Anti-diuretic drugs

Macromolecules were investigated as chemical enhancers of transdermal transport by skin electroporation. Skin electroporation increased transdermal mannitol delivery [Escobar-Chávez et al., 2009].

5.1.4. Anti-viral drugs

The use of electroporation pulses enhancing the skin permeability to deliver anti-viral drugs is in the early stages of development. A systematic study examining the parameters influencing electroporative transdermal delivery of terazosin hydrochloride to rat skin was previously reported. It was found that voltage, pulse length and number of pulses were the three most important parameters [Escobar-Chávez et al., 2009].

5.1.5. Beta-blocker agents

The studies have shown the effects of electroporation on iontophoretic transport of 2 beta-blockers, timolol (lipophilic) and atenolol (hydrophilic). The iontophoretic transport of timolol was decreased by electroporation because the high accumulation of the lipophilic cation timolol in the *s.c.* resulted in a decrease of electroosmosis. In contrast, electroosmosis was not affected by atenolol, and the iontophoretic transport of atenolol was increased by electroporation. Using two different beta-blockers, the researchers showed that lipophilicity and positive charges affected the electrotransport of drugs [Escobar-Chávez et al., 2009].

5.1.6. Insulin

The data represented that *in vivo*, non-invasive insulin delivery to therapeutic levels and glucose extraction may be achieved by combining electroporation with anionic lipids and electroosmosis [Escobar-Chávez et al., 2009]. These studies confirmed the synergistic effects of electroporation (EP) and iontophoresis (IP) on the *in vivo* percutaneous absorption of human insulin in rats [Escobar-Chávez et al., 2009].

5.1.7. Photosensitizers

Selectivity of photodynamic therapy can be improved with localized photosensitizer delivery, but topical administration is restricted by poor diffusion across the *s.c.* The researchers used the electric pulses to increase transdermal transport of D-aminolevulinic acid (ALA), a protoporphyrin IX (PpIX)-precursor for the photodynamic therapy of superficial skin cancer and cutaneous metastases of internal malignancies. A two-fold enhancement of PpIX production with electroporative delivery was observed compared to passive delivery. The application of iontophoresis also increased the ALA permeation by approximately 15-fold [Escobar-Chávez et al., 2009].

5.1.8. Folic acid antagonists

The topical administration of methotrexate (MTX) for the treatment of psoriasis and neoplastic diseases is restricted by the poor diffusion of MTX across the *s.c.* Some studies showed that electroporation is an efficient method to increase the transdermal transport of MTX. Furthermore, electroporation of MTX with an anion lipid enhancer under a mild hyperthermic environment provided a significant transdermal delivery within a short time [Escobar-Chávez et al., 2009].

5.2. Vaccine delivery

Electroporation-based immunization (especially, EP-mediated DNA vaccine) has been effective in a number of species including mice, rats, rabbits, non-human primates, pigs and sheep.

5.2.1. DNA vaccine

DNA immunization has known as an efficient strategy for vaccination [Bolhassani and Rafati, 2009]. The main disadvantage of plasmid DNA vaccines is their poor immunogenicity when administered as an unformulated intramuscular injection [Anderson and Schneider, 2007]. A number of approaches for enhancing the potency of DNA vaccines have developed over the past few years such as: **a)** Optimization of DNA constructs; **b)** Development of new DNA manufacturing processes and formulations; **c)** Augmentation of immune responses with novel encoded molecular adjuvants; and **d)** Improvement of *in vivo* DNA delivery strategies including electroporation [Sardesai and Weiner, 2011].

Among them, EP-mediated delivery has generated considerable attention and appeared to have a great impact in vaccine immunogenicity and efficacy by increasing antigen delivery up to a 1000 fold versus naked DNA delivery alone [van Drunen Littel-van den Hurk and Hannaman, 2010; Sardesai and Weiner, 2011]. In many cases, the immune responses and protection rates observed following DNA administration via EP were comparable or superior to other vaccine strategies including viral vectors and live/attenuated/inactivated virus vaccines [Sardesai and Weiner, 2011, Daemi et al., 2012; Hosseinzadeh et al., 2013]. An electroporation driven plasmid DNA vaccination strategy was studied in animal models for treatment of prostate cancer. This phPSA plasmid electroporation vaccine strategy could

effectively activate tumor specific immune responses. Optimization of the approach indicated that a four-dose regimen provided highest tumor protection. Furthermore, the four-dose regimen showed optimal and further tumor protection using co-administration of synthetic oligo CpG. Thus, the *in vivo* EP-mediated vaccination has potential to be use as a neo-adjuvant or adjuvant therapy in cancer treatment [Ahmad et al., 2010]. The effect of electroporation on DNA vaccine potency and gene delivery was studied using skin as a target tissue in larger animal species such as pig, macaque and sheep. In a macaque model, the higher cellular and humoral responses were observed to an HIV DNA vaccine harboring IL-12 gene, with electroporation compared to intradermal DNA injection alone [Hirao et al., 2008]. Furthermore, the safety and lack of integration after immunization with a high dose of a multigene HIV-1 vaccine was studied using a combination of the delivery methods jet-injection and intradermal electroporation. The data showed that plasmids persist in the skin at the site of injection for at least four months after immunization [Brave, et al., 2010]. The researchers demonstrated that mice and guinea pigs vaccinated with single-and multi-gene DNA via EP and then with recombinant gp120 protein (i.e., the synthetic DNA prime-protein boost protocol) induced significantly higher antibody binding titers [Muthumani et al., 2013]. Recently, Minicircle DNA (a new form of DNA containing only gene expression cassette but lacking backbone of bacterial plasmid DNA) is a powerful candidate of gene delivery improving the levels and the duration of transgene expression *in vivo*. A novel vaccine delivery system, including the combined *in vivo* EP and the minicircle DNA carrying codon-optimized HIV-1 gag gene was prepared to evaluate the immunogenicity of this system. The use of EP delivery further increased minicircle-based gag gene expression led to the augmentation of humoral and cellular immune responses. Increased immunogenicity of EP-assisted minicircle-gag may benefit from increasing local antigen expression, up-regulating inflammatory genes and recruiting immune cells [Wang et al., 2014]. In sheep, the significantly higher antibody responses to plasmid-encoded HBsAg were observed after IM delivery followed by electroporation in comparison with conventional IM or ID injection. Importantly, these antibody responses were sustained for 25 weeks after vaccination [van Drunen Littel-van den Hurk et al., 2008]. Moreover, various reports have illustrated that cytokine adjuvants have significant effects on modulating the immune responses to DNA vaccination. Indeed, the co-delivery of plasmid encoded cytokines is able to quantitatively and qualitatively modulate the immune responses in a large animal following *in vivo* electroporation of a DNA vaccine [Yen and Scheerlinck, 2007]. Although, intra-tumor delivery does not generally result in detectable serum transgene expression, intramuscular electroporation does result in serum expression. However, intratumor delivery is more successful than intramuscular delivery in eradicating primary tumors and in generating systemic immunity. For instance, a number of studies have demonstrated long-term, complete tumor regression, using delivery of plasmids encoding IL-12 or IFN-γ as a single agent in melanoma and squamous cell carcinoma (SCC) [Heller and Heller, 2006]. Complete regression after IL-12 gene therapy in combination with herpes simplex virus (HSV) thymidine kinase, bleomycin, or recombinant bacillus Calmette-Guérin (rBCG) has been observed in several experimental models. Electrically mediated bleomycin delivery combined with IL-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF) gene therapy also induced long-term complete regression in a small percentage of mice with

melanomas. Furthermore, complete responses have been observed in a fibrosarcoma model after delivery of a plasmid encoding GM-CSF and B7.1 [Heller and Heller, 2006]. One of the main challenges for efficient electroporation in larger animals is to ensure correct match between the electrical field and the injected DNA. Intramuscular injection of plasmid DNA followed by electrical stimulation (electroporation) is an efficient method for achieving therapeutic levels of encoded proteins or eliciting efficient immune responses in smaller animals such as mice and rats [Tjelle et al., 2006]. Application of short electrical pulses can be used to enhance gene delivery and DNA vaccination in large animals led to improved cellular and humoral immune responses. In addition, lowering the electrical field will therefore be important for reducing electroporation-induced pain. Increasing the number of electrodes and/or injection volume, could enhance the transfection efficiency of the conventional electroporation devices [Tjelle et al., 2006]. It will be interesting to electroporate different plasmids that were mixed together, plasmids mixed with proteins, or mixed proteins to understand the immune response intensity. It was reported that there is no interference with two different DNA vaccines, implying that it is possible to co-administrate vaccines directed against different pathogens at one time [Yuan, 2008; Yuan, 2008]. Induction of a humoral response against amyloid-β peptide may be beneficial for Alzheimer's disease (AD) patients. The potency of an AD DNA epitope vaccine (DepVac) delivered intramuscularly by EP and intradermally by gene gun (GG) was evaluated for treatment and prevention of AD. The results indicated that both delivery methods are effective at promoting potent antibodies specific for Aβ [Davtyan et al., 2012].

Gene delivery into solid tumors after direct injection of formulated or naked DNA preparations is generally low due to a large number of delivery barriers e.g., tumor complexity. Tumor electroporation significantly enhanced DNA delivery into solid tumors. Electroporation of *luciferase* DNA into mouse and human tumors produced 10-to 1200-fold increases in luciferase expression compared to tumors injected with *luciferase* DNA alone [Anwer, 2008]. Tumor electroporation by six-needle electrodes (100-μs pulses, 1,500 V/cm) produced a 21-fold enhancement over control while tumor electroporation by caliper electrodes (5,000-μs pulses, 800 V/cm) produced a 42-fold increase. The transfection efficiency of DNA electroporation was compared with that of non-electroporation methods including, liposome-DNA complexes and integrin-liposome-DNA complexes in different tumors [Anwer, 2008]. The electroporation delivery was found to be superior to all other test methods. The maximal enhancement in transfection efficiency by electroporation was up to 30-fold over naked DNA, 5-to 10-fold over liposome-DNA complexes, and over 100-fold over integrin-liposome-DNA complexes. Electroporation produced detectable gene expression in every tumor type while non-electroporated methods were effective only in some tumors [Anwer, 2008]. Moreover, electroporation enhancement of luciferase transfection was up to 16-fold in mouse skin and up to 83-fold in pig skin, as compared to that in non-electroporated groups. In another study, the delivery and anticancer efficacy of MBD2 antisense DNA in electroporated tumors were comparable to the *adenovirus*-treated groups [Anwer, 2008].

Intracellular targeting of tumor antigens through its linkage to immunostimulatory molecules such as calreticulin (CRT) can improve antigen processing and presentation through the MHC

class I pathway and increase cytotoxic CD8+T cell production. However, even with these strategies, the efficacy of such immunotherapeutic strategies is dependent on the identification of an effective route and method of DNA administration [Best et al., 2009]. Intramuscular administration of HPV DNA vaccines followed by electroporation increased the number of antigen-loaded dendritic cells resulting in the enhancement of gene expression. In a comparison study of the HPV DNA vaccine administered by different methods, electroporation has been shown to elicit the highest number of E7-specific cytotoxic CD8+T cells and greatest antitumor immune response compared to intramuscular injection and intradermal gene gun delivery [Best et al., 2009; Monie et al., 2010]. Generally, electroporation can be considered as a promising method for delivery of HPV DNA vaccines in human clinical trials [Best et al., 2009]. For instance, electroporation has been successfully used to administer several HPV DNA vaccines to mice as well as rhesus macaques, which has prompted its use in an ongoing Phase I clinical trial of VGX-3100, a vaccine that includes plasmids targeting E6 and E7 proteins of both HPV subtypes 16 and 18, for treatment of patients with CIN 2 or 3 [Monie et al., 2010].

Regarding to *in vivo* EP is predominantly carried out intramuscularly, currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ-producing T cells, and protective efficacy to infection. Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+T-cell immune responses while limiting invasiveness of delivery [Daemi et al., 2012]. It was shown that subcutaneous administration of HPV16 E7 DNA linked to C-terminal fragment of gp96 followed by electroporation can significantly enhance the potency of DNA vaccines [Daemi et al., 2012, Bolhassani et al., 2011; Bolhassani et al., 2009].

5.2.2. Peptide/ protein vaccine

Larger molecules, including heparin, polylysine, antisense polynucleotides, lactalbumin, and IgG, have been delivered by transdermal electroporation with proper enhancers. The transport of calcium-regulating hormones was found to be increased by applying electroporation and iontophoresis. Anionic lipid formulation has shown significant synergistic effect with electroporation on delivering insulin *in vitro* and *in vivo* and has the potential to lower the voltage threshold to a small level [Hui, 2008]. The reports showed an enhanced transport of human luteinizing hormone releasing hormone through heat-stripped human epidermis by electroporation. Furthermore, the presence of an ionic surfactant such as sodium dodecyl sulfate (SDS) reduced the electroporation threshold and significantly improved the transdermal transport of molecules by electroporation. Indeed, saturated anionic lipids tend to be preferentially retained in the epidermis during electroporation and result in disrupting the lamellar structure of the *sc* lipids, leading to prolonged lifetime of electropores. Using this method, the transport of both charged and neutral macromolecules was enhanced [Hui, 2008]. Recently, peptides and mini-gene vaccines are of particular interest since several epitopes of tumor-associated antigens have been employed as therapeutic and prophylactic cancer vaccines. Although, small molecular size antigens may be delivered into and through the skin by

diffusion or by iontophoresis methods, but, higher molecular weight antigens (>1 kDa), such as peptides, DNA, carbohydrates, as well as vaccine adjuvants need to deliver using an efficient rout of administration. Needle-free non-adjuvant skin immunization by electroporation has been reported [Hui, 2008]. For example, delivering the antigenic peptide MYR to mice by electroporation resulted in mucosal immunity and specific lymph node cell proliferation. Also, the others indicated that antigen-specific CTL response to the peptide vaccine delivered by needle-free electroporation/electroosmosis was equivalent to that delivered by intradermal injection with Freund's Complete Adjuvant. In this experiment, the Kb-binding OVA peptide SIINFEKL was used as an example to induce the peptide-specific cytotoxic T lymphocyte (CTL) response in mice [Hui, 2008; Escobar-Chávez et al., 2009].

Protein-based vaccines have emerged as a potentially promising approach for the generation of antigen-specific immune responses. However, due to their low immunogenicity, there is a need for novel approaches to enhance protein-based vaccine potency. One approach to enhance protein-based vaccine potency is the use of toll-like receptor ligands, such as CpG oligonucleotides, to activate the antigen-specific T cell immune responses [Kang et al., 2011]. Another approach involves employing a method capable of improving the intramuscularly delivery of protein-based vaccine led to the slow release of the protein. The studies showed that intramuscular injection of protein (OVA)-based vaccines in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8+T cell immune responses and antitumor effects in vaccinated mice. Similar results were observed using the HPV-16 E7 protein-based vaccination system [Kang et al., 2011].

5.2.3. RNA-based vaccines

RNA-based vaccines represent an interesting immunization modality, but suffer from poor stability and a lack of efficient and clinically feasible delivery technologies. A study evaluated the immunogenic potential of naked *in vitro* transcribed Semliki Forest virus replicon RNA (RREP) delivered intradermally in combination with electroporation [Johansson et al., 2012]. Replicon-immunized mice showed a strong cellular and humoral response, compared to mice immunized with regular mRNA. RREP-elicited induction of interferon-γ secreting CD8+T cells and antibody responses were significantly increased by electroporation. The immune response during the contraction phase was further increased by a booster immunization, and the proportion of effector memory cells increased significantly. These results demonstrated that naked RREP delivered via intradermal electroporation can constitute an immunogenic, safe and attractive alternative immunization strategy to DNA-based vaccines [Johansson et al., 2012].

5.2.4. DC-based vaccine

Designing effective strategies to load human dendritic cells (DCs) with tumor antigens is a challenging approach for DC-based tumor vaccines. In a study, a cytoplasmic expression system based on mRNA electroporation to efficiently introduce tumor antigens into DCs was described. Preliminary experiments in K562 cells revealed that mRNA electroporation compared to plasmid DNA electroporation showed improved transfection efficiency and

induced a strikingly lower cell toxicity. Next, mRNA electroporation was used for non-viral transfection of different types of human DCs, including monocyte-derived DCs (Mo-DCs), CD341 progenitor-derived DCs (34-DCs) and Langerhans cells (34-LCs). High-level transgene expression by mRNA electroporation was obtained in more than 50% of all DC types [Van Tendeloo et al., 2001]. In addition, mRNA-electroporated DCs retained their phenotype and maturational potential. Strikingly, a non-specific stimulation of CTL was observed when DCs were transfected with plasmid DNA. The data clearly demonstrated that Mo-DCs electroporated with mRNA efficiently present functional antigenic peptides to cytotoxic T cells. Therefore, electroporation of mRNA-encoding tumor antigens was a powerful technique to charge human dendritic cells with tumor antigens and could serve applications in future DC-based tumor vaccines [Van Tendeloo et al., 2001].

5.3. Gene therapy

Much intensive research has gone into the development of safe and efficient methods for the delivery of therapeutic genes [Tamura and Sakata, 2003]. Recently, an improved electroporation protocol was established by optimizing the electroporation parameters including plasmid concentration, voltage and pulse duration, to deliver DNA into dental follicle cells to study the roles of candidate genes in regulating tooth eruption [Yao et al., 2009]. Using this approach, highly efficient gene transfer has already been achieved in muscle and liver as well as in tumors [Tamura and Sakata, 2003]. Electroporation of mouse muscle with secretory *alkaline phosphatase* (*SEAP*) plasmid produced systemic levels of SEAP that were up to 120-fold higher than those achieved with *SEAP* plasmid alone. Intramuscular injection of *erythropoietin* plasmid in mouse leg produced systemic levels of erythropoietin that were 100-fold higher than those from *erythropoietin* plasmid alone. Electroporation of IL-5 plasmid DNA into mouse tibialis muscle produced 20 ng IL-5/mL while the non-electroporated delivery generated only 0.2 ng IL-5/mL in the blood. Electroporation of mouse muscle with IL-12 plasmid produced 1500 pg of IL-12 per injected muscle and 170 pg IL-12/ mL in the blood. The huge improvement in muscle delivery (up to 10,000-fold over naked DNA) compared with other non-viral gene delivery systems (10-fold over naked DNA) opens new opportunities for muscle-based gene therapy [Anwer, 2008].

5.3.1. DNA delivery

Numerous studies on gene transfer have been published in a wide variety of tissues from animal models [Gehl, 2008]. Most of the studies investigated the treatment of protein deficiencies and cancers using cytokines. DNA formulations were designed to minimize tissue damage or enhance expression at weaker electric pulses. These formulations were prepared with the addition of transfection reagents, membrane permeating agents, tissue matrix modifiers, targeted ligands, or agents modifying electrical conductivity or membrane stability to enhance delivery efficiency or reduce tissue damage. These advancements in DNA formulation could prove to be useful in improving the safety of electroporation protocols for human applications [Anwer, 2008]. In addition, several DNA formulations have been described for *in vivo* gene electroporation. DNA electroporation in saline were shown to enhance transfection

efficiency in several tissues, producing both local and systemic levels of therapeutic proteins. The enhancement of gene electroporation is associated with significant tissue damage directly related to electroporation intensity. Milder electroporation conditions, although less toxic, are transfectionally inefficient. Several formulation strategies have been examined to reduce electroporation toxicity without affecting transfection activity [Anwer, 2008]. Naked DNA in saline is the most commonly used formulation for *in vivo* gene electroporation. In skeletal muscle, electroporation enhancement of *luciferase* gene transfer was 10,000-fold over non-electroporated control. The enhancement of luciferase activity was observed in both small and large animal species. Histochemical analysis of *b-galactosidase* plasmid electroporated muscle showed a larger transfection area per muscle and a higher plasmid copy number per muscle cell when compared with non-electroporated muscle. Muscle electroporation with *FGF*1 plasmid also indicated significantly larger transfection area in electroporated muscle as compared to non-electroporated muscle [Anwer, 2008]. Also, electroporation enhanced intra-arterial administration of a transgenic construct in rats resulted in expression in mesengial cells [Stokman et al., 2010]. A report demonstrated the feasibility of electroporating genes into intact nerve to modify Schwann cell gene expression [Aspalter et al., 2009]. Gene therapy may represent a promising alternative strategy for cardiac muscle regeneration. *In vivo* electroporation with an optimized protocol was also a safe and effective tool for non-viral gene delivery to the beating heart [Ayuni et al., 2010]. This method was used to examine whether introduction and expression of PPARγ gene could differentiate skeletal muscle satellite cells to adipocytes *in vivo* [Bonamassa and Liu, 2010]. The studies indicated that the cricket (Gryllus bimaculatus) is a hemimetabolous insect that is emerging as a model organism for the study of neural and molecular mechanisms of behavioral traits. However, research strategies have been limited by a lack of genetic manipulation techniques that target the nervous system of the cricket. The development of a new method for efficient gene delivery into cricket brains was studied using *in vivo* electroporation. Plasmid DNA harboring an enhanced green fluorescent protein (EGFP) gene was injected into adult cricket brains, followed by electroporation at a sufficient voltage. Expression of EGFP was observed within the brain tissue [Matsumoto et al., 2013]. Gene therapies for cancer utilizing *in vivo* electroporation have been proved effective in a number of experimental murine tumor models. The therapeutic genes delivered in those cases were diverse including cytokine genes (IL-12) and cytotoxic genes (TRAIL), making a wide range of therapeutic strategies [Tamura and Sakata, 2003]. Generally, cancer gene therapy has been studied using *in vivo* electroporation including suicide genes (e.g., combination of HSV-TK and prodrug GCV: TK-GCV), apoptosis inducing genes (e.g., TRAIL), immuno-stimulatory genes (e.g., IFN-gamma, IL-12 and IL-18) and anti-angiogenic genes (e.g., Endostatin) [Tamura and Sakata, 2003].

5.3.2. Protein delivery

A substantial improvement in muscle delivery with the use of electroporation has renewed interest in muscle tissue for systemic protein therapy. Several therapeutic proteins have been expressed from skeletal muscle and secreted into systemic circulation at substantial concentrations with the use of electroporation [Tamura and Sakata, 2003].

5.3.3. SiRNA delivery

There are increasing interests in physical methods for delivery of siRNA [Oh and Park, 2009]. Among physical methods, electroporation has been frequently studied to stimulate the cellular and *in vivo* localized delivery of siRNA by electric pulses [Oh and Park, 2009]. An electroporation method was established to involve a constant voltage and ''plate and fork'' type electrodes and use it for *in vivo* delivery of siRNA. The electric current correlated to the microvascular density and vascular endothelial growth factor (VEGF) expression and exhibited a threshold that assures efficient delivery. VEGF siRNA electroporation suppressed the growth of tumors exhibiting high VEGF expression to less than 10% of the control level, but it had no effect on low VEGF-expressing tumors. Notably, a long interval (20 days) of electroporation was enough to obtain a satisfactory effect. Systemically injected siRNA could also be delivered into tumors by this method [Valero et al., 2008]. In atopic dermatitis mouse model, the intradermal delivery of cyclooxygenase specific siRNA into the skin by electroporation resulted in the silencing of the target gene in the skin, and reduced the scratching behavior of mice [Oh and Park, 2009]. The delivery of tumor necrosis factor α-specific siRNA via electroporation was shown to inhibit inflammation in mice with collagen-induced arthritis. Moreover, the *in vivo* silencing of target genes by electrically mediated siRNA delivery was reported in mice bearing solid tumors [Oh and Park, 2009]. Some studies reported the successful use of electroporation of siRNA delivery to renal tissue. In rats, injection of siRNA into the renal artery followed by electroporation led to predominant knockdown of the target protein in the glomeruli [Stokman et al., 2010]. A number of studies have demonstrated the feasibility of targeted delivery of oligonucleotides, small interfering RNA (siRNA), plasmid DNA, and viral vectors to the corneal cells *in vivo*, specifically stromal keratocytes and corneal epithelial cells, via intrastromal injection, iontophoresis, electroporation, and gene gun. The combination of iontophoresis and electroporation was found to be effective in delivering siRNA but not plasmid DNA into the corneal epithelium [Hao et al., 2010]. Altogether, there is great interest in platforms which efficiently deliver RNA molecules such as messenger RNA and small interfering RNA (siRNA) to mammalian tissues [Broderick et al., 2012]. However, the *in vivo* delivery of RNA enhanced by EP has not been extensively characterized.

6. Efficient agents involved in electroporation

The type of a nucleic acid and the type of the transfected cell generally affect the efficiency of electroporation [Stroh et al., 2010]. Skeletal muscle is a preferable target tissue for a number of reasons including long-term secretion of therapeutic proteins for systemic distribution and promotion of strong humoral and cellular immune responses post-vaccination. Numerous factors impact plasmid uptake and expression after intramuscular injection followed by EP. Briefly, they include: species, targeted muscle, age, plasmid formulation, plasmid concentration and dose, pulse pattern, electric field intensity (current, voltage and resistance), pulse length, lag time, electrode configuration and orientation. These improvements in the conditions of EP can increase the efficacy of plasmid transfer and lower the total amount of plasmid

and DNA vaccines required to generate targeted levels of biologically active proteins or antibodies [Draghia-Akli et al., 2005].

7. Advantages, disadvantages and solutions

The electroporation can be applied equally to all cell types and at all stages of the cell cycle [Escobar-Chávez et al., 2009]. Collateral damage by electroporation can be serious, compared with some other physical methods. When electroporation field is applied through the skin using surface plate electrodes, the major potential drop develops across the skin instead of across the targeted subcutaneous tissues. Skin edema is a common consequence. Most electroporation protocols aim to permeate only the plasma membranes. Electroporation of the nucleus requires a further step, using higher threshold voltage and shorter pulse length (nucleoporation) [Hui, 2008]. Although the principle of electroporation is applicable to all cell types, its efficiency depends on the electrical properties of the cells. Smaller cells require higher field to permeate. This is an important consideration for *ex vivo* gene delivery especially to hematopoietic cells. Cells with less conductive contents (such as adipocytes) are less susceptible. The thresholds for different cells in a heterogeneous tissue would thus vary [Hui, 2008].

DNA formulation with certain types of polymers has been found to enhance electroporation efficiency and, in some cases, reduce treatment-related toxicity. Anionic polymers, including poly-L-glutamate, polyacrylic acid, poly-L-aspartate, dextran sulfate, and pectin have been examined for their ability to enhance electroporation mediated gene transfer in skeletal muscle. In addition, DNA complexes of cationic liposomes were electroporated into several histologically distinct mouse subcutaneous tumors, and the efficiency of gene transfer was compared with that of naked DNA electroporation [Anwer, 2008, Lai et al., 2008]. Liposomal formulations were transfectionally superior to naked DNA in B16 melanoma, P22 carcinoma, and SaF sarcoma but not in T24 human bladder carcinoma or MC2 mammary carcinoma. This variation in tumor response could be due to differences in the state of tumor necrosis, tumor conductivity, or matrix complexity between the different tumors [Anwer, 2008]. A higher interaction of positively charged lipid-DNA complexes with negatively charged cell surfaces could be one of the underlying mechanisms in the lipid enhancement of the electroporation. Addition of anionic liposomes into the electroporation medium has been found to enhance the delivery of macromolecules into cells. For example, dextran uptake during electroporation was enhanced by 80-fold with the addition of phosphatidylglycerol and phosphatidylcholine into the transfection medium. The magnitude of liposome enhancement was dependent on the degree of lipid saturation but independent of polar head group [Anwer, 2008]. DNA delivery by electroporation is not target-specific. Several attempts have been made to improve tissue-specific targeting of electroporated DNA with the use of cell-specific ligands. Antibodies and other molecular entities that recognize specific cell surface receptors have been conjugated to delivery vehicles to achieve high cell specificity during electroporation. The technical feasibility of *in vivo* DNA targeting by electroporation has not been fully established. For example, electroporation of integrin conjugated liposome-DNA complexes yields much lower transfection efficiency than do the non-targeted systems [Anwer, 2008]. This failure of tumor targeting

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in vivo could be attributed to poor stability of the targeted complexes in extracellular milieu, altered integrin receptor affinity for integrin ligand or suboptimal transfection conditions. Hence, the use of targeted ligands is an attractive approach to improve target specificity of electroporation, but its *in vivo* application has not been fully established.

DNA dispersion in muscle is highly restricted because of the rigid collagen-and hyaluronan-rich matrix surrounding muscle fibers. Pretreatment of tissue with hyaluronidase has been shown to improve gene delivery into liver and skeletal muscles [Anwer, 2008]. Hyaluronidase treatment prior to electroporation in skeletal muscle produced a substantial increase both in levels and extent of gene transfer in skeletal muscle. Hyaluronidase treatment enhanced transfection efficiency at low electric pulses without significantly damaging the muscle structure or function. This tissue-protective effect of hyaluronidase has been observed in ischemic myocardium and tissue edema. These results demonstrated that hyaluronidase treatment is a useful approach to improve electrogene transfer in higher species where rigid interstitium is a major limitation to plasmid delivery [Anwer, 2008]. Application of electromigration field (3 V for 30 s) has been shown to enhance the uptake of DNA-modified gold nanoparticles during cell electroporation. Gold nanoparticles devoid of DNA coating were not taken up by cells during electroporation. Formulations that can enhance DNA binding to cell surface *in vivo* may also enhance electroporation efficiency at weak electric pulses [Anwer, 2008].

Currently used methods to introduce foreign DNA into mammalian cells are based on bulk procedures in which large cell numbers are simultaneously transfected, electroporated or virally infected. All of these methods have a number of specific limitations, such as limited control over the amount of DNA uptake, the intracellular half-life and fate of the introduced DNA, and site of genomic integration [Valero et al., 2008]. These limitations represent a serious drawback in situations where genetically modified stem cells have to be produced for therapeutic application, including gene therapy and regenerative medicine, especially when these cells are hard to isolate in large enough numbers. Recently, microfluidic devices have shown great benefits for studying a variety of cell processes. Of particular importance is the use of such devices for electroporation, enabling high efficiency transfer of a variety of macromolecules into cells [Valero et al., 2008]. However, further optimization of DNA vaccine delivery is needed for this vaccine modality to ultimately be efficacious in humans [Hallengärd et al., 2012]. The "plate and fork" electrodes were used for the transfer of a plasmid vector for erythropoietin expression into rat skin and were compared with needle-type and disc-type electrodes. Therefore, the electroporation conditions for significant efficacy vary with the molecule to be delivered [Takei et al., 2008].

In general, there are differences in effective variables between a drug and a gene for delivery by electroporation. High field strength and a short pulse length gave good results, at least with some of the drugs investigated (e.g., bleomycin), whereas electroporation for genes benefits from a combination of a low electric field and a long pulse length [Takei et al., 2008].

Membrane poration methods, such as electroporation and sonoporation, are an attractive alternative in some applications. Indeed, electroporation has demonstrated its efficacy in a number of DNA and RNA delivery applications for previously difficult-to-transfect primary

cells. However, this method can cause cell death and has been shown to damage sensitive materials such as quantum dots, which aggregate due to exposure to electric fields. There have also been limited reports of successful protein delivery by this mechanism [Sharei et al., 2013].

Electroporation is a technique that increases the permeability of cell membranes by changing the transmembrane potential and subsequently disrupting the lipid bilayer integrity to allow transportation of molecules across the cell membrane *via* nano-size pores. This process when used in a *reversible* fashion has been used in medicine and research for drug or macromolecule delivery into cells [Guo et al., 2010; Heish et al., 2011; Phillips et al., 2012; Li et al., 2012; Niessen et al., 2013; Narayanan et al., 2013]. Irreversible electroporation (IRE) is a new minimally invasive tumor ablation technique which induces irreversible disruption of cell membrane integrity by changing the transmembrane potential resulting in cell death. Irreversible electroporation is currently undergoing clinical investigation as local tumor therapy for malignant liver and lung lesions [Niessen et al., 2013].

The use of *irreversible* electroporation (IRE) has been introduced by Rubinsky's group as a method to induce irreversible disruption of cell membrane integrity subsequently causing cell death. IE can effectively create tissue death in micro-to millisecond ranges of treatment time compared to conventional ablation techniques, which require at least 30 minutes to hours. Additionally, it is possible to treat a considerably larger lesion with shorter treatment times than available with current techniques [Guo et al., 2010; Heish et al., 2011; Phillips et al., 2012; Li et al., 2012; Niessen et al., 2013; Narayanan et al., 2013]. A higher electric voltage leading to a larger potential gradient to create irreversible electroporation has been studied using *in vitro* and *in vivo* studies. Irreversible electroporation is technically simple to use and suitable for minimally invasive surgery [Rubinsky, 2007]. Irreversible electroporation is an innovative local-regional therapy that involves delivery of intense electrical pulses to induce nano-scale cell membrane defects for tissue ablation. The purpose of this study was to investigate the feasibility of using irreversible electroporation as a liver-directed ablation technique for the treatment of hepatocellular carcinoma (HCC) in the N1-S1 rodent model. The findings suggested that IRE was effective for targeted ablation of liver tumors in the N1-S1 rodent model; IRE may offer a promising new approach for liver-directed treatment of HCC [Guo et al., 2010]. The advantage of this technique is that it is drug-free and is targeted [Heish et al., 2011]. In an experiment, it was shown that direct IRE completely ablated the tumor cells in osteosarcoma-bearing rats. A significant increase in peripheral lymphocytes, especially CD3+and CD4+cells, as well as an increased ratio of CD4+/CD8+were detectable after the IE application. As compared to the surgical resection group, the IRE group exhibited a stronger cellular immune response. These findings indicated that IRE could not only locally destroy the tumor but also change the status of cellular immunity in osteosarcoma-bearing rats [Li et al., 2012]. Some reports indicate that this novel procedure can be used for abdominal cancer treatment while minimising collateral damage to adjacent tissues because of the unique ability of the ablation method to target the cell membrane [Phillips et al., 2012]. Irreversible electroporation (IRE) is a new ablative technology that uses high-voltage, low-energy DC current to create nanopores in the cell membrane, disrupting the homeostasis mechanism and inducing

cell death by initiating apoptosis in patients with Hepatocellular carcinoma (HCC) [Narayanan et al., 2013].

8. Clinical trials

One of the methods that improve DNA penetration of the cell is electroporation [Bolhassani and Rafati, 2011]. EP itself works as an adjuvant to enhance the necessary "danger signals" that become detectable by the immune system. The tissue damage caused by the application of EP causes inflammation and recruits DCs, macrophages and lymphocytes to the injection site inducing significant immune responses, including antibody and T-cell responses [Fioretti et al., 2014; Saade and Petrovsky, 2012]. *In vivo* use of electroporation is done by injecting naked DNA followed by electric pulses from electrodes that are located *in situ* in the target tissues. Successful use of electroporation was observed in transfecting muscles, brain, skin, liver, and tumors. Since every tissue is specific and has its own characteristics, there are no generally accepted optimal conditions of electroporation that are suitable for effective transfection. These are dependent both on the amplitude and duration of the electric pulses and on the amount and concentration of DNA [Bolhassani and Rafati, 2011]. Up to now, several clinical trials have been planned using the electroporation with DNA vaccines for cancer therapy such as: a) Intratumoral IL-12 DNA plasmid (pDNA) [ID: NCT00323206, phase I clinical trials in patients with malignant melanoma, Heller and Heller, 2006; Daud et al., 2008]; 2) Intratumoral VCL-IM01 (encoding IL-2) [ID: NCT00223899; phase I clinical trials in patients with metastatic melanoma]; 3) Xenogeneic tyrosinase DNA vaccine [ID: NCT00471133, phase I clinical trials in patients with melanoma]; 4) VGX-3100 [ID: NCT00685412, phase I clinical trials for HPV infections], and 5) IM injection prostate-specific membrane antigen (PSMA)/ pDOM fusion gene [ID: UK-112, phase I/II clinical trials for prostate cancer, Low et al., 2009; Fioretti et al., 2010] [Saade and Petrovsky, 2012; Bolhassani and Rafati, 2011]. Furthermore, Hepatitis C virus DNA vaccine showed acceptable safety when delivered by Inovio Biomedical's electroporation delivery system in phase I/II clinical study at Karolinska University Hospital. ChronVac-C is a therapeutic DNA vaccine being given to individuals already infected with hepatitis C virus with the aim to clear the infection by boosting a cell-mediated immune response against the virus. This vaccination was among the first infectious disease DNA vaccine to be delivered in humans using electroporation based DNA delivery [Bolhassani and Rafati, 2011]. Recent patents have been focused on the use of genetic immunomodulators, such as “universal” T helper epitopes derived from tetanus toxin, *E. coli* heat labile enterotoxin and vegetable proteins, as well as cytokines, chemokines or co-stimulatory molecules such as IL-6, IL-15, IL-21 to amplify immunity against cancer. Electroporation-based DNA delivery technology dramatically enhances cellular uptake of DNA vaccines [Fioretti et al., 2014]. Preliminary data from an ongoing clinical trial showed electroporation enhanced the frequency and the magnitude of the anti-HIV-1 T-cell response [Saade and Petrovsky, 2012].

Hemorrhagic fever with renal syndrome (HFRS) is endemic in Asia, Europe and Scandinavia, and is caused by infection with the hantaviruses Hantaan (HTNV), Seoul (SEOV), Puumala (PUUV), or Dobrava (DOBV) viruses. The candidate DNA vaccines were developed for HFRS

expressing Gn and Gc genes of HTNV or PUUV and evaluated in a Phase I study. Three groups of nine subjects each were vaccinated on days 0, 28 and 56 with the DNA vaccines for HTNV, PUUV, or mixture of both vaccines using the Ichor Medical Systems TriGrid™ Intramuscular Delivery System (TDS-IM) [Hooper et al., 2012]. All vaccinations consisted of a total dose of 2.0 mg DNA in an injected volume of 1 mL saline. For the combined vaccine, the mixture contained equal amounts (1.0 mg) of each DNA vaccine. There were no study-related serious adverse events (SAEs). Neutralizing antibody responses were detected in 5/9 and 7/9 of individuals who completed all three vaccinations with the HTNV or PUUV DNA vaccines, respectively. In the combined vaccine group, 7/9 of the volunteers receiving all three vaccinations developed neutralizing antibodies to PUUV. The three strongest responders to the PUUV vaccine also had strong neutralizing antibody responses to HTNV. These results demonstrated that the HTNV and PUUV DNA vaccines delivered by electroporation separately or as a mixture are safe. In addition, both vaccines were immunogenic, although when mixed together, more subjects responded to the PUUV than to the HTNV DNA vaccine [Hooper et al., 2012]. Figue 2 shows several important EP-mediated DNA vaccines used in clinical trials.

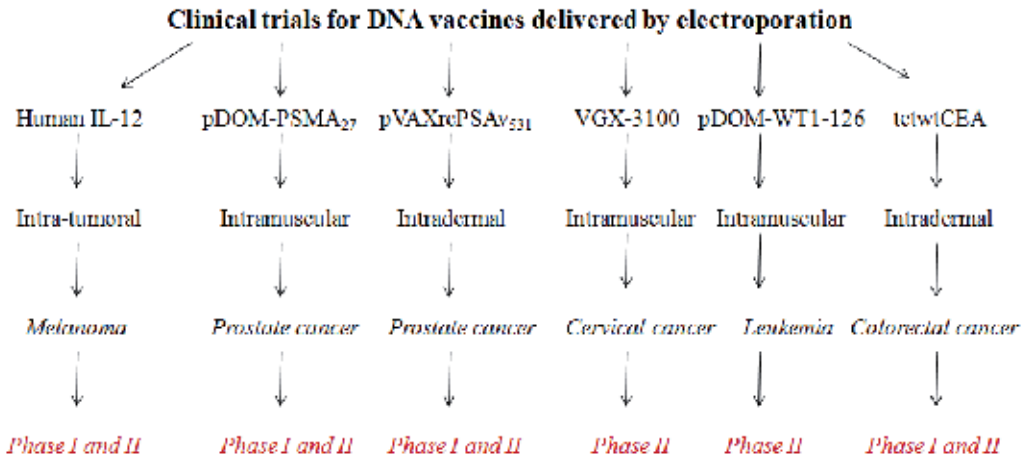

Figure 2. EP-mediated DNA vaccines used in cancer clinical trials

Drug delivery by electroporation has been in experimental use for cancer treatment since 1991 as shown in 11 studies of electrochemotherapy (ECT) of malignant cutaneous or subcutaneous lesions, e.g., metastases from melanoma, breast or head-and neck cancer. The treatment was well tolerated and could be performed on an out-patient basis [Gothelf et al., 2003]. At the Institut Gustave-Roussy, France, the fist clinical trial of ECT with bleomycin in eight patients with recurrent or progressive head and neck squamous cell carcinoma was published in 1991. After that, several clinical studies have been performed in different tumors. Clinical trials have been performed in the treatment of basal cell carcinoma, head and neck cancer (squamous cell carcinoma, adenocarcinoma and adenoid cystic carcinoma), adenocarcinoma of the breast, and malignant melanoma. In addition, a case report was published in which metastatic lesions from a bladder cancer have been successfully treated [Gothelf et al., 2003].

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9. Conclusions

Electroporation is a widely recognized method of gene delivery into mammalian tissues. It is a highly efficient method, with delivery efficiency better than many non-viral vectors. The preclinical development of electroporation in vivo is focused on tissues that are easily accessible to electroporation and can resist to electric pulsation. The standard DNA formulation for electroporation is DNA in physiological saline. Under optimal conditions, DNA electroporation in saline yields a 10- to 10,000-fold enhancement in gene delivery efficiency over non-electroporated controls. This enormous increase in transfection activity, however, accompanies significant tissue damage and local inflammation, which might not be a disadvantage, if the target is cancer. However, for applications in which expression from normal tissues is desired, tissue damage and inflammatory response are not favorable to therapeutic objectives and, therefore, must be minimized. Several formulation strategies have been designed to enhance electroporation efficiency and minimize toxicity. Hopeful results have been obtained with some approaches, which must be further developed into clinically viable formulations for non-cancer applications. Some progresses, such as HIV vaccine, West Nile virus vaccine have been made; however, these also propose some questions: What are the differences for best parameters when conduct electroporation on various muscle cells with distinct morphology and membrane properties that are also different among species? How to reduce the pain during electroporation? How long can gene expression be maintained after electrotransfer? Many experiments showed that electroporation is a safe and potent method, thus electroporation-mediated anticancer gene therapy represents a great therapeutic potential. The further improvements of electrodes including shape or arrangement of electrodes and electric conditions, by which more efficient and reliable gene transfer is achieved, are important especially in clinical trials. Furthermore, electroporation is an efficient method for enhancing transdermal drug delivery in vitro and in vivo and expands the range of compounds delivered transdermally. The combined use of electroporation with other physical enhancers such as iontophoresis is likely to yield useful and interesting data, to further explore electroporation as an efficient method of transdermal drug delivery. The technique of electroporation to enhance anticancer drug (such as bleomycin) delivery to tumor cells, so-called as electrochemotherapy, is already being applied clinically against head and neck cancers with little or no side effects. In summary, electroporation is one of the physicochemical methods for gene and drug delivery. It is superior in some aspects but also has several drawbacks. Pulse protocol and electrode design need to be optimized to reduce the main side effects e.g., muscle contraction.

Author details

Azam Bolhassani*, Afshin Khavari and Zahra Orafa

*Address all correspondence to: azam.bolhassani@yahoo.com; A_bolhasani@pasteur.ac.ir

Department of Hepatitis and AIDs, Pasteur Institute of Iran, Tehran, Iran

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Chapter 12

Prodrugs for Masking the Bitter Taste of Drugs

Rafik Karaman

Additional information is available at the end of the chapter

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1. Introduction

The palatability of the active ingredient of a drug is a significant obstacle in developing a patient friendly dosage form. Organoleptic properties, such as taste, are an important factor when selecting a certain drug from the generic products available in the market that have the same active ingredient. It is a key issue for doctors and pharmacists administering the drugs and particularly for the pediatric and geriatric populations. Nowadays, pharmaceutical companies are recognizing the importance of taste masking and a significant number of techniques have been developed for concealing the objectionable taste [1].

The word “medicine” for a child is considered a bad thing to administer because of its aversive taste. Medicines dissolve in saliva and bind to taste receptors on the tongue giving a bitter, sweet, salty, sour, or umami sensation. Sweet and sour taste receptors are concentrated on the tip and lateral borders of the tongue respectively. Bitter taste is sensed by the receptors on the posterior part of the tongue and umami taste receptors are located all over the tongue. A short period after birth, infants reject bitter tastes and prefer sweet and umami tastes[1]. Children have larger number of taste buds than adults which are responsible for sensitivity toward taste. These taste buds regenerate every two weeks. Taste becomes altered as a function of the aging process, which explains why most children find certain flavors to be too strong when adults do not. The American Academy of Pediatrics estimates that compliance in children is as low as 53%, indicating that children frequently fail to take medications properly. Noncompliance can lead to: (1)persistent symptoms, (2) need for additional doctor visits or even hospitalizations, (3) worsening of condition, (4) need for additional medications, (5) increased healthcare costs and (6) development of drug-resistant organisms in cases of infectious diseases [2].

In mammals, taste buds are groups of 30-100 individual elongated "neuroepithelial" cells which are often embedded in special structure in the surrounding epithelium known as papillae. Just below the taste bud apex, taste cells are joined by tight junctional complexes that prevent gaps

between cells. Food molecules cannot therefore squeeze between taste cells and get into the taste bud.

Taste papillae located on the tongue appear as little red dots, or raised bumps, particularly at the front of the tongue called "fungi form" papillae. There are three other kinds of papillae, foliate, circumvallates and the non-gustatory fili form. In mammals taste buds are located throughout the oral cavity, in the pharynx, the laryngeal epiglottis and at the entrance of the esophagus. Taste perception fades with age; on average, people lose half their taste receptors by time they turn 20 [3].The sensation of taste can be categorized into five basic tastes: sweetness, sourness, saltiness, bitterness, and umami. Taste buds are able to differentiate among different tastes through detecting interaction with different molecules or ions. Sweet, umami, and bitter tastes are triggered by the binding of molecules to G protein-coupled receptors on the cell membranes of taste buds. Saltiness and sourness are perceived when alkali metal or hydrogen ions enter taste buds, respectively [4].As taste senses both harmful and beneficial things, all basic tastes are classified as either aversive or appetitive, depending upon the effect the things they sense have on our bodies [5].Sweetness helps to identify energy-rich foods, while bitterness serves as a warning sign of poisons [6].

For a long period, it was commonly accepted that there is a finite and small number of "basic tastes" of which all seemingly complex tastes are ultimately composed. As of the early twentieth century, physiologists and psychologists believed there were four basic tastes: sweetness, sourness, saltiness, bitterness. At that time umami was not proposed as a fifth taste but now a large number of authorities recognize it as the fifth taste [7]. In Asian countries within the sphere of mainly Chinese and Indian cultural influence, pungency (piquancy or hotness) had traditionally been considered a sixth basic taste. Today, the consensus is that sweet,amino acid (umami), and bitter taste converge one common transduction channel, the transient receptor potential channel TRPM5, *via* phospholipase C (PLC). TRPM5 is a newly discovered TRP related to other channels in sensory signaling systems. It has been shown that PLC, a major signaling effect or of G-protein coupled receptors (GPCRs), and TRPM5 are co expressed with T1Rs and T2Rs and are vital for sweet, amino acid, and bitter taste transduction. Activation of T1R or T2R receptors by their respective taste molecules would stimulate G proteins, and in turn PLC (PLC-ß2). The activation of PLC generates two intracellular messengers-IP3 and di-acyl glycerol (DAG)-from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2)and opens the TRPM5 channel, resulting in the generation of a depolarizing receptor potential. Other additional pathways may modulate sweet, amino acid, or bitter taste reception but would not, themselves, trigger a taste response. It is not at present known how PLC activates TRPM5 or whether DAG is involved [8-18].

2. Taste masking

There are numerous pharmaceutical and over the counter (OTC) preparations that contain active ingredients, which are bitter in taste. With respect to OTC preparations, such as cough and cold syrups, the bitterness of the preparation leads to lack of patient compliance. Among

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examples that are commonly used drugs with bitter taste: (1) pseudoephedrine (1) (Figure 1),a sympathomimetic drug of the phenethylamine (2) (Figure 1) and amphetamine (3) (Figure 1) chemical classes. It may be used as a nasal/sinus decongestant, as a stimulant, or as a wakefulness-promoting agent [19], (2) dextromethorphan (4) (Figure 1), an antitussive (cough suppressant) drug. It is one of the active ingredients in many over-the-counter cold and cough medicines. Dextromethorphan has also found other uses in medicine, ranging from pain relief to psychological applications. It is sold in syrup, tablet, spray, and lozenge forms. In its pure form, dextromethorphan occurs as a white powder [20], (3) dyphylline (5) (figure1) also known as dipprophyllinea xanthine derivative withbronchodilatorandvasodilator effects. It is used in the treatment of respiratory disorders like asthma, cardiac, and bronchitis. It acts as an adenosine receptor antagonist and phosphodiesterase inhibitor [21].(4) phenylephrine (6) (Figure 1), is a selective α1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure [22]. Phenylephrine is marketed as a substitute for the decongestant pseudoephedrine, (5) chlorhexidine (7) (Figure 1), a chemical antiseptic. It is effective on both Gram-positive and Gram-negative bacteria, although it is less effective with some Gram-negative bacteria. It has both bactericidal and bacteriostatic mechanisms of action, the mechanism of action being membrane disruption, not ATPase inactivation as previously thought [23]. It is also useful against fungi and enveloped viruses, though this has not been extensively investigated, (6) atorvastatin (8) (Figure 1), a member of the drug class known as statins, used for lowering blood cholesterol. It also stabilizes plaque and prevents strokes through anti-inflammatory and other mechanisms. Like all statins, atorvastatin works by inhibiting HMG-CoA reductase, an enzyme found in liver tissue that plays a key role in production of cholesterol in the body [22], (7) loperamide (9) (Figure 1),a piperidine derivative, is an opioid drug used against diarrhea resulting from gastroenteritis or inflammatory bowel disease. In most countries it is available generically [24].(8) terfenadine (10) (Figure 2), was an antihistamine formerly used for the treatment of allergic conditions. It was brought to market by Hoechst Marion Roussel (now Sanofi-Aventis) and marketed under various brand names. According to its manufacturer, terfenadine had been used by over 100 million patients worldwide as of 1990 [25]. It was superseded byfexofenadine (11) (Figure 2) in the 1990s due to the risk of a particular type of disruption of the electrical rhythms of the heart (specifically cardiac arrhythmia caused by QT interval prolongation) [22], (9) prednisolone (12) (Figure 2), is a synthetic glucocorticoid, a derivative of cortisol, which is used to treat a variety of inflammatory and auto-immune conditions. It is the active metabolite of the drug prednisoneand is used especially in patients with hepatic failure, as these individuals are unable to metabolize prednisone into prednisolone [22], (10) salbutamol (13) (Figure 2), or albuterol (USAN) is a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease. It is marketed as Ventolin among other brand names. Salbutamol was the first selective β2-receptor agonist to be marketed – in 1968. It was first sold by Allen & Hanburys under the brand name Ventolin. The drug was an instant success, and has been used for the treatment of asthma ever since [26]. (11) guaifenisen (14) (Figure 2), or guaiphenesin (former BAN), also glyceryl guaiacolate, is an expectorant drug sold over the counter and usually taken orally to assist the bringing up (expectoration) of phlegm from the airways in acute respiratory tract infections

[22] and (12) amoxicillin (15) (Figure 2), a moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is one of the most common antibiotics prescribed for children. The drug became available in 1972 [22].

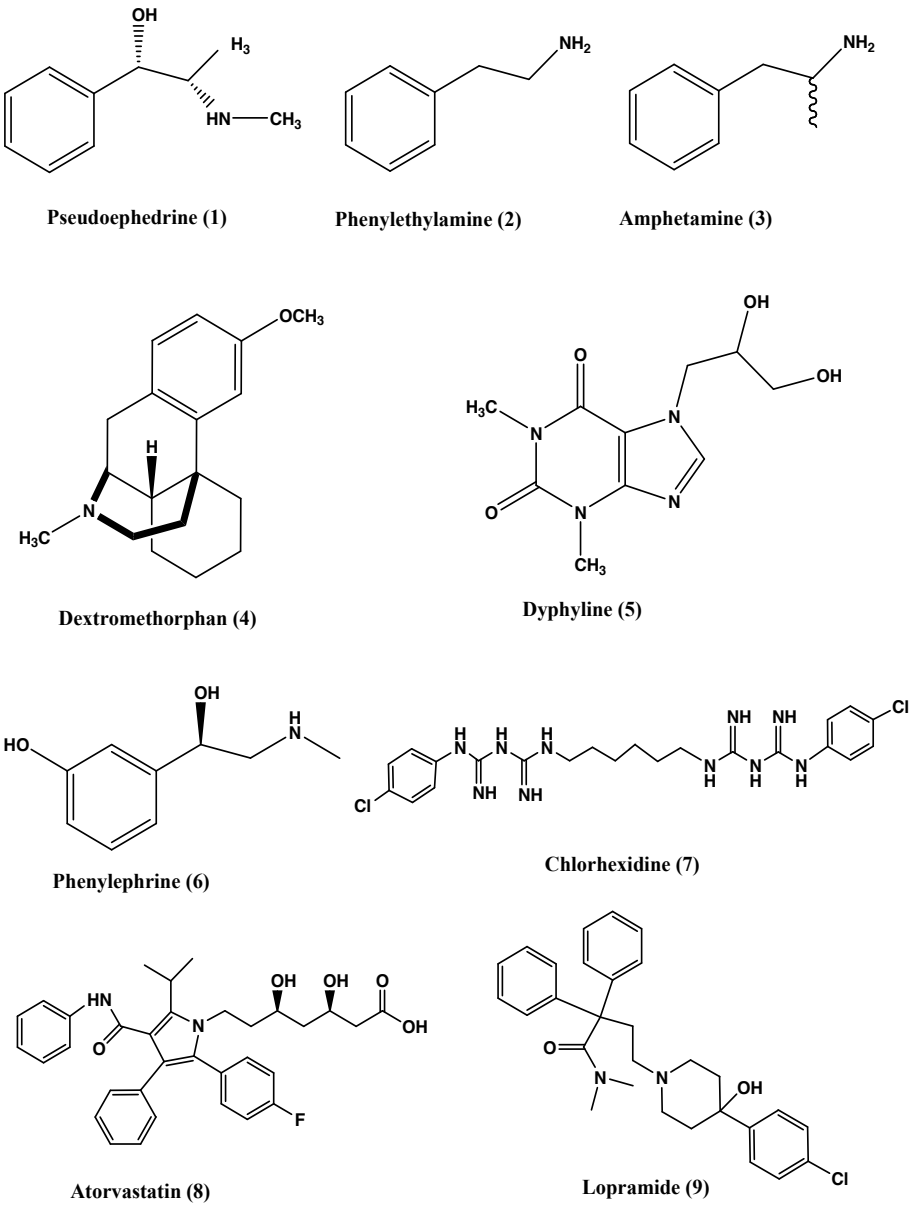


Figure 1. Chemical structures for **1-9.**

3. Challenges and criteria for pursuing masking bitter taste approaches

The most significant challenges that facing developers when pursuing masking bitter taste drugs approaches are: (i) Safety, tolerability and efficacy of the compound which are based on non-clinical testing, and physicochemical properties such as solubility, permeability and stability, (ii) lack of robust and reliable techniques for early taste screening of compounds with limited toxicity data, (iii) structure–taste relationships of pharmaceutically active molecules is limited, (iv) The perception of taste of pharmaceuticals has been shown to be different between adults and children and it might differ between healthy and patient children [4] and (v) ethical concerns to perform taste studies in healthy children unless the study is a 'swill and spit' one with drugs known to have a good safety profile [27-29].

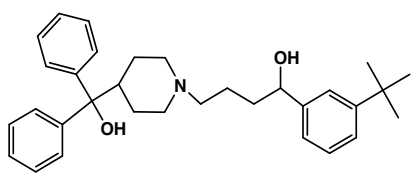
4. Bitter taste masking approaches (techniques)

A variety of taste masking approaches has been used to address the patient compliance problem. With strongly bad tasting medications even a little exposure is sufficient to perceive the bad taste. Conventional taste masking methods such as the use of sweeteners, amino acids and flavoring agents alone are often inadequate in masking the taste of highly bitter drugs. Drugs such as macrolide antibiotics, non-steroidal anti-inflammatory such as ibuprofen (**16**) (Figure 2), quinine (**17**) (Figure 2), celecoxib (**18**) (Figure 3), etoricoxib (**19**) (Figure 3), levofloxacin (**20**) (Figure 3)and penicillins have a pronounced bitter taste [30]. Masking the taste of water soluble bitter drugs, especially those given in high doses, is difficult to achieve by using sweeteners alone. As a consequence, several approaches have been investigated and have resulted in the development of more efficient techniques for masking the bitter taste of active ingredients. All of the developed techniques are based on the physical modification of the formulation containing the bitter tastant. Among the approaches used to mask bitter taste of pharmaceuticals are: (1) taste masking using flavors, sweeteners, and amino acids. This technique is the foremost and the simplest approach for taste masking, especially in the case of pediatric formulations, chewable tablets, and liquid formulations. However, it is not an ideal to be used for highly bitter and highly water soluble drugs. An example for such approach is the use of monosodium glycyrrhizinate together with flavors to mask the bitter taste of guaiphenesin (**14**) (an expectorant drug) Taste masked lamivudine (antiretroviral drug) was prepared by using lemon, orange and coffee flavors [3,31]; (2) taste masking with lipophilic vehicles such as: i) Lipids; acetaminophen granules are sprayed with molten stearyl stearate, mixed with suitable tablet excipients, and incorporated into a taste masked, chewable tablet formulation and (ii) lecithin and Lecithin-like substances; formulations with lecithin or lecithin-like substances in large quantities are believed to efficiently mask bitter taste of pharmaceuticals [3]. An example of a drug formulation containing lecithin-like substance is the one composed of magnesium aluminum silicate with soybean lecithin and talampicillin HCl (**21**) (antibiotic drug) (Figure 3); (3) coating is one of the most efficient and commonly used taste mask-

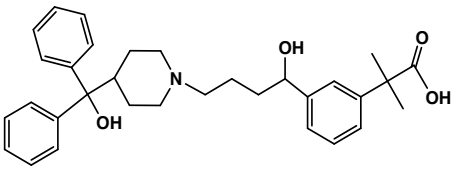
ing techniques. It is more efficient technology for aggressively bitter drugs even though coating imperfections, if present, reduce the efficiency of the technique. Coating of tablets, pellets or any other kind of particles with a film-forming polymer is a successful approach to provide a physical barrier, concealing unpleasant odors and bitter taste. Additionally, it can prevent penetration of moisture into the formulation. Coating materials can be selected from a wide range of hydrophobic and hydrophilic polymers such as polyvinylpyrrolidone, polyvinyl alcohol and cellulose derivatives. The ideal polymer for taste-masking, odor suppression and moisture protection should prevent dissolution of the dosage form in the mouth, but should be readily soluble in the stomach. Coating is classified based on the type of coating material, coating solvent system, and the number of coating layers. Taste masked famotidine (a drug for ulcer treatment) formulated by using a combination of water soluble polymers like polyvinylpyrrolidone and insoluble polymers like cellulose acetate is an example of such technique. Other various inert coating agents can be used to coat bitter drugs. These coating agents simply provide a physical barrier over the drug particles. Examples for such coating agents are starch, povidone, gelatin, methylcellulose, ethyl cellulose and etc. One of the most efficient Method of drug particle coating is the fluidized bed processor [4]. In this approach, powders as fine as 50 um are fluidized in an expansion chamber by means of heated, high-velocity air, and the drug particles are coated with a coating solution introduced usually from the top as a spray through a nozzle. Increasing the length of the coating cycle can increase coating thickness. Taste masking of Ibuprofen (**16**)(Figure 2) has been successfully achieved by this technique [4]; (4) microencapsulation is a technique applicable to protect materials from oxidation, volatilizing as well as to mask their bitter tastes [6]. Microencapsulation processes are commonly based on the principle of solvent extraction or evaporation. Microencapsulation as a process has been defined by Bakan [6] as a means of applying relatively thin coating to small particles of solid, droplets of liquid and dispersion. This process can be used for masking the bitter taste of drugs by microencapsulating drug particles with various coating agents. Coating agents employed includes gelatin, povidone, HPMC, ethyl cellulose, Bees wax, carnauba wax, acrylics and shellac. Bitter-tasting drugs can be first encapsulated to produce free flowing microcapsules, which are then blended with other excipients and compressed into tablets. Microencapsulation also increases the stability of the drug. It can be accomplished by a variety of methods, including air suspension, coacervation-phase separation, spray drying and congealing, pan coating, solvent evaporation and multi-orifice centrifugation techniques; (5) taste suppressants and potentiators such as the Linguagen's bitter blockers (e.g. adenosine monophosphate) are used for masking bitter taste of various compounding by competing with the latter on binding to the G-protein coupled receptor sites (GPCR) [32]; (6) ion exchange resins are water insoluble, cross-linked polymers containing salt forming groups in repeating position on the polymer chain. Drug can be bound to the ion exchange resin by either repeated exposure of the resin to the drug in a chromatographic column or by prolonged contact of resin with the drug solution. The resins forms insoluble adsorbates or resinates through weak ionic bonding with oppositely charged drugs. The exchange of counter ions from resin is competitive.

Most of the bitter drugs have amine as a functional group, which is the cause of their obnoxious taste. If the functional groups are blocked by complex formation the bitterness of the drug reduces drastically. A drug-resin complex is made from the bitter drugs and ion-exchange resins. The nature of the drug-resin complex is such that the average pH of 6.7 and cation concentration of about 40 meq/ lit in saliva are not able to break the drug-resin complex but it is weak enough to be broken down by the hydrochloric acid present in the stomach. Thus the drug: resin complex is absolutely tasteless and stable, with no after taste, but at the same time its bioavailability is not affected. Ion exchange resin like Amberlite was used to formulate taste masked fast dissolving orally consumable films of dextromethorphan (cough suppressant drug) [33,34]; (7) inclusion complexes in which the drug molecule fits into the cavity of a complexing agent forming a stable complex. The obtained complexing agent has the potential to mask the bitter taste of a drug by either decreasing its oral solubility on ingestion, or decreasing the amount of drug particles exposed to taste buds, thus reducing the perception of bitter taste. The inclusion complexes with cyclodextrin owe their existence to van der Waals forces between the host and guest. Cyclodextrin is the most widely used complexing agent for inclusion type complexes. It is a sweet, nontoxic, cyclic oligosaccharide derived from starch. Cyclodextrin forms inclusion complexes with organic molecules both in solid state and in solution [35]; (8) pH modifiers are capable of generating a specific pH microenvironment in aqueous media that has the ability to facilitate *in situ* precipitation of the bitter drug compound in saliva thus reducing the overall taste sensation for liquid dosage forms like suspension [36]; (9) adsorbates which are commonly used with other taste masking technologies to mask pharmaceuticals bitterness. The pharmaceutical may be adsorbed or/and entrapped in the matrix of the adsorbate porous, which may result in a delayed release of the bitter tastant during the passage through the oral cavity and hence achieving taste masking [37]; (10) chemicals; the solubility and absorption of drugs can be modified by the formation of molecular complexes. Lowering drug solubility through molecular complexation can decrease the intensity of bitterness. Higuchi and Pitman [38] reported that caffeine (**22**) (Figure 3) forms complexes with organic acids that are less soluble than xanthenes and as such can be used to decrease the bitter taste of caffeine; (11) solid dispersions; solid dispersion have been defined as dispersion of one or more active ingredients in an inert carrier or matrix at solid state prepared by melting (fusion) solvent or melting solvent method. Solid dispersion is also called as co precipitates for those preparation obtained by solvent method such as co precipitates of sulphathiazole (**23**) (Figure 3) and povidone. Solid dispersions using insoluble matrices or bland matrices may be used to mask the bitter taste of drugs. Also using them as absorbates on various carriers may increase the stability of certain drugs [39]; (12) multiple emulsions; a novel technique for taste masking of drugs employing multiple emulsions has been prepared by dissolving drug in the inner aqueous phase of w/o/w emulsion under conditions of good shelf stability. The formulation is designed to release the drug through the oil phase in the presence of gastrointestinal fluid [40]; (13) using liposomes is another way of masking the unpleasant taste of therapeutic agent is to entrap them into liposome. For example, incorporating it into a liposomal

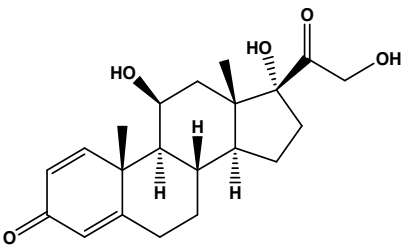
formulation prepared with egg phosphatidyl choline masked the bitter taste of an antimalarial, chloroquine phosphate(**24**) (Figure 4)in HEPES (N-2-hydroxyetylpiperzine-N′-2-ethane sulfonic acid) buffer at pH 7.2 [41];and (14) prodrugs; chloramphenicol palmitate ester (**25**) (Figure 4), clindamycin palmitate ester (**26**) (Figure 4)and triamcinolone diacetate ester (**27**)(Figure 4) [42].



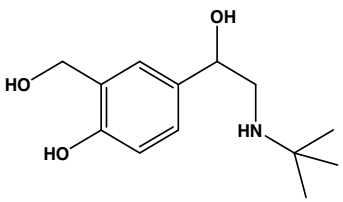
erfenedine (10)



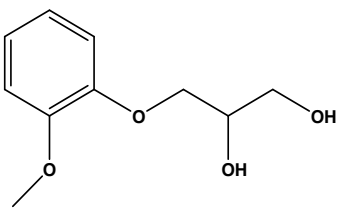
Fexofenadine (11)



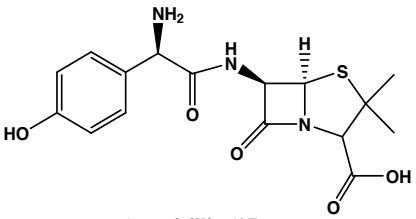
Prednisolone (12)



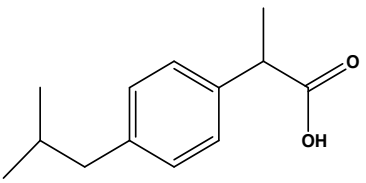
Salbutamol (13)



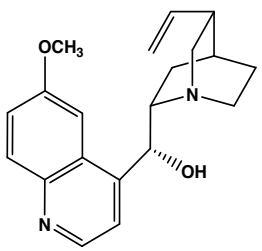
Guaifenesin (14)



Amoxicillin (15)



Ibuprofen (16)



Quinine (17)

Figure 2. Chemical structures for **10**-**17**.

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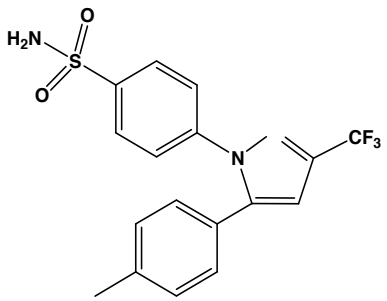
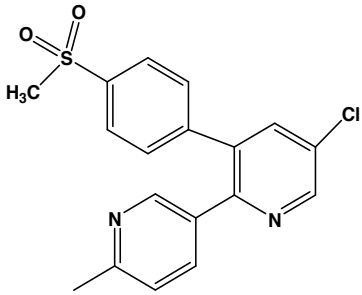
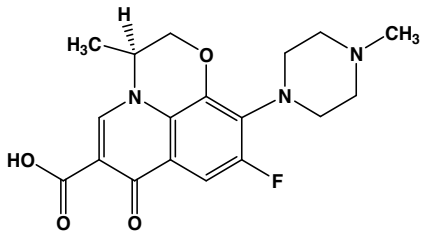


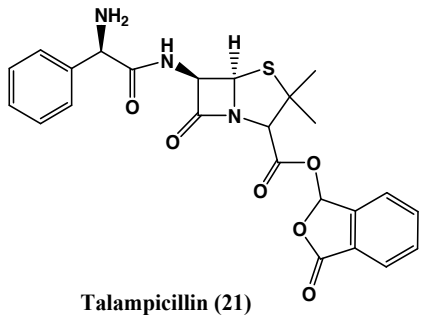
Celecoxib (18)



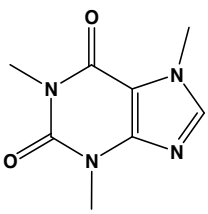
Etoricoxib (19)



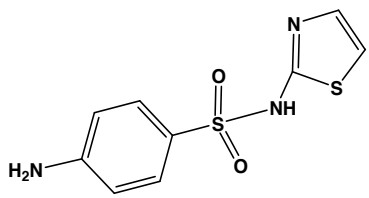
Levofloxacin (20)



Talampicillin (21)



Caffeine (22)



Sulfathiazole (23)

Figure 3. Chemical Structures for **18**-**23**.

Although the mentioned approaches have helped to improve the taste of some drugs formulations, the problem of the bitter taste of drugs in pediatric and geriatric formulations still creates a serious challenge to pharmacists. Thus, different strategies should be developed in order to overcome this serious problem. The novel chemical approach discussed in this chapter involves the design of prodrugs for masking bitter taste of pharmaceuticals based on intramolecular processes using density functional theory (DFT) and ab initio methods [43] and correlations of experimental and calculated reactions rates. No enzyme is needed to catalyze the interconversion of a prodrug to its corresponding

drug. The rate of drug release is controlled by the nature of the linker bound to the drug. Bitter tastant molecules interact with taste receptors on the tongue to give bitter sensation. Altering the ability of the drug to interact with bitter taste receptors could reduce or eliminate its bitterness. This could be achieved by an appropriate modification of the structure and the size of a bitter compound. Bitter molecules bind to the G-protein coupled receptor-type T2R on the apical membrane of the taste receptor cells located in the taste buds [44,45].

Due to the large variation of structural features of bitter tasting molecules, it is difficult to generalize the molecular requirements for bitterness. Nevertheless, it was reported that a bitter tastant molecule requires a polar group and a hydrophobic moiety. A quantitative structure activity relationship (QSAR) model was developed and has been established for the prediction of bitterness of several tastant analogues. For example, it was reported that the addition of a pyridinium moiety to an amino acid chain of a variety of bitter amino acid compounds decreases bitterness, such as in the case of glycine. Other structural modifications, such as an increase in the number of amino groups/residues to more than 3 and a reduction in the poly-hydroxyl group/ COOH, have been proven to decrease bitterness significantly. Moreover, changing the configuration of a bitter tastant molecule by making isomer analogues was found to be important for binding affinity to enhance bitterness agonist activity (e.g. L-tryptophan is bitter while D-tryptophan is sweet) [46].

Our recent studies on intramolecularity have demonstrated that there is a necessity to further explore the mechanisms for the intramolecular processes to be utilized in the design for determining the factors playing dominant role in determining the reaction rate. Unraveling the reaction mechanism would allow for an accurate design of an efficient chemical device to be used as a prodrug linker that can be covalently linked to a drug which can chemically, and not enzymatically be cleaved to release the active parent drug in a controlled manner. For instance, exploring the mechanism for a proton transfer in Kirby's acetals [47] has led to a design and synthesis of novel prodrugs of aza-nucleosides to treat myelodysplastic syndromes [48] and statins to treat high cholesterol levels in the blood [49]. In the above mentioned examples, the prodrug moiety was attached to the hydroxyl group of the active drug such that the drug promoiety (prodrug) has the potential to degrade upon exposure to physiological environment such as stomach, intestine, and/or blood circulation, with rates that are solely dependent on the structural features of the pharmacologically inactive promoiety (Kirby's enzyme model). Other different linkers such as Kirby's maleamic acid enzyme model [50] was also investigated for the design of some prodrugs such as tranexamic acid prodrugs to treat bleeding conditions [51] and acyclovir as anti-viral drug to treat Herpes Simplex [52]. Menger's Kemp acid enzyme model [53] was also utilized for the design of dopamine prodrugs for the treatment of Parkinson's disease [54]. Prodrugs for dimethyl fumarate to treat psoriasis were also designed, synthesized and currently under *in vitro* and *in vivo* kinetic studies [55].

The same approach was utilized for masking the bitter taste of antibacterial drugs such as cefuroxime (**28**) (Figure 4), atenolol (**29**) (Figure 4), paracetamol (**30**) (Figure 4), amoxicil-

lin (**15**) (Figure 2) and cephalexin (**31**) (Figure 4) [56]. The role of the promoiety in the antibacterial (cefuroxime) prodrugs is to block the free amine or the hydroxyl group which is responsible for the drug bitter taste, and to enable the release of a drug in a programmable manner. The only difference between the proposed prodrugs and their parent drugs is that the amine group in the parent drug is replaced with an amide moiety. Replacing the amine group with an amide eliminate the capability of the molecule to hydrogen bond with the bitter taste receptor, thus masking the bitter taste of the parent drug. For example, paracetamol, a widely used pain killer and fever-reducer found in the urine of patients who had taken phenacetin (**32**) (Figure 4) has a very unpleasant bitter taste. Phenacetin, on the other hand, lacks or has very slight bitterness. The difference in the structural features of both drugs is only in the nature of the group in the *para* position of the benzene ring. While in the case of paracetamol the group is hydroxyl, in phenacetin it is ethoxy. Acetanilide (**33**) (Figure 4) has a chemical structure similar to that of paracetamol and phenacetin but lacks the group in the *para* position of the benzene ring, making it lack the bitter taste characteristic of paracetamol. These combined facts suggest that the presence of the hydroxy group on the *para* position of paracetamol is the major contributor for its bitter taste. It is believed that paracetamol interacts with the bitter taste receptors *via* hydrogen bonding which involves its phenolic group. Blocking the phenolic hydroxyl of paracetamol is expected to inhibit its binding to the bitter taste receptor and hence to eliminate its bitterness. Similarly, it is expected that blocking the free amine group in atenolol, amoxicillin or cephalexin with a suitable linker might inhibit the interaction between the amine group of the parent drug and its bitter taste receptors and hence masks its bitterness. The nature of the bitter taste receptors with paracetamol (via the phenolic group) or atenolol, amoxicillin or cephalexin (via the amine group) is likely to be as a result of hydrogen bonding between the substrate and the receptor.

In this chapter, the novel prodrug approach to be presented is based on enzyme models that have been made to understand the mechanism by which enzymes catalyze biochemical reactions. The tool exploited in the design is computational calculations using molecular orbital and molecular mechanics methods and correlations between experimental and calculated rate values for some intramolecular processes. In this approach, no enzyme is needed to catalyze the conversion of a prodrug to its active parent drug. The conversion rate is solely determined by the factors affecting the rate limiting step in the intramolecular (conversion) process. Knowledge gained from the mechanisms of the previously studied enzyme models was exploited in the design.

It is believed that the use of this approach might eliminate all disadvantages related to prodrug conversion by the metabolic (enzyme catalyzed process) approach. The bioconversion of prodrugs is perhaps the most vulnerable link in the chain, because there are many intrinsic and extrinsic factors that can affect the process. For example, the activity of many prodrug activating enzymes may be varied due to genetic polymorphisms, age-related physiological changes, or drug interactions, leading to adverse pharmacokinetic, pharmacodynamic, and clinical effects. In addition, there are wide interspecies variations in both the expression and

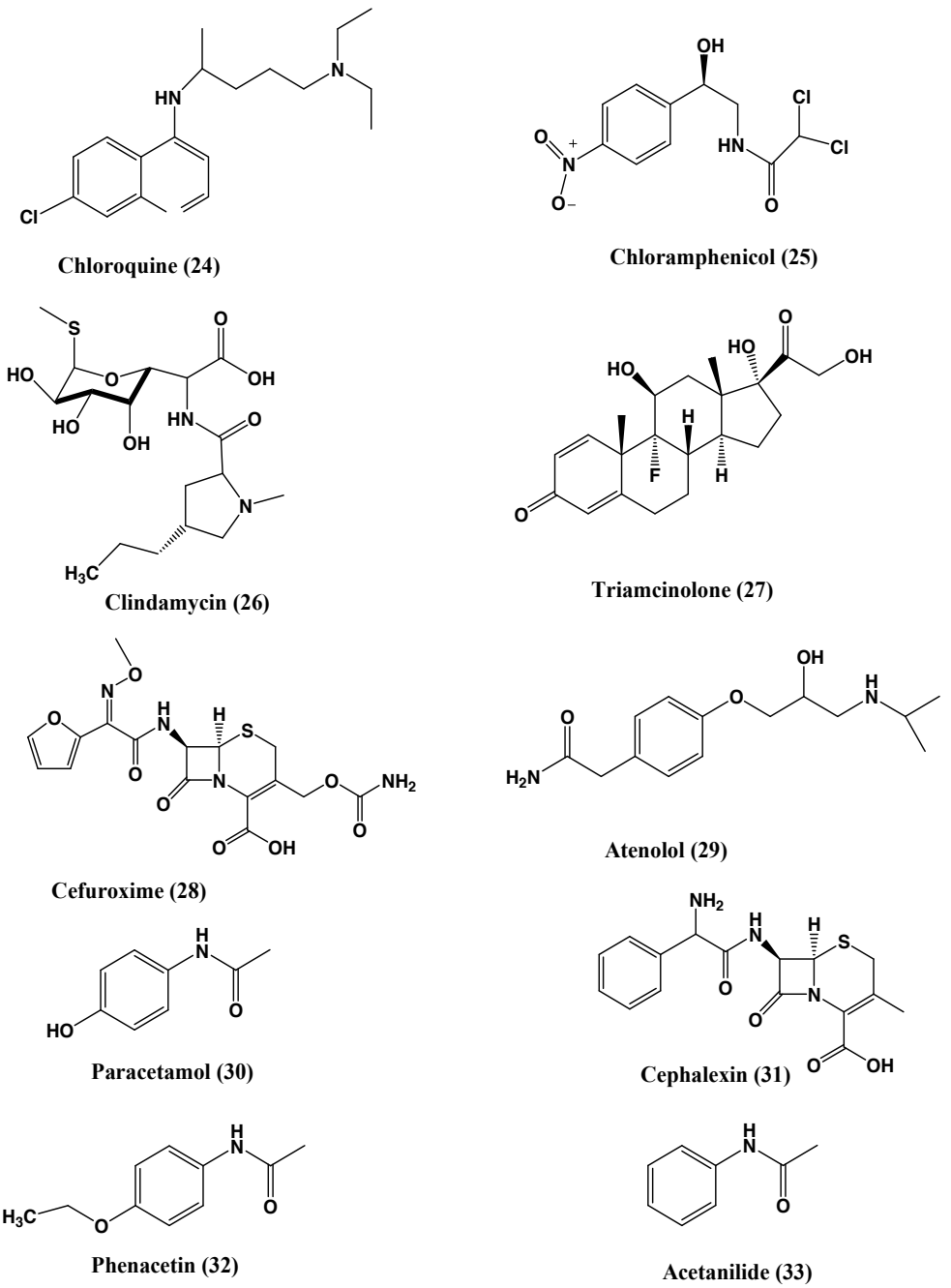


Figure 4. Chemical structures for 24-33.

function of the major enzymes activating prodrugs, and these can pose some obstacles in the preclinical optimization phase.

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5. Enzyme models utilized for the design of potential bitterless prodrugs for bitter drugs such as atenolol, amoxicillin, cephalexin, paracetamol and guaiphenesin

Scholar studies of enzyme mechanisms by several chemists and biochemists, over the past five decades, have had a significant contribution for understanding the mode and scope of enzymes catalysis.

Nowadays, the scientific community has reached to the conclusion that enzyme catalysis is based on the combined effects of the catalysis by functional groups and the ability to reroute intermolecular reactions through alternative pathways by which substrates can bind to preorganized active sites. It is believed that rate accelerations by enzymes can be proceed by (i) covalently enforced proximity, as seen in the case of chymotrypsin, [57] (ii) non-covalently enforced proximity, as represented in the catalysis of metallo-enzymes, [58] (iii) covalently enforced strain, [59], and (iv) non-covalently enforced strain, which has been extensively studied on models mimicking the lysozyme enzyme which is most closely associated with rate acceleration due to this kind of strain [60].

Rates for the majority of enzymatic reactions ranges between 10^{10} and 10^{18} fold their non-enzymatic bimolecular counterparts. For instance, biochemical reactions involving the catalysis of the enzyme cyclophilin are enhanced by10^{5} and those by the enzyme orotidine monophosphate decarboxylase are accelerated by 10^{17} [61]. The significant enhancement in rate manifested by enzymes is a result of the substrate binding within the confines of the enzyme active site. The substrate-enzyme binding energy is the dominant driving force and the major contributor to catalysis. A consensus has been reached that in all enzymatic processes binding energy is used to overcome physical and thermodynamic factors that make barriers to the reaction (free energy). These factors are: (1) the change in entropy (ΔS°), in the form of the freedom of motions of the reactants in solution; (2) the hydrogen bonding net around bio-molecules in aqueous solution; (3) a proper alignment of catalytic functional groups on the enzyme; and (4) the distortion of a substrate that must occur before the reaction takes place [62,63].

Scholarly studies have been done by Bruice, Cohen, Menger, Kirby and others to design enzyme models having the potential to reach rates comparable to rates of biochemical reactions catalyzed by enzymes. Examples for such models are those based on rate enhancements driven by covalently enforced proximity. The most cited example is the intramolecular cyclization of dicarboxylic semi esters to anhydrides advocated by Bruice *et al.*[64,65]. Bruice et al. has demonstrated that a relative rate of anhydride formation can reach 5×10^{7} upon cyclization of a dicarboxylic semi ester when compared to a similar counterpart's bimolecular process.

Other examples of rate acceleration based on proximity orientation include: (a) acid-catalyzed lactonization of hydroxy-acids as studied by Cohen et al.[66-68] and Menger [63, 69-75], (b) intramolecular S_N2-based cyclization reactions as researched by Brown et al. [76] and Mandolini's group [77], (c) proton transfer between two oxygens in Kirby's acetals [78-84], and proton transfer between nitrogen and oxygen in Kirby's enzyme models [78-84], (d) proton transfer

between two oxygens in rigid systems as investigated by Menger [63, 69-75],and (e) proton transfer from oxygen to carbon in some of Kirby's enol ethers [78-84]. The conclusions emerged from these studies are (1) the driving force for enhancements in rate for intramolecular processes are both entropy and enthalpy effects. In the cases by which enthalpy effects were predominant such as ring-closing and proton transfer reactions proximity or/and steric effects were the driving force for rate accelerations. (2) The nature of the reaction being intermolecular or intramolecular is determined on the distance between the two reacting centers. (3) In S_N2-based ring-closing reactions leading to three-, four-and five-member rings the *gem*-dialkyl effect is more dominant in processes involving the formation of an unstrained five-member ring, and the need for directional flexibility decreases as the size of the ring being formed increases. (4) Accelerations in the rate for intramolecular reactions are a result of both entropy and enthalpy factors. (5) An efficient proton transfer between two oxygens and between nitrogen and oxygen in Kirby's acetal systems were affordable when a strong hydrogen bonding was developed in the products and the transition states leading to them [85-103].

In the past few years some prodrugs based on the trimethyl lock system have been reported. Borchardt et al. has shown that the pro–prodrug 3-(2′-acetoxy-4′, 6′-dimethyl dimethyl)-phenyl-3, 3-dimethylpropionamide is capable of releasing the biologically active amine drug upon acetate hydrolysis by enzyme triggering. Another successful example exploiting a stereopopulation control model is the prodrug Taxol which enhances the drug water solubility and hence affords it to be administered to the human body *via* intravenous injection. Taxol is the brand name for paclitaxel, a natural diterpene, approved in the USA for use to treat cancer [104-108].

6. Computational methods used in the design of bitterless prodrugs for bitter tastant drugs

Nearly sixty five years ago, organic, bioorganic and medicinal chemists alike have started using computational methods for calculating molecular properties of ground and transition states. These computational methods use principles of computer science to aid in solving chemical problems. Theoretical results emerged from these methods, incorporated into efficient computer programs, for calculating the structures and physical and chemical properties of molecules.

Equilibriums energy-based and reactions rates calculations for systems having medicinal interests are of a vast importance to the health community. Today, quantum mechanics (QM) such as *ab initio*, semi-empirical and density functional theory (DFT), and molecular mechanics (MM) are commonly and increasingly being used and broadly accepted as precise tools for predicting structure-energy calculations for drugs and prodrugs alike [109-112].

Ab initio methods typically are adequate only for small systems. Ab initio methods are based entirely on theory from first principles. The ab initio molecular orbital methods (QM) such as HF, G1, G2, G2MP2, MP2 and MP3 are based on rigorous use of the Schrodinger equation with

a number of approximations. Ab initio electronic structure methods have the advantage that they can be made to converge to the exact solution, when all approximations are sufficiently small in magnitude and when the finite set of basis functions tends toward the limit of a complete set. The convergence is usually not monotonic, and sometimes the smallest calculation gives the best result for some properties. The disadvantage of ab initio methods is their enormous computational cost. They take a significant amount of computer time, memory, and disk space [109-112]. On the other hand, empirical or semi-empirical methods are less accurate because they employ experimental results, often from acceptable models of atoms or related molecules, to approximate some elements of the underlying theory. Example for such methods is the semi-empirical quantum chemistry methods based on the Hartree–Fock formalism, but make many approximations and obtain some parameters from empirical data. These methods are especially important for calculating large molecules where the full Hartree–Fock method without the approximations is too expensive. Semi-empirical calculations are much faster than their ab initio counterparts. Their results, however, can be imprecise if the molecule being computed is not similar enough to the molecules in the database used to parameterize the method. Among the commonly used semiempirical methods are MINDO, MNDO, MINDO/3, AM1, PM3 and SAM1. Calculations of molecules exceeding 60 atoms can be handled using semiempirical methods [113-116].

Another widely used quantum mechanical method is the density functional theory (DFT). With this theory, the properties of many-electron systems can be determined by using functionals, i.e. functions of another function, which in this case is the spatially dependent electron density. Therefore, the name density functional theory comes from the use of functionals of the electron density. DFT is among the most popular and versatile methods available in condensed-matter physics, computational physics, and computational chemistry. The DFT method is adequate for calculating structures and energies for medium-sized systems (30-60 atoms) of biological, pharmaceutical and medicinal interest and is not restricted to the second row of the periodic table [43].

Although the use of DFT method is significantly increasing some difficulties still encountered when describing intermolecular interactions, especially van der Waals forces (dispersion); charge transfer excitations; transition states, global potential energy surfaces and some other strongly correlated systems. Incomplete treatment of dispersion can adversely affect the DFT degree of accuracy in the treatment of systems which are dominated by dispersion.

On the other hand, molecular mechanics is a mathematical approach used for the computation of structures, energy, dipole moment, and other physical properties. It is widely used in calculating many diverse biological and chemical systems such as proteins, large crystal structures, and relatively large solvated systems. However, this method is limited by the determination of parameters such as the large number of unique torsion angles present in structurally diverse molecules [117].

Molecular mechanics simulations, for example, use a single classical expression for the energy of a compound, for instance the harmonic oscillator. The database of compounds used for parameterization, i.e., the resulting set of parameters and functions is called the force field, is crucial to the success of molecular mechanics calculations. A force field parameterized against

a specific class of molecules, for instance proteins, would be expected to only have any relevance when describing other molecules of the same class. These methods can be applied to proteins and other large biological molecules, and allow studies of the approach and docking of potential drug molecules Since the size of the system which *ab initio* calculations can handle is relatively small despite the large sizes of biomacromolecules surrounding solvent water molecules such as in the cases of enzymes and receptors, isolated models of areas of proteins such as active sites have been investigated using *ab initio* calculations. However, the disregarded proteins and solvent surrounding the catalytic centers have also been shown to contribute to the regulation of electronic structures and geometries of the regions of interest. To overcome these discrepancies, quantum mechanics/molecular mechanics (QM/MM) calculations are used, in which the system is divided into QM and MM regions where QM regions correspond to active sites to be studied and are described quantum mechanically. MM regions correspond to the remainder of the system and are treated molecular mechanically. The pioneer work of the QM/MM method was accomplished by Warshel and Levitt [118], and since then, there has been a significant progress on the development of a QM/MM algorithm and applications to biological systems [119,120].

7. Mechanistic study of the acid-catalyzed hydrolysis of maleamic acids 34-42 used for the design of atenolol, amoxicillin and cephalexin prodrugs

The acid-catalyzed hydrolysis of **34**-**42** (Figure 5) was kinetically investigated by Kirby et al. [84]. The study demonstrated that the amide bond cleavage is due to intramolecular nucleophilic catalysis by the adjacent carboxylic acid group and the rate-limiting step is the tetrahedral intermediate breakdown (Figure 6) [84]. In 1996, the reaction was computationally investigated by Katagi using AM1 semiempirical calculations. In contrast to what was suggested by Kirby, Katagi's study demonstrated that the rate-limiting step is the formation of the tetrahedral intermediate and not its dissociation [121]. Later on Kluger and Chin have experimentally researched the mechanism of the intramolecular hydrolysis process utilizing several N-alkylmaleamic acids derived from aliphatic amines with a wide range of basicity [122]. The study findings demonstrated that the identity of the rate-limiting step is a function of both the basicity of the leaving group and the solution acidity.

In order to utilize Kirby's enzyme model [84] for the design of prodrugs of the following drugs: atenolol, amoxicillin and cephalexin, a mechanistic study using DFT calculation methods at B3LYP/6-31G (d,p), B3LYP/311+G (d,p) levels and hybrid GGA (MPW1k) on an intramolecular acid catalyzed hydrolysis of maleamic (4-amino-4-oxo-2-butenoic) acids (Kirby's N-alkylmaleamic acids) **34**-**42** was conducted. The calculations confirmed that the reaction involves three steps:(1) proton transfer from the carboxylic group to the adjacent amide carbonyl oxygen, (2) nucleophilic attack of the carboxylate anion onto the protonated carbonyl carbon; and (3) dissociation of the tetrahedral intermediate to provide products (Figure 6). Moreover, the calculations demonstrate that the rate-limiting step is dependent on the reaction medium. When the calculations were run in the gas phase the rate-limiting step was the tetrahedral intermediate formation, whereas when the calculations were conducted in the presence of a

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cluster of water the dissociation of the tetrahedral intermediate was the rate-limiting step. When the leaving group (methylamine) in **34**-**42** was replaced with a group having a low pKa value the rate-limiting step of the hydrolysis in water was the formation of the tetrahedral intermediate. In addition, the calculations revealed that the efficiency of the intramolecular acid-catalyzed hydrolysis by the carboxyl group is remarkably sensitive to the pattern of substitution on the carbon–carbon double bond. The rate of hydrolysis was found to be linearly correlated with the strain energy of the tetrahedral intermediate or the product. Systems having strained tetrahedral intermediates or products experience low rates and vice versa [51,52,54,91].

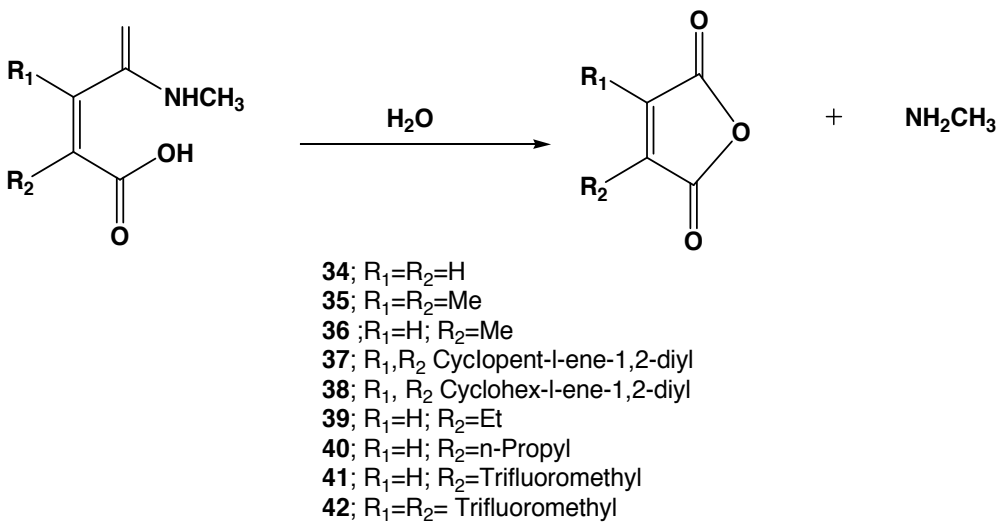

Figure 5. Acid-catalyzed hydrolysis of maleamic acids **34**-**42**.

8. Bitterless atenolol prodrugs based on Kirby's maleamic acids enzyme model

Atenolol is a relatively polar hydrophilic compound with water solubility of 26.5 mg/mL at 37 ^{0}C and a log partition coefficient (octanol/ water) of 0.23. Atenolol is a selective ß$_1$-adrenoceptor antagonist, applied in the treatment hypertension, angina, acute myocardial infarction, supraventricular tachycardia, ventricular tachycardia, and the symptoms of alcohol withdrawal. The net effect of atenolol on controlling both the heart rate and blood pressure is the reduction in myocardial work and oxygen requirement which reduces cardiovascular stress, thereby preventing arrhythmia and angina attacks.

Atenolol has a pKa of 9.6; it undergoes ionization in the stomach and intestine thus its oral bioavailability is low due to inefficient absorption through membranes.

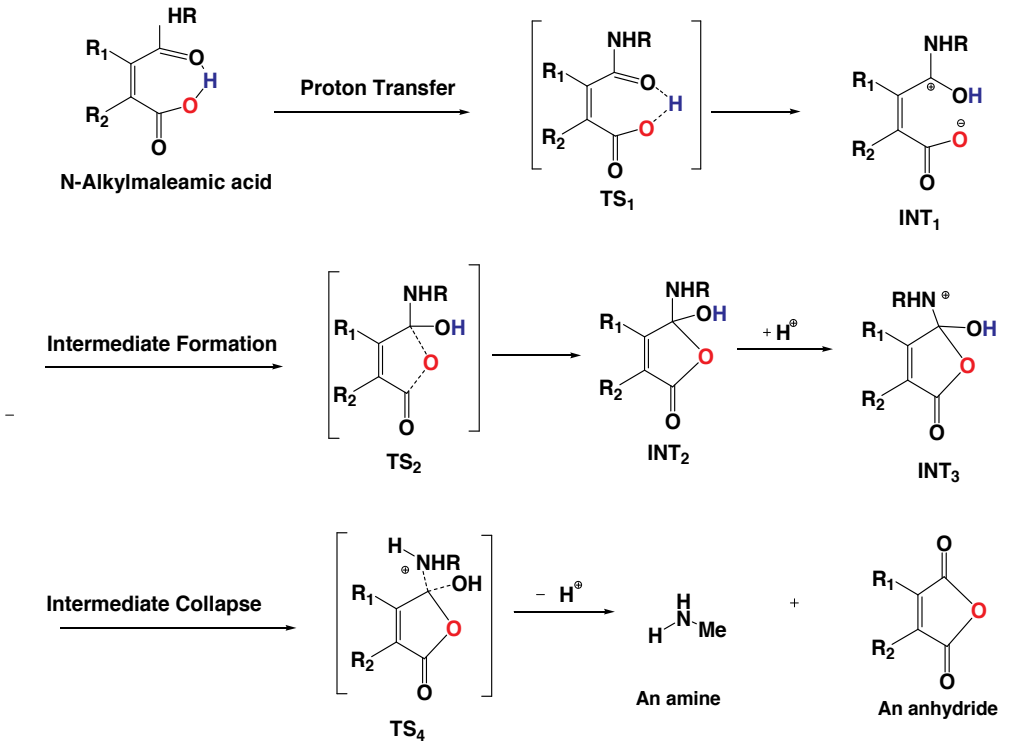

Figure 6. Proposed mechanism for the acid-catalyzed hydrolysis of maleamic acids.

The bioavailability of atenolol is 45%-55% of the given dose and is not increased by administration of the drug in a solution form [123-125].About 50% of administered atenolol is absorbed; however, most of the absorbed quantity reaches the systemic circulation. Atenolol peak blood levels are reached within two to four hours after ingestion. Differently from propranolol or metoprolol, atenolol is resistant to metabolism by the liver and the absorbed dose is eliminated by renal excretion. More than 85% of I.V. dose is excreted in urine within 24 hours compared with 50% for an oral dose. Only 6-16% is protein-bound resulting in relatively consistent plasma drug levels with about a four-fold inter-patient variation. The elimination half-life of atenolol is between 6 to 7 hours and there is no alteration of kinetic profile of a drug by chronic administration.

Atenolol is one of the most important medicines used for prevention of several types of arrhythmias in childhood, but unfortunately it is still unlicensed [126]. On the other hand, atenolol is indicated as a first-step therapy for hypertension in elderly patients, who have difficulty in swallowing and, thus, tablets and capsules are frequently avoided.

Atenolol is available as 25, 50 and 100 mg tablets for oral administration. However, most of these medicines are not formulated for easy or accurate administration to children for the migraine indication or in elderly patients who may have a difficulty swallowing tablets. Attempts to prepare a liquid formulation was challenging because atenolol is unstable in solutions. Studies showed that the degradation rate of atenolol is dependent on the temperature, indicating higher stability at 4 ºC. Atenolol syrup is stable only for 9 days. Furthermore, oral doses of atenolol are incompletely absorbed (range 46-62%), even when formulated as a solution. Furthermore, atenolol bitterness is considered as a great challenge to health sector when used among children and geriatrics [125]. The main problem in oral administration of bitter drugs such as atenolol is incompliance by the patients [1] and this can be overcome by masking the bitterness of a drug either by decreasing its oral solubility on ingestion or eliminating the interaction of drug particles to taste buds [2].Thus the development of bitterless and more lipophilic prodrug that is stable in aqueous medium is a significant challenge. Improvement of atenolol pharmacokinetic absorption properties and hence its effectiveness may increase the absorption of the drug *via* a variety of administration routes. The aims of the study described in this section were: (1) design of atenolol prodrugs that can be (i) formulated in aqueous solutions and be stable over a long period of time, (ii) bitterless compounds having the capability to convert in physiological environment to the parent active drug, atenolol, in a controlled manner and (2) synthesis, characterization and *in vitro* kinetic study of the conversion of the designed prodrugs to their parent drug in different pHs (physiological media).

The proposed atenolol prodrugs that were designed based on the acid-catalyzed hydrolysis reactions of N-alkyl maleamic acids **34-42** (Figure 5)are depicted in Figure 7.

As shown in Figure 7, the only difference exists between the proposed atenolol prodrugs and their parent drug is that the amine group of atenolol was replaced with an amide moiety. Replacing the free amine in atenolol with an amide is expected to increase the stability of the prodrug thus formed due to general chemical stability for tertiary alcohols over amine alcohols. In addition, recent stability studies on atenolol esters have demonstrated that the esters were more stable than their corresponding alcohol, atenolol, when formulating in aqueous solutions. Furthermore, kinetic study on atenolol and propranolol demonstrated that increasing the lipophilicity of the drug leads to an increase in the stability of its aqueous solutions. Based on that, it is expected that atenolol prodrugs shown in Figure 7 will have the potential to be more resistant to heat or/oxidation when formulated in aqueous solutions [128-131]. Atenolol's bitter-taste can be masked by using the prodrug chemical approach. For example, paracetamol (**30**), a widely used pain killer found in the urine of patients who had taken phenacetin has a very unpleasant bitter taste. Phenacetin (**31**), on the other hand, lacks or has very slight bitter taste. The difference in the structural features of both drugs is only the group in the *para* position of the benzene ring. While in the case of paracetamol the group is hydroxyl, in phenacetin it is ethoxy. On the other hand, acetanilide (**32**)is a bitterless compound with a chemical structure similar to that of paracetamol and phenacetin but lacks the group in the *para* position of the benzene ring. These facts suggest that the presence of the hydroxyl group on the *para* position of the benzene ring is the major contributor for the bitterness of paracetamol. It is likely that paracetamol bitterness is a result of interactions *via* hydrogen bonding

of the phenolic group in paracetamol with the bitter taste receptors. Similarly, it is expected that blocking the amine group in atenolol with a suitable linker might inhibit the hydrogen bonding between the amine group in atenolol and its bitter taste receptors and hence masking the drug's bitterness [132].

The proposed atenolol prodrugs, atenolol **ProD 1** and atenolol **ProD 2**, have a hydroxyl and carboxylic acid groups (hydrophilic moiety) and the rest of the prodrug molecule is a lipophilic moiety (Figure 7), where the combination of both groups ensures a moderate hydrophilic lipophilic balance (HLB).

It is worth noting that the HLB value of atenolol prodrug will be largely determined on the pH of the physiological environment by which the prodrug is exposed to. For example, in the stomach pH, the atenolol prodrugs, **ProD 1** and **ProD 2,** will exist in the free carboxylic acid form whereas in the blood circulation the carboxylate form will be dominant. It was planned that atenolol **ProD 1**-**ProD 2** (Figure 7) will be formulated as sodium salts since the carboxylate form is expected to be quite stable in neutral aqueous medium. However, upon dissolution in the stomach (pH less than 3) the proposed prodrugs will exist mainly as a carboxylic acid form thus enabling the acid-catalyzed hydrolysis to commence.

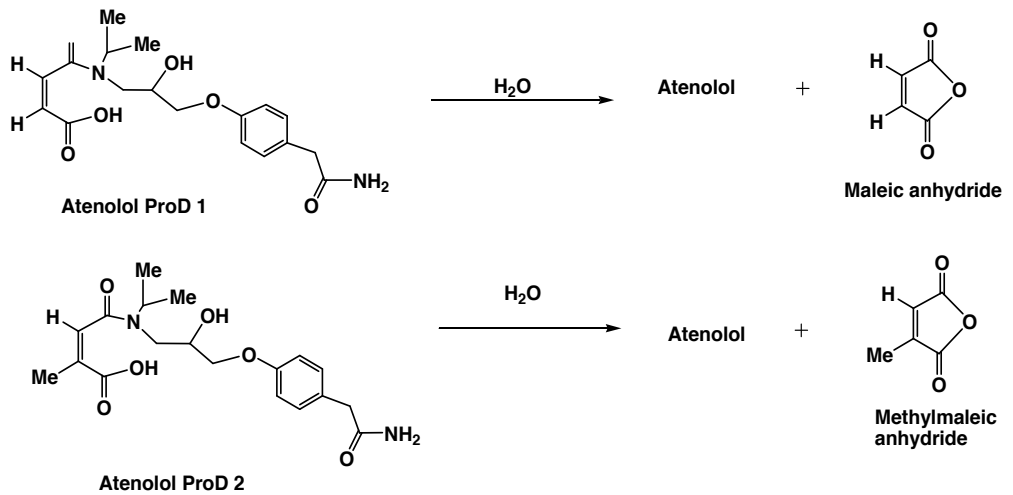

Figure 7. Acid-catalyzed hydrolysis for atemolol ProD 1 and atenolol ProD 2.

9. Calculation of the $t_{1/2}$ values for the cleavage reactions of atenolol prodrugs ProD 1-2

The effective molarity (EM) parameter is a commonly tool used to predict the efficiency of intramolecular reactions when bringing two functional groups such as an electrophile and a nucleophile in a close proximity. Intramolecularity is usually measured by the effective

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molarity parameter. The effective molarity is defined as the rate ratio (k_{intra}/k_{inter}) for corresponding intramolecular and intermolecular processes driven by identical mechanisms. Ring size, solvent and reaction type are the major factors affecting the EM value. Ring-closing reactions *via* intramolecular nucleophilic addition are much more efficient than intramolecular proton transfer reactions. EM values in the order of 10^9-10^{13} M were determined for intramolecular processes occurring through nucleophilic addition. Whereas for proton transfer processes values of less than 10 M were measured for proton transfer processes until recently where values of 10^{10} was documented by Kirby on the hydrolysis of some enzyme models [60,78-84].

For obtaining the EM values for processes **34**-**42** and atenolol **ProD1**-**2** the kinetic and thermodynamic parameters for their corresponding intermolecular process, **Inter** (Figure 8) were calculated.

Using equations 1-4, equation 5 was derived, and describes the EM term as a function of the difference in the activation energies of the intra-and the corresponding inter-molecular processes. The calculated EM values for processes **34**-**42** and **ProD 1**-**2** were calculated using equation 5.

$$EM = k_{intra}/k_{inter} \quad (1)$$

$$\Delta G^{\ddagger}_{inter} = -RT \ln k_{inter} \quad (2)$$

$$\Delta G^{\ddagger}_{intra} = -RT \ln k_{intra} \quad (3)$$

$$\Delta G^{\ddagger}_{intra} - \Delta G^{\ddagger}_{inter} = -RT \ln k_{intra}/k_{inter} \quad (4)$$

$$\ln EM = -\left(\Delta G^{\ddagger}_{intra} - \Delta G^{\ddagger}_{inter}\right)/RT \quad (5)$$

Where T is the temperature in Kelvin and R is the gas constant.

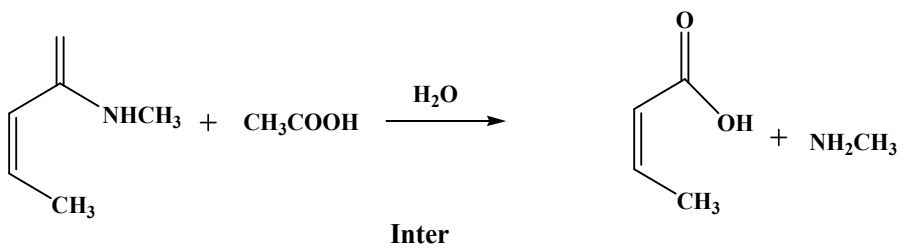

Figure 8. Acid catalyzed hydrolysis for process **Inter**.

The calculated EM values from eq. 5 for processes**34**-**38** were correlated with the corresponding EM values [101] (Figure 9a). Good correlation with a correlation coefficient of r=was obtained. The correlation results demonstrate that processes **35** and **37**werethe most efficient among **34**-**38**, whereas process **4** was the least. The discrepancy in the rates of processes **35** and **38**on one hand and process **37** on the other hand is might be attributed to strain effects.

In addition, for further support to the credibility of our DFT calculations the calculated free activation energies ($\Delta G_{BW}^{\ddagger}$) were correlated with the corresponding experimental free activation energies (Exp $\Delta G^{\ddagger}$). Good correlation was obtained with R value of 0.96 (Figure 9b).

Utilizing eq. 6 obtained from the correlation of log k_{rel}vs. $\Delta G^{\ddagger}$ and the experimental $t_{1/2}$ value measured for process **2** ($t_{1/2}$=1 second) [103], the $t_{1/2}$ values for atenolol **ProD 1** and atenolol **ProD 2** at pH 2 were calculated and their values were65.3 hours and 11.8 minutes, respectively.

$$log\ k_{rel} = -\ 0.44\ \Delta G^{\ddagger} + 13.53 \tag{6}$$

10. In vitro intraconversion of atenolol ProD 1 to the parent drug atenolol

Kinetics of the acid-catalyzed hydrolysis for atenolol **ProD 1** was carried out in an aqueous buffer in a similar manner to that done by Kirby on N-alkylmaleamic acids **34**-**38**. This is in order to examine whether atenolol prodrug is hydrolyzed in aqueous medium and to what extent, suggesting its fate in the system. Acid-catalyzed hydrolysis of atenolol **ProD 1** was investigated in four different aqueous media: 1 N HCl and buffers pH 2, pH 5 and pH 7.4. Under the experimental conditions, atenolol **ProD 1** was hydrolyzed to release the parent drug, atenolol, (Figure 10) as was evident by HPLC measurements. At constant pH and temperature, the reaction displayed strict first order kinetics as the k_{obs} was fairly constant and a straight line was obtained on plotting log concentration of residual atenolol prodrug verses time. The rate constant (k_{obs}) and the corresponding half-lives ($t_{1/2}$) for atenolol prodrug **ProD 1** in the different media were calculated from the linear regression equation correlating the log concentration of the residual prodrug verses time. The kinetic data, k_{obs} and $t_{1/2}$ values, are listed in Table 1. 1N HCl, pH 2 and pH 5 were selected to examine the intraconversion of atenolol **ProD 1**in pH as of stomach, because the mean fasting stomach pH of adult is approximately 1-2 and increases up to 5 following ingestion of food. In addition, buffer pH 5 mimics the beginning of the small intestine environment. The medium at pH 7.4 was selected to examine the intraconversion of the tested prodrug in the blood circulation system. Acid-catalyzed hydrolysis of atenolol **ProD 1** was found to be higher in 1N HCl than at pH 2 and 5 (Figure 10). At 1N HCl atenolol **ProD 1** was intraconverted to release the parent drug in 2.53 hour. On the other hand, at pH 7.4, the prodrug was entirely stable and no release of the parent drug was observed. Since the pK_a of the carboxylic group of atenolol **ProD1** is in the range of 3-4, it is expected at pH 5 the anionic form of the prodrug will be dominant and the percentage of the free acid form that expected to undergo hydrolysis will be relatively low. At 1N HCl and pH 2 most of the prodrug will exist as the free acid form, whereas at pH 7.4 most of the prodrug will be in the anionic form. Thus, the difference in rates at the different pH buffers.

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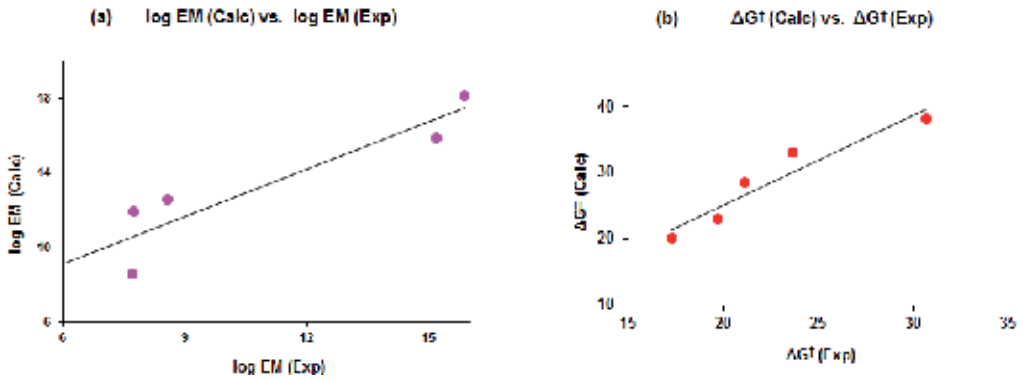

Figure 9. (a) log calculated effective molarity vs. experimental effective molarity for processes **34-38**. (b) DFT calculated activation energy (kcal/mol) vs. experimental activation energy (kcal/mol) for processes **34-38**.

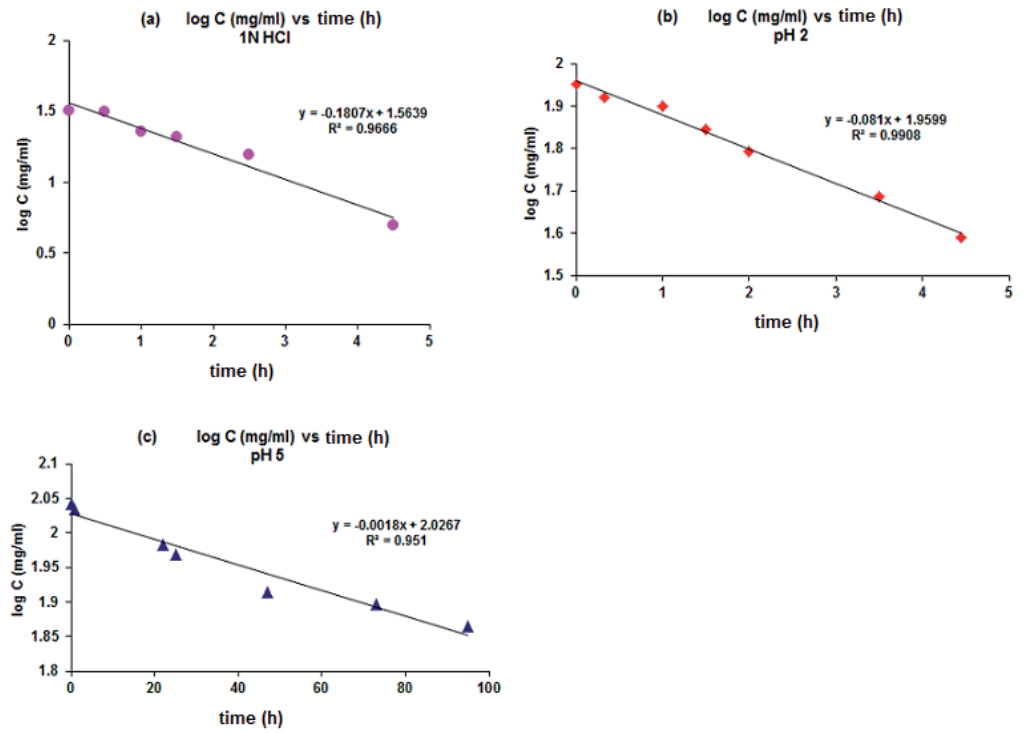

Figure 10. First order hydrolysis plot of atenolol **ProD 1** in (a) 1N HCl, (b) buffer pH 2 and (c) buffer pH 5.

$t_{1/2}$ (h)	k_{obs} (h^{-1})	Medium
2.53	4.95×10^{-4}	1 N HCl
3.82	2.22×10^{-4}	Buffer pH 2
133	2.75×10^{-6}	Buffer pH 5
-----	No Reaction	Buffer pH 7.4

In 1N HCl and at pH 2, 5 and 7.4

Table 1. The observed k value and $t_{1/2}$ of atenolol **ProD 1**

11. Bitterless amoxicillin and cephalexin prodrugs based on Kirby's maleamic acids enzyme model

Most of the antibacterial agents that are commonly used suffer unpleasant taste and a respected number of them are characterized with bitter taste. For example, amoxicillin, cephalexin and cefuroxime axetil have an extremely unpleasant and bitter taste which is difficult to mask. This is a particular problem in geriatric patients who cannot swallow whole tablets or when small doses are required. Even the antibacterial suspension is difficult for pediatrics to administer due to its better and unpleasant taste [133-139].

It is widely assumed that the extremely bitter and unpleasant taste of these antibacterial drugs is due to a formation of intermolecular force/s between the drug and the active site of the bitter taste receptor/s. The intermolecular bond/s is/are most likely due to formation either *via* hydrogen bond or ionic bond of the amido (in cefuroxime)or amine (in amoxicillin and cephalexin) group to the active site of the bitter taste receptors.

Antimicrobial agents are classified according to their specific mode of action against bacterial cell. By which these agents may interfere with cell wall synthesis, inhibit protein synthesis, interfere with nucleic acid synthesis or inhibit a metabolic pathway. They have a broad spectrum of activity against both gram-positive and gram-negative bacteria. Among these agents, β-lactams – penicillins, cephalosporins, carbapenems and monobactams, by which represent 60% of all antimicrobial use by weight. They are preferred because of their efficacy, safety, and because their activity can be extended or restored by chemical manipulation. Inevitably, however, their usage has been restricted because of their bacterial resistance.

11.1. Amoxicillin

Amoxicillin is an oral semi-synthetic penicillin, moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms by which it is susceptible to the action of the β-lactamases. Amoxicillin has a bactericidal action and acts against both Gram positive and Gram-negative microorganisms by inhibiting the biosynthesis and repair of the bacterial mucopeptide wall. It is usually the drug of choice within its class because it is well absorbed following oral administration. Amoxicillin presents some outstanding advantages in comparison with other amino-penicillins, such as: a better absorption

from the intestinal tract, better capacity for reaching effective concentrations at the sites of action and a more rapid capacity for penetrating the cellular wall of Gram-negative microorganisms. Amino-penicillins are frequently prescribed agents for the oral treatment of lower respiratory tract infections and are generally highly effective against S. pneumonia and non-β-lactamase-producing H. influenza. Amoxicillin is mostly common antibiotics prescribed for children. It has high absorption after oral administration which is not altered and affected by the presence of food. Amoxicillin dose reaches C_{max} about 2 hours after administration and is quickly distributed and eliminated by excretion in urine (about 60%-75%). The antibacterial effect of amoxicillin is extended by the presence of a benzyl ring in the side chain. Because amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a broad spectrum of β-lactam antibiotics, such as penicillin, for this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor. This increases effectiveness by reducing its susceptibility to β-lactamase resistance. Amoxicillin has two ionizable groups in the physiological range (the amino group in α-position to the amide carbonyl group and the carboxyl group). Amoxicillin has a good pharmacokinetics profile with bioavailability of 95% if taken orally, its half-life is 61.3 minutes and it is excreted by the renal and less than 30 % biotransformed in the liver [140-142].

11.2. Cephalexin

Cephalexin is a first-generation cephalosporin antibiotic, which was chosen as the model drug candidate to obtain dosage with improved stability, palatability and attractive pediatric elegance, cost effective with ease of administration. Cephalosporins are the most widely used for treatment of skin infections because of their safety profile, and their wide range of activity against both gram positive and gram negative microorganism. Cephalexin is also used for the treatment of articular infections as a rational first-line treatment for cellulitis, it is a useful alternative to penicillins hypersensitivity, and thought to be safe in a patient with penicillin allergy but caution should always be taken, that's because cephalexin and other first-generation cephalosporins are known to have a modest cross-allergy in patients with penicillin hypersensitivity. In addition, cephalexin is also effective and used in the treatment of group A β-hemolytic streptococcal throat infections. Cephalexin works by interfering with the bacteria's cell wall formation, causing it to rupture, and thus killing the bacteria. The compound is zwitterion by which it contains both a basic and an acidic group, the isoelectric point of cephalexin in water is approximately 4.5 to 5. Cephalexin has a good pharmacokinetic profile by which it is well absorbed, 80% excreted unchanged in urine within 6 hours of administration. Cephalexin's half-life is 0.5-1.2 hours and it is excreted *via* the renal. It is used for the treatment of infections including otitis media, streptococcal pharyngitis, bone and joint infections, pneumonia, cellulitis and UTI, and so it may be used to prevent bacterial endocarditis [142-145].

11.3. Cefuroxime axetil

Cefuroxime axetil is a semi-synthetic, broad-spectrum cephalosporin antibiotic for oral administration. Cefuroxime axetil is an orally active antibacterial agent though its absorption is incomplete. The range of its bioavailability is 25-52%. The axetil moiety is metabolized to acetaldehyde and acetic acid. Peak plasma concentration is reached 2-3 hours after an oral

administration. Up to 50% of cefuroxime in the circulation is bound to plasma proteins. The plasma half-life is about 70 minutes and is prolonged in patients with renal impairments and in neonates. Cefuroxime axetil is widely distributed in the body including plural fluid, sputum bone synovial fluid, and aqueous humor, but only achieves therapeutic concentration in the CSF when the meninges are inflamed. It crosses the placenta and has been detected in breast milk. Cefuroxime is excreted unchanged, by glomerular filtration and renal tubular secretion, and high concentration is achieved in urine [146].

Amoxicillin, cephalexin and cefuroxime axetil as mentioned before suffer low stability and bitter taste sensation. Several attempts were made in order to enhance their aqueous solubility and bioavailability. Among several research approaches, the prodrug approach has been widely used for an improvement of drugs delivery to their site of action by physicochemical modulation properties that affect absorption or by targeting to specific enzymes or membrane transporters [147,148]. Generally, enzymatic catalysis is required for most of prodrugs that are in clinical use in order to be converted into the parent drug. This is mostly particular for those prodrugs designed to liberate the parent drug in the blood stream following gastro-intestinal absorption. These prodrugs are typically ester derivatives of drugs containing carboxyl or hydroxyl groups which are converted into the parent drug by esterase catalyzed hydrolysis. However, a high chemical reactivity that precludes either liquid or solid formulation of the prodrug (e.g. some phenol esters) or low chemical reactivity, resulting in reduced regeneration of the parent drug due to enzymatic activation for other functional groups. Thus, non-enzymatic pathways for some prodrugs that can regenerate the parent drug, have emerged as an alternative approach by which prodrug activation is not influenced by inter-and intra-individual variability that affects the enzymatic activity. In particular, since the middle-1980s, cyclization-activated prodrugs have been capturing the attention of medicinal chemists, and reached maturity in prodrug design in the late 1990s. Activation of prodrugs *via* a cyclization pathway allows a fine tuning of the rate of drug release through the appropriate choice of the functional groups involved in ring closure and stereoelectronic constraints in the course of the cyclization step. As noticed from the history of prodrugs mostly in preclinical and clinical consideration of prodrug bioconversion, the most common that several hydrolyses-activated prodrugs of penicillins, cephalosporins, and angiotensin-converting enzyme inhibitors have less than complete absorption which was observed and highlights yet another challenge with prodrugs susceptible to esterase hydrolysis. The oral bioavailability of these mentioned types of prodrugs is typically around 50% since these prodrugs undergo premature hydrolysis during the absorption process in the enterocytes of the gastrointestinal tract [149]. Another approach which has been utilized to enhance bioavailability of antibacterial drugs is by making the corresponding prodrugs with optimum lipophilicity. Some drugs remain poorly absorbed from most of the administration routes due to their poor lipophilicity. Two approaches were utilized to enhance the bioavailability of antibacterial drugs by increasing their lipophilicity: (a) membrane/water partition coefficient of the lipophilic form of a drug has been enhanced as compared to the hydrophilic form, thus favoring passive diffusion such as in the cases of pivampicillin, bacampicillin and talamipicillin (prodrugs of ampicillin)which are more lipophilic and better absorbed than amoxicillin and are rapidly interconverted and (b) the

lipophilic prodrugs have poor solubility in gastric fluids and thus greater stability and absorption example for such approach is erythromycin esters [150].

Some ampicillin esters were prepared for improving the bioavailability of ampicillin. For example, the pivaloyloxyethyl (pivampicillin), phthalidyl (talampicillin), and ethoxycarbonyloxyethyl (bacampicillin)were found to have two fold the oral bioavailability of their parent drug, ampicillin. Complete hydrolysis of these esters was occurred in the gastrointestinal mucosa, whereas methoxymethyl ester of ampicillin was partially hydrolyzed by gut and hepatic first-pass metabolism and appears in the systemic circulation and tissues as intact ester [151-154].

12. In vitro intraconversion of amoxicillin and cephalexin prodrugs to their parent drugs

Based on our previously reported DFT calculations and on experimental data for the acid-catalyzed hydrolysis of amide acids **34**-**42** (Figure 5) [84,91], two amoxicillin and cephalexin prodrugs were proposed (Figures11 and 12, respectively). As shown in Figures11 and 12, the antibacterial prodrugs, amoxicillin **ProD 1** and cephalexin **ProD 1** molecules are composed of an amide acid promoiety, containing a carboxylic acid group (hydrophilic moiety) and the rest of the antibacterial prodrug molecule (a lipophilic moiety).

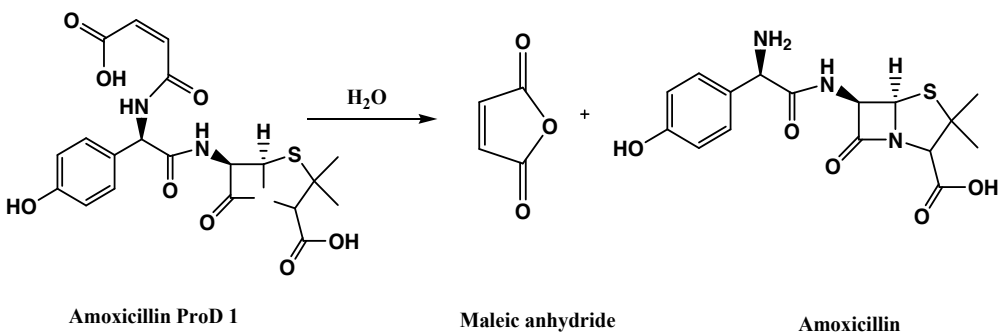

Figure 11. Acid-catalyzed hydrolysis of amoxicillin ProD 1.

The combination of both, the hydrophilic and lipophilic groups provides a prodrug entity with a potential to be with a high permeability (a moderate HLB). It should be emphasized, that the HLB value of the prodrug entity will be determined upon the pH of the target physiological environment. In the stomach where the pH is in the range 1-2, it is expected that prodrugs, amoxicillin **ProD1** and cephalexin **ProD1** will be in a free carboxylic acid form (a relatively high hydrophobicity) whereas in the blood stream circulation where the is pH 7.4 a carboxylate anion (a relatively low hydrophobicity) is expected to be predominant form. Our strategy was to prepare amoxicillin **ProD 1** and cephalexin **ProD 1** as sodium or potassium carboxylates due to their high stability in neutral aqueous medium. It should be indicated that compounds

34-**42** undergo a relatively fast hydrolysis in acidic aqueous medium whereas they are quite stable at neutral pH.

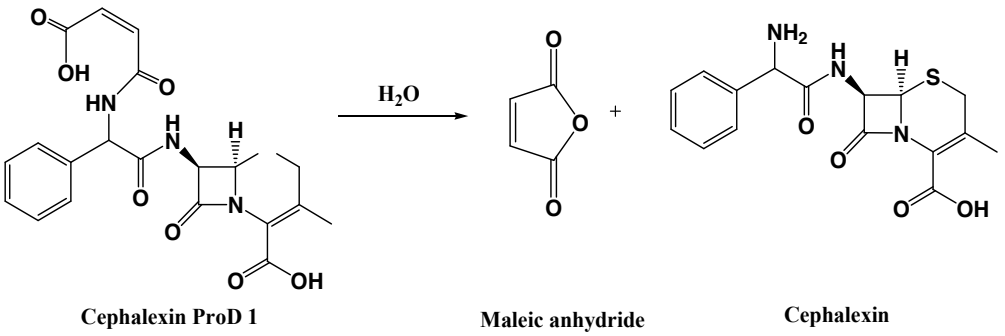

Figure 12. Acid-catalyzed hydrolysis of cephalexin ProD 1.

The hydrolysis kinetic studies for amoxicillin **ProD 1** and cephalexin **ProD 1** were carried out in aqueous buffers in the same manner to that executed by Kirby *et al.* on maleamic acids **34**-**40**. This is to investigate whether the antibacterial prodrugs undergo hydrolysis in aqueous medium and to what extent or not, suggesting the fate of the prodrugs in the system. The kinetics for the acid-catalyzed hydrolysis of the synthesized amoxicillin **ProD 1** and cephalexin **ProD 1** were carried out in four different aqueous media: 1 N HCl, buffer pH 2.5, buffer pH 5 and buffer pH 7.4. Under the experimental conditions the two antibacterial prodrugs intraconverted to release the parent drugs (Figures 13 and 14) as was determined by HPLC analysis. For both amoxicillin and cephalexin prodrugs, at constant temperature and pH the hydrolysis reaction displayed strict first order kinetics as the k_{obs} was quite constant and a straight line was obtained on plotting log concentration of residual prodrug verves time. The rate constant (k_{obs}) and the corresponding half-lives ($t_{1/2}$) for amoxicillin **ProD 1**and cephalexin **ProD 1** in the different media were calculated from the linear regression equation obtained from the correlation of log concentration of the residual prodrug verses time. The kinetic data for amoxicillin **ProD 1**and cephalexin **ProD 1** are listed in Tables 2 and 3, respectively. It is worth noting that 1N HCl, pH 2.5 and pH 5 were selected to examine the intraconversion of amoxicillin **ProD 1**and cephalexin **ProD 1** in the pH as of stomach, since the mean fasting stomach pH of adult is approximately 1-2.5. Furthermore, environment of buffer pH 5 mimics that of beginning small intestine route, whereas pH 7.4 was selected to determine the intraconversion of the tested prodrugs in blood circulation system. Acid-catalyzed hydrolysis of both, amoxicillin **ProD 1**and cephalexin **ProD 1** was found to be much higher in 1N HCl than at pH 2.5 and 5 (Figures13 and 14). At 1N HCl the t ½ values for the intraconversion of amoxicillin **ProD 1**and cephalexin **ProD 1** were about 2.5 hours. On the other hand, at pH 7.4, both prodrugs amoxicillin **ProD 1**and cephalexin **ProD 1** were quite stable and no release of the parent drugs was observed. At pH 5 the hydrolysis of both prodrugs amoxicillin **ProD 1**and cephalexin **ProD 1** was too slow. This is because the pK_a of amoxicillin **ProD 1**and

cephalexin **ProD 1** is in the range of 3-4, it is expected that at pH 5 the anionic form of the prodrug will be dominant and the percentage of the free acidic form that undergoes an acid-catalyzed hydrolysis will be relatively low. At 1N HCl and pH 2.5 most of the prodrug will exist as the free acid form and at pH 7.4 most of the prodrug will be in the anionic form. Thus, the discrepancy in rates at the different pH buffers.

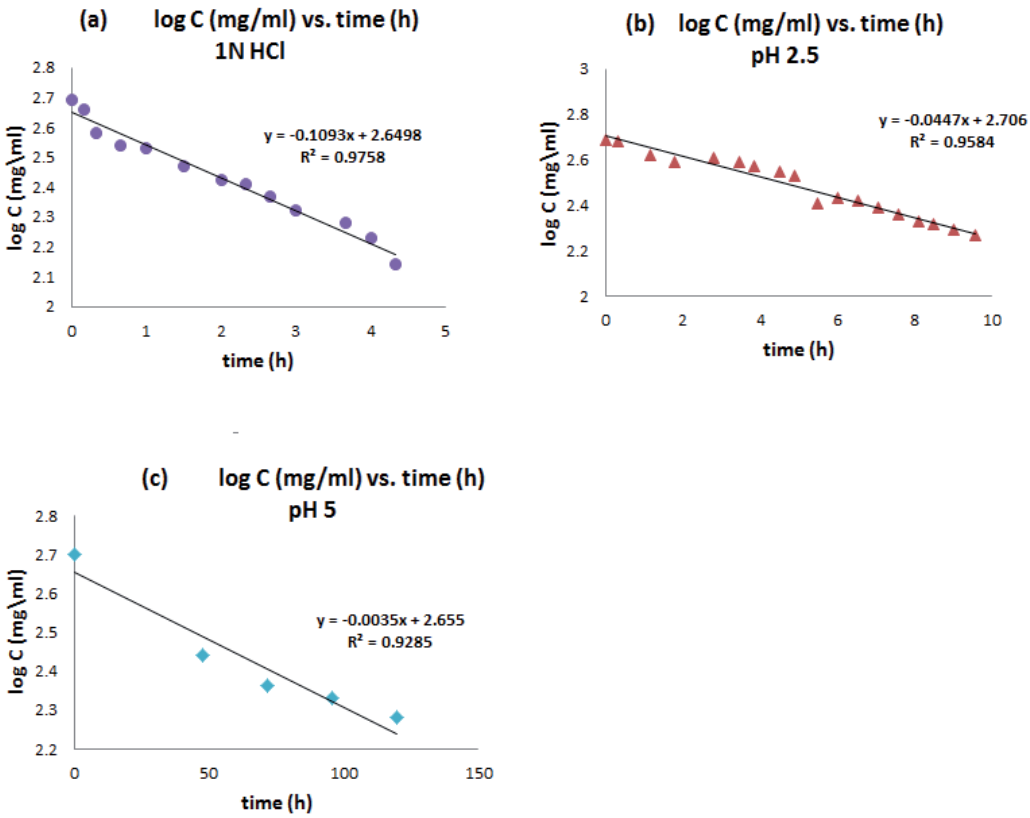

Figure 13. First order hydrolysis plot of amoxicillin **ProD 1** in (a) 1N HCl, (b) buffer pH 2.5 and (c) buffer pH 5.

$t_{1/2}$ (h)	k_{obs} (h^{-1})	Medium
2.5	2.33×10^{-4}	1 N HCl
7	9.60×10^{-5}	Buffer pH 2.5
81	7.55×10^{-6}	Buffer pH 5
----	No reaction	Buffer pH 7.4

Table 2. The observed *k* value and $t_{1/2}$ of amoxicillin **ProD 1** in 1N HCl and at pH 2, 5 and 7.4

$t_{1/2}$ (h)	k_{obs} (h^{-1})	Medium
2.4	2.41×10^{-4}	1 N HCl
14	4.17×10^{-5}	Buffer pH 2.5
---	No reaction	Buffer pH 5.5
---	No reaction	Buffer pH 7.4

in 1N HCl and at pH 2, 5 and 7.4

Table 3. The observed *k* value and $t_{1/2}$ of cephalexin **ProD 1**

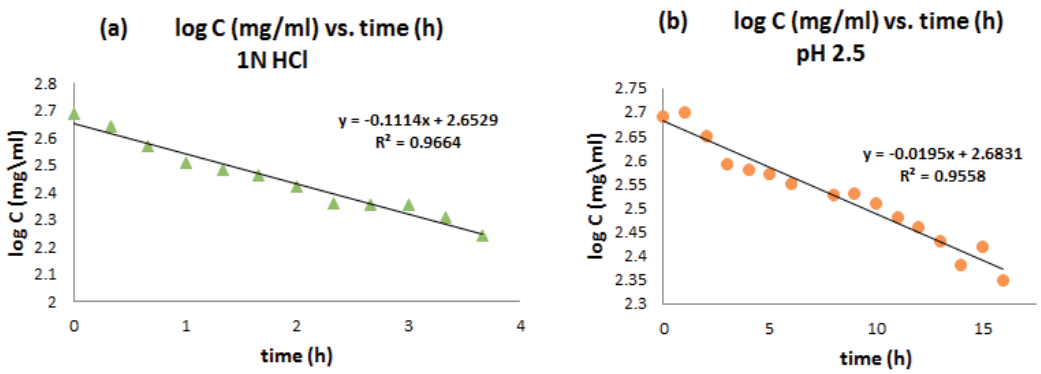

Figure 14. First order hydrolysis plot of cephalexin ProD 1 in (a) 1N HCl, (b) buffer pH 2.5 and (c) buffer pH 5.

13. Mechanistic study of Bruice's hydrolysis of di-carboxylic semi-esters 43-47 used for the design of bitterless paracetamol prodrugs

Five decades ago, Bruice and Pandit have investigated the kinetics of for the hydrolysis reaction of di-carboxylic semi-esters **43-47** depicted in Figure 15 [64,65]. Their findings revealed the relative rate (k_{rel}) for **47>46>45>44>43**. They attributed the discrepancy in rates to differences in the proximity orientation of the nucleophile to electrophile. Using the observation that alkyl substituent on succinic acid influences rotamer distributions, the ratio between the reactive gauche and the unreactive anti-conformers, they proposed that *gem*-dialkyl substitution increased the probability of the resultant rotamer adopting the more reactive conformation. Hence, for ring-closing reaction to precede, the two reacting centers, the nucleophile and electrophile, must be in the gauche conformation. In the unsubstituted reactant, the nucleophile and electrophile are almost entirely in the anti-conformation in order to minimize steric interactions [81-82]. In order to design paracetamol prodrugs, *via* linking the active drug with a di-carboxylic semi-ester linker (Bruice's enzyme model), lacking the bitterness of their parent drug, paracetamol, and have the capability to chemically and not enzymatically undergo

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hydrolysis in physiological environment we have unraveled the mechanism for the ring-closing reaction of **43-47** using DFT and molecular mechanics calculation methods [93].

Quantum molecular mechanics using DFT methods at B3LYP 6-31G (d,p) and B3LYP/311+G (d,p) levels were exploited to calculate the thermodynamic and kinetic parameters for all reactants, transition states, intermediates and products involved in the proposed mechanism for process **43-47** (Figure 16). As shown in Figure 16 the mechanism for these processes consists of two steps; (1) formation of a tetrahedral intermediate and (2) collapse of a tetrahedral intermediate to furnish a cyclic anhydride and p-bromophenolate anion.

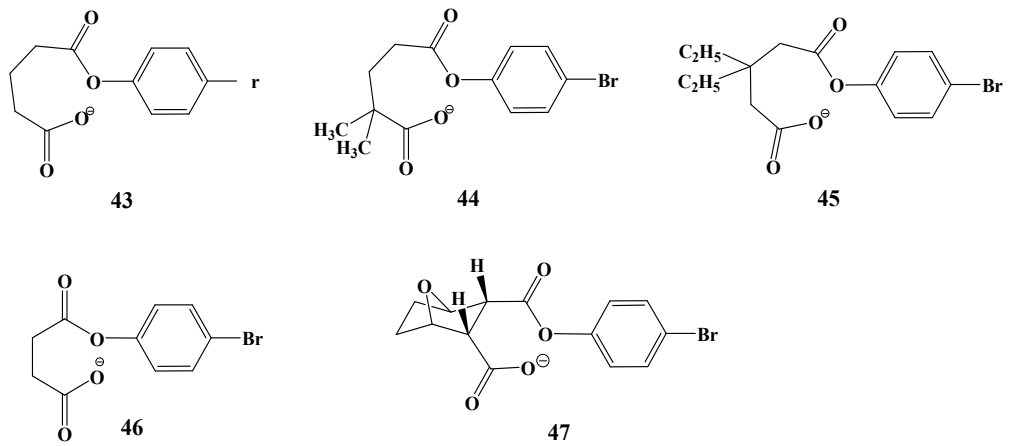

Figure 15. Hydrolysis of di-carboxylic semi-esters **43-47**.

The phenomenon of rate enhancements in several intramolecular processes was ascribed by Bruice and Menger to the importance of the proximity of the nucleophile to the electrophile of the ground state molecules [64,65,155]. Menger in his "spatiotemporal" hypothesis advocated a mathematical equation correlating activation energy to distance and based on this that, he came to the conclusion that enormous rate accelerations in reactions catalyzed by enzymes are feasible when imposing short distances between the reactive centers of the substrate and enzyme [155]. Differently from Menger, Bruice attributed the catalysis by enzymes to favorable 'near attack conformations'; systems that have a high quota of near attack conformations will have a higher intramolecular reaction rate and *vice versa*. Bruice's idea invokes a combination of distance between the two reacting centers and the angle of attack by which the nucleophile approaches the electrophile [64,65].

In contrast to the proximity orientation proposal, others proposed the high rate enhancements in intramolecular processes to steric effects (relief of the strain energy of the reactant) [156].

To test whether the acceleration in rates for processes **43-47** (Figure 15) is a result of proximity orientation or due to steric effects (difference in strain energies of the reactants), the strain energy values for the reactants and the intermediates in systems **43-47** were calculated using Allinger's MM2 method. The calculated strain energy values for **43-47** were correlated with

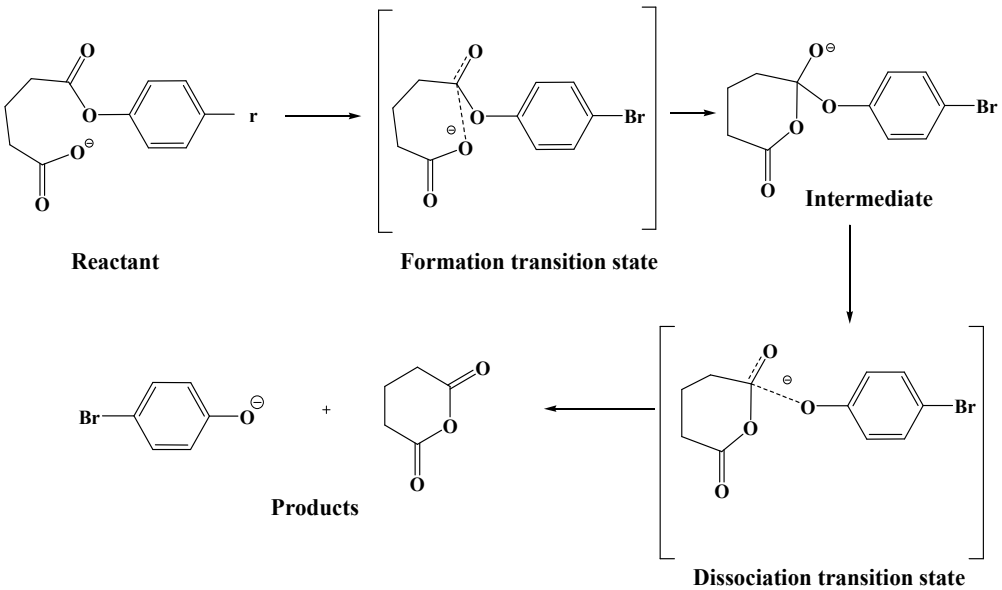

Figure 16. Proposed mechanism for the hydrolysis of di-carboxylic semi-esters **43-47**.

the corresponding experimental relative rates (log k_{rel}) [64,65]. The results demonstrated good correlation between the two parameters. On the other hand, attempts to correlate the distance between the two reactive centers and log k_{rel} failed to provide any correlation between the two parameters. This reveals that the driving force for acceleration in rates of **43-47** is driven by strain effects and not proximity orientation stemming from Bruice's near attack conformation [64,65].In addition, in accordance with Bruice and Pandit's findings [64,65] we have found that the ring-closing reactions proceed by one mechanism, by which the rate-limiting step is the tetrahedral intermediate dissociation and not its formation.

14. Paracetamol Prodrugs Based on Bruice's Enzyme Model

Paracetamol is an odorless, bitter crystalline compound used as an over the counter analgesic and anti-pyretic drug. Paracetamol is used to relief minor aches. it is used as pain killer by decreasing the synthesis of prostaglandin due to inhibiting cyclooxygenases (COX-1 and COX-2). Paracetamol is favored over aspirin as pain killer in patients have excessive gastric secretion or prolonged bleeding. It was approved to be used as fever reducer in all ages. Pharmacokinetic studies have shown that urine of patients who had taken phenacetin contained paracetamol. Later was demonstrated that paracetamol was a urinary metabolite of acetanilide. Phenacetin known historically to be one of the first non-opioid analgesics without anti-inflammatory properties lacks or has a very slight bitter taste [157,158]. Comparison of the structures of paracetamol and phenacetin shows that shows close similarity between both analgesics except of the nature of the group on the *para* position of the benzene ring. While in

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paracetamol the group is hydroxyl, in phenacetin it is ethoxy. On the other hand, acetanilide has a chemical structure similar to that of paracetamol and phenacetin but it lacks any group at the *para* position of the benzene ring. Acetanilide lacks the bitter taste characteristic for paracetamol. The comparisons of the three compounds might suggest that the presence of hydroxy group on the *para* position of the benzene ring plays a major role for paracetamol bitterness. Therefore, it is expected that masking the hydroxyl group in paracetamol with a suitable linker could inhibit the binding of paracetamol to its bitter taste receptor/s and hence masking its bitterness. It is likely that paracetamol binds to the active site of its bitter taste receptor via hydrogen bonding interactions by which its phenolic hydroxyl group is engaged. It is worth noting that linking paracetamol with Bruice's enzyme model linker *via* its phenolic hydroxyl group might hinder paracetamol bitter taste.

Based on the DFT calculations on the cyclization of Bruice's **43**-**47** (Figure 15), two paracetamol prodrugs were proposed (Figure 17). As shown in Figure 17, the paracetamol prodrugs, **ProD 1-2**,have a carboxylic acid group as a hydrophilic moiety and the rest of the prodrug, acetanilide, as a lipophilic moiety, where the combination of both groups provides a moderate HLB. It should be noted that the HLB value will be determined upon the physiologic environment by which the prodrug is dissolved. For example, in the stomach, the paracetamol prodrugs will primarily exist in the carboxylic acid form whereas in the blood circulation the carboxylate anion form will be predominant. Since Bruice's cyclization reaction occurs in basic medium paracetamol **ProD 1-2** were obtained as carboxylic free acid form, since this form is expected to be stable in acidic medium such as the stomach.

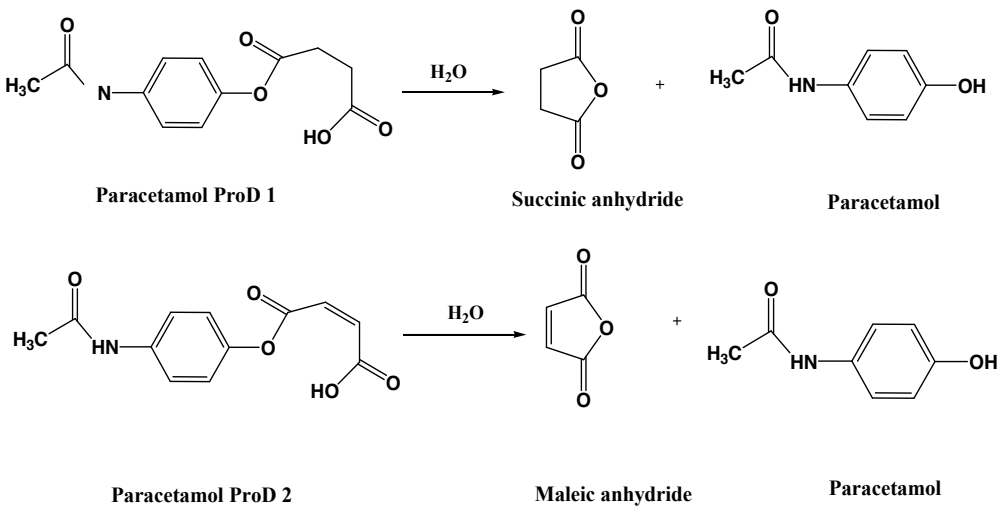

Figure 17. Hydrolysis of paracetamol ProD 1 and paracetamol Prod 2.

15. In vitro intraconverion of Paracetamol ProD 1 to the parent drug paracetamol

The hydrolysis of paracetamol **ProD 1** was studied in four different media; 1N HCl, buffer pH 3, buffer pH 6.6 and buffer pH 7.4. The prodrug hydrolysis was monitored using HPLC analysis. At constant pH and temperature the release of paracetamol from its prodrug was followed and showed a first order kinetics. k_{obs} **(h^{-1})** and $t_{1/2}$ values for the intraconversion of paracetamol **ProD 1** was calculated from regression equation obtained from plotting log concentration of residual of paracetamol **ProD 1** vs. time. The kinetics results in the different media are summarized in Table 4 and Figure 18.

Medium	k_{obs} (h^{-1})	$t_{½}$ (h)
1N HCl	No reaction	No reaction
Buffer pH 3	6.3 x 10^{-5}	3
Buffer pH 7.4	6.1 x 10^{-4}	0.3

Table 4. The observed *k* value and $t_{1/2}$ of paracetamol **ProD 1** In 1N HCl and buffers pH 3and 7.4.

As shown in Table 4 the hydrolysis rate of paracetamol **ProD 1** at pH 7.4 was the fastest among all media, followed by pH 6.6 medium. In 1N HCl no conversion of the prodrug to the parent dug was observed.

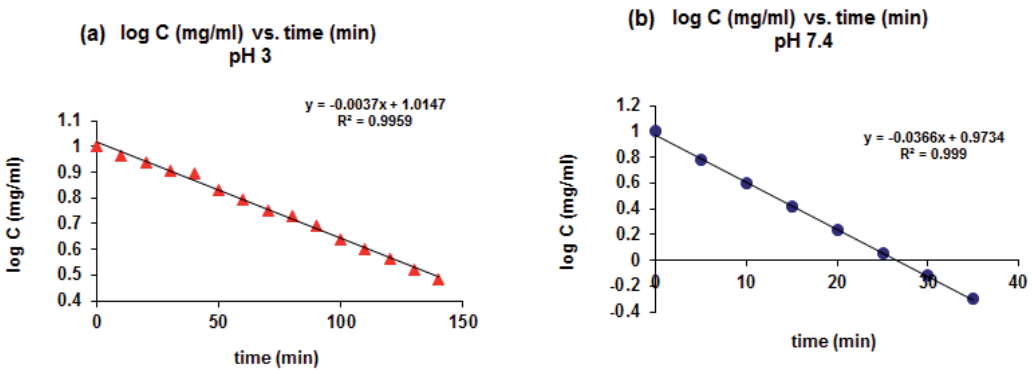

Figure 18. First order hydrolysis plot of paracetamol **ProD 1** in (a) buffer pH 3 and (b) buffer pH 7.4.

At pH 7.4 and 6.6 paracetamol **ProD 1** is mainly exists as the carboxylate anion form which is expected to undergo fast hydrolysis according to Bruice's mechanism shown in Figure 16. At pH 3, the prodrug exists in both form, the carboxylate anion and the carboxylic free acid forms since the pKa of the prodrug is about 3. In 1N HCl, the prodrug is entirely exists as the

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carboxylic free acid form and since only the carboxylate anion form undergoes Bruice's cyclization the hydrolysis rate in 1N HCl is almost negligible or zero.

16. Conclusions and future directions

The quantum mechanics (QM) calculations in different methods revealed that the acid-catalyzed hydrolysis efficiency of processes **34**-**42**, atenolol **ProD 1**-**ProD 2**,amoxicillin **ProD1** and cephalexin **ProD 1** is significantly sensitive to the pattern of substitution on the carbon-carbon double bond and nature of the amine leaving group. The linear correlation found between the reaction rate and strain energy difference between the intermediate and the reactant (E_s INT-GM) supports the notion that the reaction is governed by strain effects. Furthermore, the linear correlation of the calculated DFT and experimental EM values reinforce the credibility of using DFT methods for energy and rate predictions for the kind of processes reported in this section.

Comparisons of the calculated DFT properties for processes **34**-**40**andatenolol prodrugs **ProD1**-**ProD2** with the calculated DFT properties for the acid-catalyzed hydrolysis of acyclovir prodrugs and cefuroxime (Figure 19) demonstrate that while for processes **34**-**40** and atenolol prodrugs **ProD 1**-**ProD 2,** the rate-limiting step was the collapse of the tetrahedral intermediate in the processes of cefuroxime prodrugs and acyclovir prodrugs the rate-limiting step was the tetrahedral intermediate formation. This is might attributed to the nature of the amine leaving group involved in the tetrahedral intermediate collapse step.

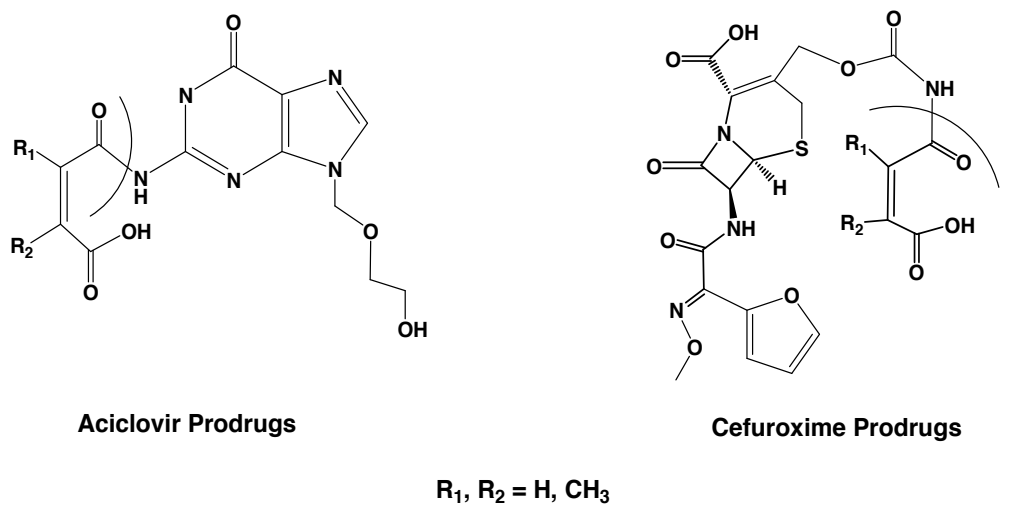

Figure 19. Chemical structures for acyclovir and cefuroxime prodrugs.

Comparison of the calculated $t_{1/2}$ value (63.2 hours) for atenolol **ProD 1** to the experimental value (3.82 hours) indicates that while the B3LYP/6-31G (d,p) value is overestimated (about 17

times larger than the experimental) the one obtained by mpwpw91/6-31+G(d,p) was much more closer (6.3 hours). The discrepancy between the calculated B3LYP/6-31G (d,p) and experimental values might be attributed to (i) B3LYP/6-31 G(d,p) is a DFT method without dispersion corrections and (ii) PCM solvation model (calculations in presence of solvent) is not capable of handling calculations in acidic aqueous solvent.

The experimental $t_{1/2}$ value for atenolol **ProD 1** at pH 5 was 133 hours and at pH 7.4 no hydrolysis was observed. The lack of the hydrolysis at the latter pH might be due to the fact that at this pH atenolol **ProD 1** exists mainly in the ionized form (pK_a about 3-4). As mentioned before the free acid form is a mandatory requirement for the acid-catalyzed hydrolysis to proceed.

In a similar manner to that observed in the intraconversion of atenolol **ProD 1, the** acid-catalyzed hydrolysis of both, amoxicillin **ProD 1**and cephalexin **ProD 1** was much faster in 1N HCl than in pH 2.5 and 5 (Figures13 and 14). At 1N HCl the t ½ values for the intraconversion of amoxicillin **ProD 1**and cephalexin **ProD 1**was in both cases about2.5hours. On the other hand, in pH 7.4, both amoxicillin **ProD 1**and cephalexin **ProD 1** were entirely stable and no intraconversion to the parent drugs was detected. The salient points emerged from our study on Bruice's system are as follows: (i) the cyclization rate of Bruice's system was found to be dependent on the difference in the strain energies of the intermediate and reactant, and no relationship was found between the reaction rate and the distance between the nucleophile and the electrophile. (ii) The reactions of strained di-carboxylic semi-esters are more efficient than the less strained ones, and the reactivity extent was linearly correlated with the strain energy difference between the intermediate and reactant. (iii) The activation energy required to give a stable transition state for a strained di-carboxylic semi-ester is less than that for the unstrained semi-ester, since the conformational change from the reactant to the transition state in the former is smaller, and (iv) based on the linearity found between the relative rate, the activation energy and the difference in strain energies of the intermediate and reactant for Bruice's di-carboxylic semi-esters we have proposed two paracetamol prodrugs, which were synthesized and their in vitro kinetics was studied. Future strategy to achieve more efficient atenolol prodrugs capable of increasing the liquid formulation stability, eliminating atenolol bitterness and releasing the parent drug in a programmable manner is synthesis of atenolol prodrugs having *pKa* around 6 (intestine pH). At the pH of the intestine the planned prodrugs will exist mainly in the acidic form which has the capability to undergo an acid-catalyzed hydrolysis to provide the active drug, atenolol.

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Author details

Rafik Karaman[1,2*]

Address all correspondence to: dr_karaman@yahoo.com

1 Bioorganic Chemistry Department, Faculty of Pharmacy, Al-Quds University, Jerusalem, Palestine

2 Department of Science, University of Basilicata, Potenza, Italy

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Chapter 13

Organic Nanotubes: Promising Vehicles for Drug Delivery

Utpal Bhadra, Manika Pal Bhadra,
Jagannadh Bulusu and J.S. Yadav

Additional information is available at the end of the chapter

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1. Introduction

1.1. Nano particles and drug delivery

Nanostructured materials, due to the 'size effect' [1] and flexible surface modifications, demonstrate unique properties. The surface chemistry and quantum effects of nanomaterials give rise to novel electronic, optical and magnetic properties and thus have become attractive for their applications *in vivo* imaging and diagnostics, regenerative medicine, infection biology, neuroelectronics and biosensors [2, 3]. Functional organic molecules self-assemble into well-defined nanostructures have become a fast developing field due to its similarity with the natural biological processes but also to produce a new range of materials with many application possibilities. Studies have shown that the self-assembling molecular scaffolds like peptides, lipids and other organic scaffolds, on the basis of different non-covalent interactions (π-π interaction, van der Waals, hydrogen bonding, hydrophilic/hydrophobic and electrostatic) can spontaneously associate to form nanomaterials with various morphologies, like nanotubes, nanospheres, nanofibrils, nanorods, nanotapes, and nanovesicles under different conditions [4, 5, 6]. These nanomaterials made from simple building blocks, can be tuned to present interesting physicochemical properties owing to their biocompatibility, capability of specific molecular recognition, easy availability, and functional flexibility. They offer several advantages as mass delivery agents due to their size, which allows them to cross biological barriers and their chemical versatility makes them suitable for loading a wide range of substances enabling multifunctionality [7].

Several biomaterials have been studied that show a greater promise in nanomedicine including quantum dots [8, 9], carbon nanotubes, coupling of quantum dots and carbon nanotubes [10]

gold nanoparticles [11], silica nanoparticles [12], organic polymers [13], bi-or multilayer liposomes (14), magnetic and magnetofluorescent nanoparticles [15], silica nanoparticles [16]. These studies have highlighted the importance of the role of nanomaterial size, shape, material composition, surface chemistry; the choice of the cell type for the study; the effect of nanomaterial-cell interactions, the fate of the nanomedicines and the resulting cellular responses [17, 18]. But each material has its own limitation in biological systems. The complexity of interaction between nanomaterials and cellular environment, biocompatibility, their progressive accumulation in live cells, inefficient bio-degradation and other pharmacokinetic properties including cell toxicity and immunogenicity presents a variety of obstacles for choosing the specific nanomaterials for testing. Considerable efforts have been directed towards surface modifications [19], multivalent attachment of small molecules [20] and coating for minimizing such effects. These measures also favour *in vivo* distribution through diversified biological organs and effective tissue specific targeting. Though use of nano-materials have been successful in *in vitro* cultured cells, its *in vivo* application by repeated injections is more challenging for shelf life, potential immunogenecity, biocompatibility and other physiological hurdles. An alternate approach for tracking the micro device through complex organs is via oral ingestion followed by better absorption and systemic spreading through body fluid instead of repeated intravenous injections.

2. Advantages of nanotubes

Nanotubes, hollow cylindrical nanostructures are promising drug carriers offering many advantages over other drug delivery systems. Nanotubes, which have separated inner and outer surfaces, can be differentially functionalized either to load desired molecules inside or by suitably designing the chemical features of the outer surface allows for site-specific drug delivery. Relevant attachments include biologically active molecules, targeting sequences, intrinsic fluorescent or other imaging devices, biocompatible coatings, and others. Major focus in the development of nanotubes for biomaterial delivery relies on three important factors, chemical modification, biocompatibility and minimal damage of the harbouring environment. To date, the potential use of drug components for synthesizing the microstructure has not been realized primarily because of lack of methods for self-assembly to form a tubular structure and coupling them with tracking fluorescence markers.

3. PABA nanotubes

The core or the building block is an important component in biomaterial development. p-aminobenzoic acid (PABA) is frequently found as a structure moiety in drugs (in a database of 12111 commercial drugs, 1.5% (184 drugs) were found to contain the PABA moiety that have a wide range of therapeutic uses [21]. To minimize the problem of biocompatibility and cell toxicity, a more reliable choice is to select building blocks that can be used for developing a library of nanomaterials, which will have functional and structural diversity. We have chosen

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PABA as the molecular building block with appropriate chemical saturated or unsaturated fatty acid substitution to build a library of PABA based nanomaterials for potential use as drug delivery systems.

We have synthesized 4-N-pyridin-2-yl-benzamides from p-nitrobenzoic acid [22]. The synthesis involved amide formation with 2-aminopyridine followed by reduction of the nitro functionality utilizing a standard protocol using Pd/C under hydrogen atmosphere as the reducing agent. Subsequently, the free amine functionality present in benzamide was coupled with seven different acid chlorides (undecanoyl chloride, C=11; undec-10-enoyl chloride, C=11;1, Lauroyl chloride, C=12, Miristoyl Chloride, C=14, Palmetoyl chloride, C=16, Stearoyl chloride, C=18 and Oleoyl chloride C=18:1) to furnish the corresponding 4-alkylamido-N-pyridin-2-yl benzamides respectively hereafter referred to as C11, C11U, C12, C14, C16, C18 and C18U based on the length of the side chains and unsaturated moieties coupled during synthesis. Synthesis of the Lauroyl chlrode and stearoyl chloride is shown in Figure 1.

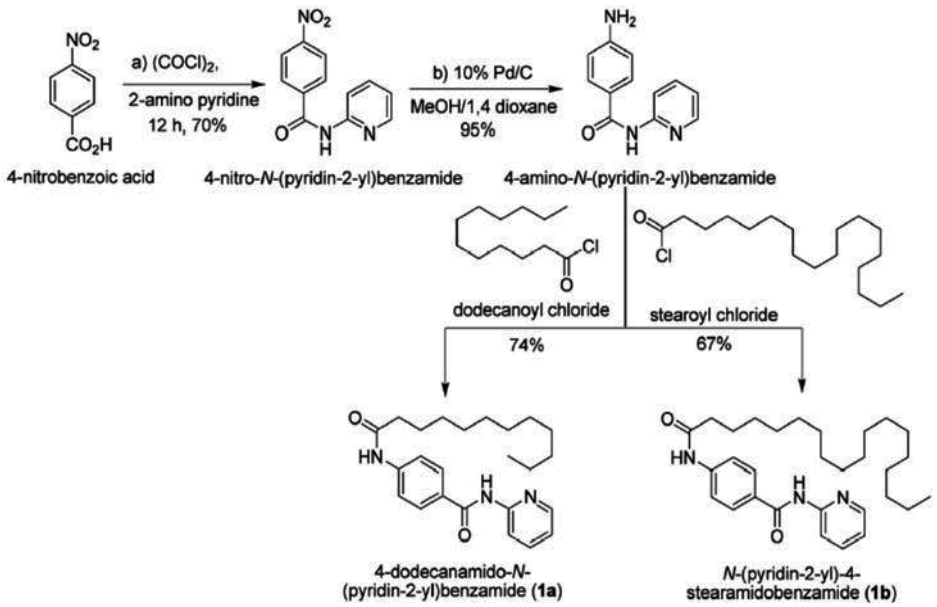

Figure 1. Synthesis of Lauroyl chlrode and stearoyl chloride

The seven alkyl benzamides (1 mg) were added to methanol (2 ml) and heated to 60 ∘C till it dissolved completely. Deionized water (2 ml) was added slowly at the same temperature to obtain a milky white solution which, upon gradually cooling to room temperature, furnished cotton-like white aggregates. Three of the nanoaggregates exhibited intrinsic fluorescence (C11, C6 and C18) and to prepare rhodamine-B embedded Benzamide nanotubes (C11U, C12,C14, C18U) compounds, rhodamine B solution (0.1 ml, 1 mg of rhodamine B in 5.0 ml of deionized water) was added prior to the addition of deionized water (2 ml) which, on cooling,

produced pink-coloured aggregates. The aggregates were isolated under centrifuged conditions (4500 rpm for 20 min) followed by overnight drying at 60 ◦C to afford 0.5 mg of the final nanomaterials. All the PABA based nanomaterials C11, C11U, C12, C14. C16, c18 and C18U studied obtained by side chain variation are shown in Figure 2.

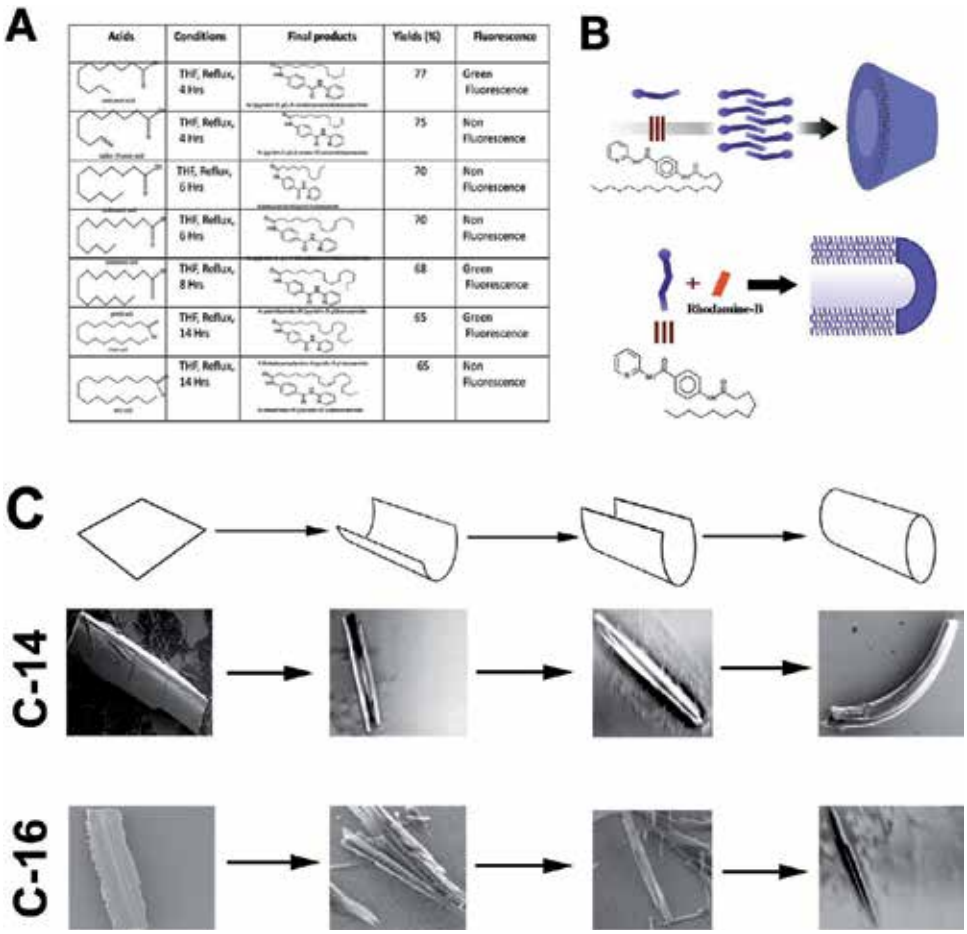

Figure 2. Design and chemical synthesis of nanomaterials. (A) chemical structure of acid side chains, final self assembled product reaction condition, percentage of yield, fluorescent dyes summarized in a table. (B) Schematic diagram showing formation of two nanoparticles (C12 and C18) was drawn (C) Routine diagram and compatible SEM images showing rollover mechanism of two nanomaterial (C-14 and C16) formation.

Prior to the study of the cellular uptake of the seven nanomaterials which use the PABA template, a study was also undertaken to observe whether the biological properties of PABA changed due to the side chain substitution and self-assembly. The biological properties of PABA in self-assembled conjugates as monitored by the growth and viability of the wild type bacterial strains (E. coli K12) in cultured media in the presence of PABA or PABA containing nanostructures. A similar level of bacterial growth in culture media containing PABA or PABA

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nanomaterials revealed that modifications and subsequent formation of PABA based biomaterials did not result in change of properties.

Scanning electron microscopy (SEM), transmission electronic microscopy (TEM) and atomic force microscopy (AFM) and dynamic light scattering (DLS) were used to investigate the structural and morphological properties of the self-assembled nanomaterials. The TEM and SEM characterization suggest that the nanomaterials self-assembled using saturated acid side chains mainly form tubular structures with a hollow space inside, while those self-assembled from unsaturated side chains produced cube shaped particles (Figure 3). The mechanism of nanotubes formed by acid chlorides with saturated side chains is believed to that initially, they aggregate into sheets which later transform to curved structures and join to form nanotubes. This nanotube formation mechanism was also confirmed from a low-angle powder X-ray diffraction studies carried out on the of self-assembly of para-terphenylen-1,4''-ylenebis (dodecanamide) and the results have shown that self-assembly results in layered sheets or rolled up nanotubes dependent on the experimental conditions [23]. Recently a frustrated aggregate internal rearrangement (FAIR) mechanism was also proposed for organic nanotube formation [24]. The authors suggested from density functional calculations that self-assembly takes place by forming sheet like structures driven by nonspecific and nondirectional intermolecular interactions with weak intermolecular H-bonds providing additional stability to the structure. Instead of the fully formed H-bonded structure, the partially formed hydrogen bonded layers to avoid kinetic energy traps transform to curved structures. Mechanism by which the self-assembly of C11U and C18U results in the formation of cubic structures is also not known. It can be surmised however that like the tubular structure formation, the cubic structures also form by unidirectional growth of the sheets by the chemical subunits, followed by the folding of sheets into cubes by penalties that are compensated by favoured binding energies [25].

To ascertain the size, a Dynamic Light-Scattering (DLS) study was carried out using different nanoparticles produced by side chain variation. In all cases, freshly prepared nanomaterials were mostly uniform in size with very few submicron sized aggregates, while materials examined after prolonged storage (after 3 days) contains more micron sized aggregates. DLS studies from fresh preparations estimated an average size in the range of 100 to 200 nm but prolonged storage leads to the formation of submicron-sized structures. The average height of each nanoparticle as measured by 3 D reconstituted AFM images is 3-5 nm.

4. Characterisation of nanoparticles

Laser confocal microscopic images showed that three nanostructures, C-11, C-16 and C-18 emitted intrinsic green fluorescence, while remaining four nanomaterials (C-11U, C-12, C-14, C-18U) do not emit any intrinsic fluorescence (Figure 3). To verify the fluorescence enhancement, induced by self-assembly nanostructure, the fluorescence emission of the monomer and the self-assembled nanoparticles were compared using Nanodrop 3300 fluoro-spectrometer. The fluorescence intensity of the nanostructures (determined by a methanol/water solution)

using blue diode option (maximum excitation 477 nm) was much stronger and found in 510 nm than that of the non-fluorescent monomer (studied in CH2Cl2, where it does not aggregate) under the same 0.3 wt % concentration.

5. Relative uptake of nanomaterials in insect and human cell lines

We have carried out a systematic and comparative study on the relative uptake and estimation of the accumulated nanomaterials of varying composition and size inside the subcellular organelles by fluorescence microscopy, confocal laser scanning microscopy, and fluorometry. Three model cell lines representing different physiological function (insect and human) were chosen for the investigation. Drosophila S2 was chosen as a model as they are phagocytic cells and have a high cotransfection rate and for the mammalian cell lines HeLa cancer cells and the nonneoplastic Human Embryonic Kidney (HEK-293) were chosen for the study. The cell lines were cultured in media containing different concentrations of the nanomaterial; 10 μg/ml, 30 μg/ml and 60 μg/ml in 0.01% DMSO. In all cases, nanomaterial containing media to a final concentration 60 μg/ml in 0.01% DMSO showed no adverse effect on cell physiology.

In general, basic cell physiology and cell surveillance do not allow easy accessibility of foreign particles inside the cells. Exhaustive efforts are being carried out for engineering smooth delivery vehicles, synthesized from biocompatible and biodegradable materials. Though use of nano-materials has been successful in in vitro cultured cells [26], in practice, its adaptability in in vivo organ tracking by repeated injections is more challenging because of its limited self-life, delivery hurdles, and compatibility to fragile cell environment and potent immunogenicity [19]. Major improvements on chemical modifications of nano-materials play a fundamental role in cell uptake and live tissue distribution [27]. The surface texture by using small molecules, side chains and other conjugates alter the biological properties of nanoparticles [20]. We therefore hypothesized that such variation could increase smooth transition to shuttle inside live cells. To date, efforts for surface modifications of organic nanostructures have been rare. It is mainly due to lack of self-assembled organic molecules and compatibility of small molecules with nanoskeleton [29. 30].

Accumulation of nanomaterials varied widely based on the side chains of PABA conjugates inside both insect (Drosophila S2) and human (HEK293, HeLa) cells. It was observed that nanoparticles, which emit intrinsic green fluorescence (C-11, C-16 and C-18) accumulate almost equally in all three cell types despite the differences in the length of carbon side chains (Figure 4). These results suggest that the tubular shape of all three nanostructures is more important than the length of the acid chains for cell entry. The accumulation increased proportionately to the concentration of incubated nanoparticles and time. Moreover, uptake of nanotubes C-12 and C-14, are more intense relative to unsaturated acid chains (C-11U and C-18U) in human cells. It is possible that PANA nanomaterials with unsaturated side chain might hinder the cellular entry. In contrast, a distinct internal cell environment of Drosophila S2 cells increases the uptake of unsaturated C-11U particles. These results demonstrated that three major factors; shape, properties associated with unsaturated side chain and cross species cell physiology are involved in the rate of cellular uptake.

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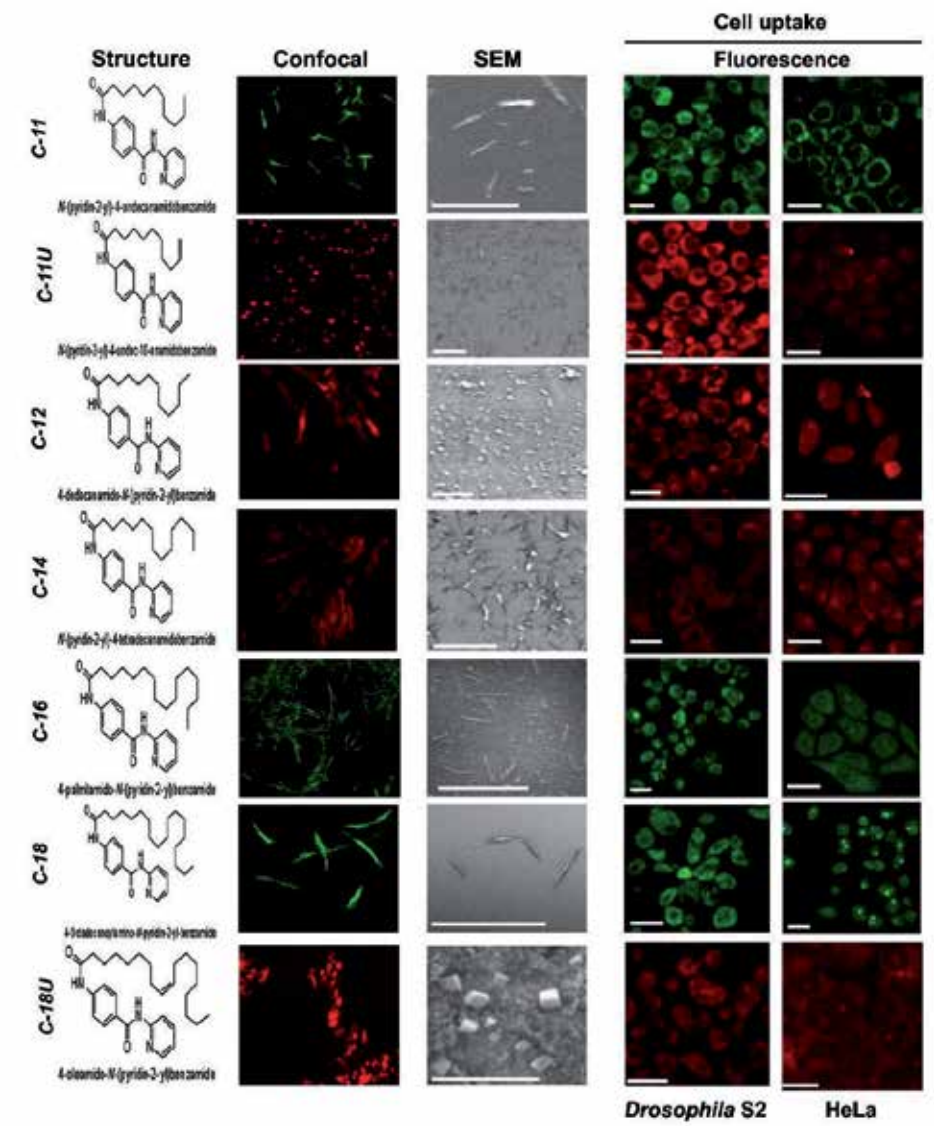

Figure 3. Physico-chemical properties and microscopic views of seven PABA anomaterials. elative Uptake of several nanomaterials in insect (Drosophila S2) and human tumour cells (HeLa) were shown. The differences in chemical structure, shape and surface texture of nanomaterials leads to a variation in cell uptake. Scale-250 nm (SEM), 50 µm (cells)

Since rhodamine was not covalently bonded with nanostructures C11U, C12, C14 and C18U, we cannot rule out the possibility that they might leach the dye from the nanostructures during cell uptake. Total fluorescence intensity in cells following exposure to the nanoparticle solutions could be due to the presence of nanoparticles through the cell, rather than correctly assigned to either a combination of free-dye and nanoparticle-bound dye, or even entirely to free dye, we incubated both the insect and human cells with rhodamine dye as well as

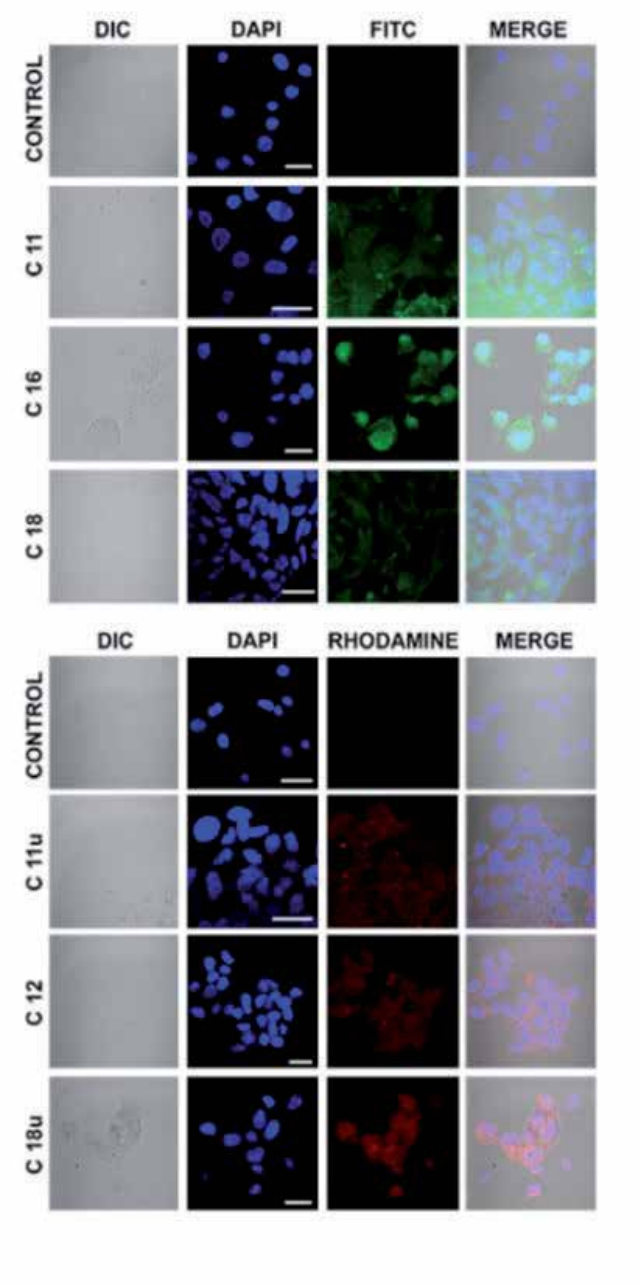

Figure 4. Biocompatibility of nanoparticles in nonneoplastic (HEK-293) Human Embryonic Kidney cells. Specificity of cellular uptake of different nanoparticles in HEK-293 cells was shown. The cells were incubated in 0.1% DMSO (control) and 60mg/ml of each nanoparticle containing culture media separately for 12 hrs prior to process. The cells were counterstained with DAPI. The DIC images and merge figures were shown in the left and right sides of the panel. Scale 40µm

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rhodamine bound nanomaterials (C-11U and C-14) separately under same experimental conditions. After equal period of incubation, cells from both conditions were processed and viewed under confocal microscope. Cells cultured with only rhodamine showed accumulation at the outer periphery with negligible amount inside, while an intense fluorescence was seen inside the cells cultured with rhodamine containing nanoparticles indicating that rhodamine dye did not leached and was entrapped or contained in the nanotubes and nanocubes. (can we make this statement If all of the dye is nanoparticle bound then the nanoparticles are localized in sub-cellular oraganelles and where as if these system contains a significant amounts of free dye then fluorescence is distributed throughout the cell across cell barriers and into the cytoplasm.)

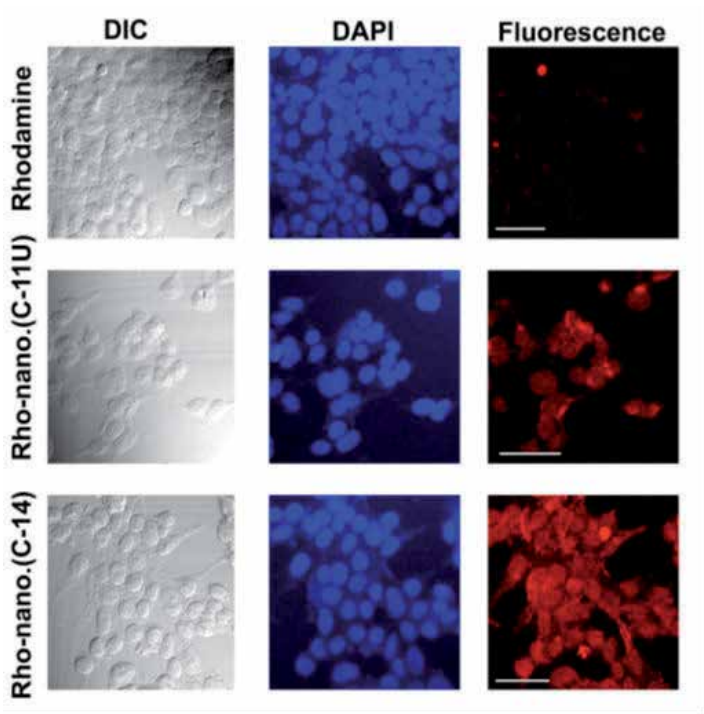

Figure 5. Uptake of raw rhodamine were compared to rhodamine containing nanoparticles uptake in HEK-293 cultured cell lines. Three separate sets of HEK-293 cells were cultures with raw rhodamine, C-11U and C-14 at 60 µg/ml concentration. The cells were fixed, processesd and viewed in a confocal microscope. Scale 50 µm.

The fruit fly, Drosophila melanogaster, is a good model for study cell biology with emphasis on toxicology (Peterson RT, Nass R, Boyd WA, Freedman JH, Dong K, Narahashi T. Use of non-mammalian alternative models for neurotoxicological study. Neurotoxicology. 29,546–555,2008). Here, we employed the Drosophila model to investigate nanoparticle interactions at different hierarchical scales of organization on Drosophilla at the egg, larval, and adult stages. PABA nanotubes and nanomaterials were mixed with yeast the standard Drosophila food at different concentrations, The food was seeded with 50 eggs of Drosophila melanogast-

er. After egg hatching (24 h), the larvae were observed to crawl through the food and ingest the suspended nanomaterials. Two of the fluorescent nanotubes were chosen for oral delivery. An important aspect of toxicity and genotoxicity studies is the selection of the assay system. Though In vitro approaches are preferred, the in vivo eukaryotic model, Drosophila appears as an ideal model organism. This has been already used to evaluate the internalization of nanoparticles and to solve open questions concerning cell uptake and live tissue distribution [27, 30]. The organic nanotubes when delivered through the food to the larval stage had no detectable effect on egg to adult survivorship.

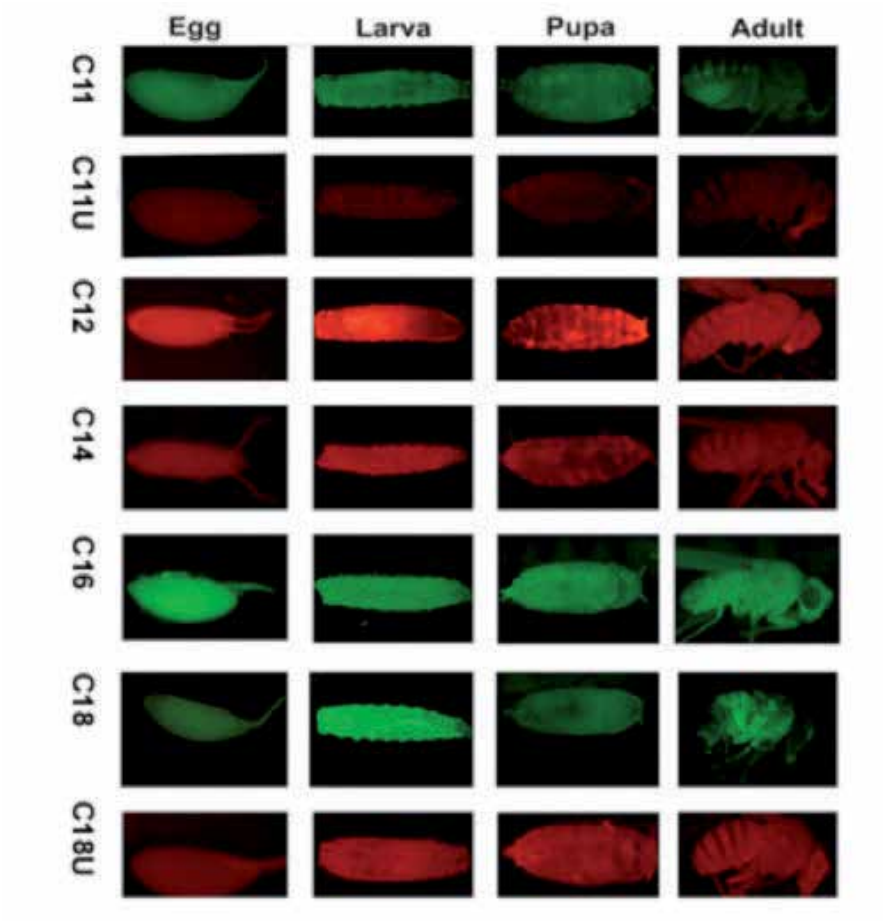

Figure 6. Biocompatibility and distribution of nanoparticles in four different developmental stages of Drosophila progeny after feeding nanomaterials containing media of the parental population.

Effect of nanoparticles on cell viability and cytotoxicity To address cell viability and cytotoxicity, colorimetric assay was performed using 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide. The cells incubated in freshly prepared nanoparticles containing media were treated with MTT. Uptake of nanoparticles in all cell types does not disturb normal cell

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propagation and showed more than 90% cell viability even at the higher nanomaterial concentration (120 μg/ml) relative to DMSO treated cells. These results suggest that nanomaterials function as efficient bio-transporters and fail to show any cytotoxicity. These findings were further verified by a parallel study using flow-cytometry measurements. The mitotic cells from confluent cultures incubated with different nanomaterials containing media were assayed. The relative progression of cells from G1 to S phase was also determined. In three separate cultures containing 0 μg/ ml, 30 μg/ml, 60 μg/ml nanoparticles, the phases of cell cycles were progressing normally based on the incubation time, but in higher concentration (120 μg/ml), a fall of G1 number with concurrent increase in G2 and S phase was noticed indicating progression towards asynchrony.

To obtain visual images of assemblies including 3D structure and measurements, many images from fluorescent laser confocal microscope, scanning electron microscope (SEM) were analyzed. The 3D images showed that self-assembled structures, Benzamide microtubes (BAMT-A and BAMT-B) form tube like structure with a hollow space inside. Tubes are nearly 0.178 to 0.207 μm in width. The BAMT-A shows green fluorescence colour that emits from self-assembled 4-alkylamido-N-pyridin-2yl-benzamides coupling with a stearic side chain (C=18). Compound BAMT-B conjugated with a shorter lauric side chain (C=12) does not emit intrinsic fluorescence colour. To add standard fluorescence dye (Rhodamine B, red), BAMT-B organic tubular structure was manufactured by mixing Rhodamine B with tube-forming benzamide during the self-assembly process. The Rhodamine B that is embedded in the surface wall of self-assembled microtubes (BAMT-B) emits red fluorescence as specific signal. Both microstructures with variable side chains are DMSO and ethyl alcohol soluble, but not in water and can be stored for prolonged periods without loosing its organic properties.

Next insect and mammalian cells were used to estimate the efficiency of cellular uptake of two microtubes in vitro. Drosophila S2 cells, nonneoplastic Human Embryonic Kidney (HEK-293) (Figure 2A, B) and neoplastic HeLa cells were grown in small dishes or cover glasses and incubated with the microstructures dissolved in 0.1% DMSO that has no adverse effect on cell physiology (14). After 24 or 48 hrs incubations, cells were fixed in 4% paraformaldehyde, followed by few gentle washes with PBS. The cells were viewed under laser confocal microscope (Olympus FV1000). The reconstituted images showed that both BAMT-A and BAMT-B was accumulated in the periphery of the nucleus of both insect and human cells incubated with 60mg/ml microtubes containing 0.1% DMSO (Figure 2 a,b). The accumulation is increased proportionately to the concentration of microtubes ingested. In contrast, a negligible amount of fluorescence signals was emitted from the cells incubated in 0.1% DMSO alone for the same period of time. These findings demonstrated that microstructures are accumulated in the cultured cell after penetrating the plasma membrane. Moreover, an identical distribution pattern of BAMT-A and BAMT-B in the insect and human cells further verify that similar to intrinsic green in BAMT-A, embedded red fluorophore remains coupled with BAMT-B tube wall after accumulation in the cytosol.

Next to test the viability of the culture cells, a colorimetric assay was performed using 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described earlier. This assay is based on the reductive capacity to metabolize the tetrazolium salt to blue coloured formazone.

Cells were incubated with various concentrations of microtubes. After incubation, cells were added with MTT and the absorbance of coloured product was monitored in a microplate reader at 570nm. In all the cases tested, the cell proliferation estimated by the absorbance and the viability of tumor HeLa and non cancerous HEK-293 cells in the presence of microtubes showed more than 95% viability relative to DMSO treated cells (Figure 2C). These results suggest that microstructures are the efficient molecular transporters for different biologically important cells with no cytotoxicity.

Further, these microsized structures were scaled up for *in vivo* use precisely to facilitate organ distribution and to combat different physiological hurdles in live organisms. To test overall viability and growth, after feeding the PABA containing microtubes to larvae, pupae and adult Drosophila were estimated. After hatching, larvae undergo an intense 4-5 day feeding, when they increase weight by 200-folds. In subsequent immobile pupal stage, they stop feeding, therefore no marked gain in adult fly weight from the larval stage is noticed. To feed larvae with highest possible doses of BAMT-A and BAMT-B, dry Baker's yeast was mixed with concentrated suspensions of microstructures (60μg in 100μl) in 0.1 % DMSO solutions, followed by centrifugation and decantation. The resulting pastes were used in equal amount as the sole food source for various batches of equal number of larvae. Indeed viability of the larvae, pupae and adult was marginally higher in sole DMSO (0.1%) fed flies relative to the flies fed on yeast paste containing microtubes (Figure 3). The feeding of low percentage DMSO (0.1%) has no apparent effect on fly physiology and viability. To investigate the effect of the microtubes on overall growth, behaviour and physiology of the flies, the size of the adults and the sexual behaviour of newly emerged flies were estimated. No size difference between DMSO and BAMT-A and BAMT-B fed male and female flies and abnormalities in their sexual behaviour were marked (Figure 3). The egg laying capacity of adult females for six consecutive days after hatching and the sex of eclosed flies were counted. No significant difference was detected in egg laying capacity and male/female ratios when compared to the wild type, DMSO or microtubes fed females (Figure 3). Taken together organic microtubes and their side alkyl chain modifications have no adverse effect on cellular physiology, behaviour, and sensitivity of fly sex and other pharmacokinetics parameters of live cells in the insects.(same as publication)

Organic nanotubes claimed a greater stability and better self-life and biocompatibility. To verify such claim of orally ingested microtube in live organisms, we reared the newly hatched adult flies, generated from microtubes or DMSO fed larvae, in the normal culture media for 7 consecutive days and examined under fluorescence microscope. Nearly equal level of fluorescence intensity was found from body parts of the adult flies reared in normal food media for 0 to 7 days (Figure 3d). Culture in food media without microtubes does not reduce the stability and self-life of the fluorescence organic microtubes for a limited period. The fluorescence intensity was reduced conspicuously after extending the culture on an average of 23-25 days and was eliminated within 47 days from the organs of the adult flies reared in the normal media. We further examined fluorescence intensity of the mature fertilized eggs laid by adult females reared in normal food for 7 days after hatching. No fluorescence signal was emitted from the eggs (Figure 3d). It eliminates the rare possibility of genetic inheritance of micro-

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structures in the next successive generations through germ cells. Therefore, lack of heritable transfer of microtubes leads to an ineffective route of microtube spreading in the environment and their natural entry into the foodchain via participating consumers.

In live insects, majority of the internal organs are submerged in haemolymph – a blood equivalent of human. The haemolymph circulate through the open vessel and pump the fluid in a fixed direction at the posterior body cavity by using a series of valves that prevent opposite haemolymph flow. As reported earlier by feeding single walled carbon nanotubes in intact fly postulated that the fluorescent methods are ideal for delivery and diagnostic application. Feeding of microtubes in larvae and adults causes systemic spreading of signals by the gut peristaltic movement to cross the cell membrane barrier. The intensity of the dye associated with the microsized materials is proportionate to the amount of accumulated tubes that were incorporated in the gut cells. Variable intensity of fluorescent dyes in different parts of the body demonstrates different amounts of microtube accumulation in the different organs. Internal organs of larvae and adult tissues were dissected, fixed in 4% paraformaldehyde, processed and scanned under Confocal Fluorescence Microscope. At least five samples of each organ from larvae fed on microtubes were viewed and intensity of fluorescence scored (Supplementary Material). The amount of fluorescence of each tissue of the DMSO and microtube fed larvae and adults was estimated (Gray value/pixel) using Metamorph software. An increased accumulation of fluorescence dye was found in the digestive track (83.06+4.51 in BAMT-A), Malpighian tubules (57.19+3.81), fat bodies (38.41+2.19) compared to the salivary glands (27.73+1.81) and rapidly dividing cells of two imaginal discs (11.52+0.56 in wing discs and 14.32+0.41 in eye discs) (Figure 4). However, variable lengths of the side chains lead to the conspicuous changes in microtubes distribution at the different body parts. Shorter length of lauric side chain exhibited greater accumulation of microstructure. The cells of different discs and larval brain were devoid of any BAMT-A tubes containing longer side chain (C=18) but a considerable amount of BAMT-B (C=12) penetrated the same tissues after feeding equal amount of microtubes (Figure 4). In adults, a distinct pattern of BAMT-A and BAMT-B distribution in different organs was found. BAMT-A mainly accumulated in the abdomen, thorax including haltere and leg assuming their prolonged retention in the body fluid, while BAMT-B conjugated with C-12 side chain widely distributed in eye, antennae, proboscis and adult brain (Figure 4). To examine the preferential penetration of two side chains in adult brains, the amount of dyes in the brain tissue were tested. The BAMT-B with short side chain has a clear advantage in the entry of the brain tissue over BAMT-A in equal concentration. Interestingly, no change in fly behaviour after oral digestion suggest that accumulation of PABA microstructure do not produce any permanent damage in the brain because p-amino-benzoic acid functions in the improvement of neuro-degerative damages by inhibiting acetylcholinesterase.

Further high concentrations of fluorescence conjugated with BAMR-B were observed in insect cells and in all the organs of Drosophila after feeding. Enlarged view of insect cells and wing disc (Figure 4) verified the accumulation of micro-structure in the cytoplasm of cultured cells and fixed tissues. These finding allowed us to confirm the physical accumulation of microtubes in both cell types. In contrast, low abundance of the BAMT-A

microtubes in the salivary glands and rapidly dividing cells of imaginal discs probably represent the secondary uptake after the microtubes enter into the haemolymph. These findings demonstrate that differential uptake and specificity of live cell targeting by the microtubes depend on the cellular physiology, chemical modification of carrier to travel and enter live cells.

Here, several critical questions relevant to biocompatibility and application of p-aminozbenzoic containing microstructures are addressed. (1) It determines the parameters of chemical and biological modifications suitable for oral administration. However, the impact on specific cellular physiology and efficient uptake of microstructures by carrier molecules based on their chemical modifications cannot be ignored. It is evident that distribution and accumulation is prone to shorter (C=12) alkyl side chain. (2) These organic microtubes overcome cell physiological, pharmacokinetic barriers and show an efficient cellular uptake in animal cultured cells (5,19). (3) It has no adverse effects in physiology, behaviour and growth of insects that shows strong similaries with human at molecular level (3) Finally, modifications in alkyl side chain play an important role in biodistribution. The side chain modifications in BAMT-A and BAMT-B alter the shape of microstructures by changing self assembly properties and discriminate tissue specific distribution specifically in adult eye, its precursor cells, wing imaginal discs and neuronal tissues in larval and adult brain but do not show any conspicuous changes of their biodistribution in cultured insect and human cells. The chemical modifications of self assembled p-aminobenzoic moiety also show a better stability and long self-life before degradation but no short-term toxicity, impaired growth of Drosophila larvae and adults after feeding solely on microtubes containing media. Moreover, no obvious effect on fecundity or impairment of fertility was noticed in the adult female flies. During self assembly, chemical modifications of PABA, and their longer stability in the internal organs provides the microtubes as better-suited and sustainable cargo in live organisms, unlike inorganic nanomaterials that neither accumulate in the live cells nor produce cytotoxicity in the cellular environment. Most logical extension of this work would be the cell specific target delivery in which coupled small molecule on the surface wall have define properties for attachment of specific cell surface and/or stringent proteins for facilitating antigen-antibody reactions. This approach could be used not only to deliver small regulatory RNA and DNA as therapeutic materials but also to optimize pill like properties for oral ingestion with no pharmacokinetic barriers. Though this study used only one structure moiety of drug molecules, it is likely that the method would work with other types organic microstructure as long as three major criteria of orally administered molecule biocompatibility, pharmacokinetics and a capacity for multivalency are considered. Therefore chemical modulations on the surface should be incorporated in the design to attach the multivalency of small ligands. These changes in organic microstructure were described recently. Such attempts for synthesizing tissue or cell guided orally ingested microstructure may favour to make the next generation micropill to deliver biomaterials for effective gene therapy and novel cargo useful for molecular diagnostics.

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6. Mode of uptake of PABA nanomaterials

Broadly, there are two modes of entries, either PABA nano-materials transverse the cell membrane via endocytosis orenergy independent nonendocytotic mechanism. We have carried out a series of investigations on uptake mechanism and cellular internalization for PABA conjugates. Endocytosis is an energy dependent mechanism. The process is hindered at a low temperature (at 4°C instead of 37°C) or in ATP deficient environment. Cells incubated in media containing nanoparticles were either cultured at 4°C or pretreated with NaN3 for inhibiting the production of ATP, thereby hampering the endocytosis process. The level of fluorescent intensity in the cytosol of each cultured cells was reduced dramatically relative to cells cultured in regular standard conditions (Figure 3). This reduction determines that PABA conjugates enter in the sub-cellular compartment of cultured cells via endocytosis. We also sub-categorized the endocytosis pathway including phagocytosis, pinocytosis, clathrin dependent receptor mediated and clathrin independent mechanisms.Internalization often occurs when the clathrin coat on the plasma membrane forms conspicuous invagination in the cell membrane leading to the budding of clathrin-coated vesicles. As a result, extracellular species located on the cell membrane are trapped within the vesicles and invaginated inside the cells. To disrupt the formation of clathrin coated vesicles on the cell membranes, cells were preincubated in sucrose (hypertonic) soluton or K+-depleted media before treatment with all seven nano-particles. Data showed a drastic reduction in PABA nano-particle uptake (Figure 3C), which suggests that a clathrin dependent endocytosis process is involved in entry mechanism.

7. Uptake of PABA nanomaterials by clathrin dependent endocytosis

To rule out the possibility of cellular uptake of PABA conjugates via caveolae or lipid rafts pathway, we pre-treated the cells with drug filipin and nystatin, which disrupt cholesterol distribution within the cell membrane. In contrast to clathrin blocking experiments, pre-treatment of the drugs had a negligible blockage on the cellular uptake, which suggests a little or no involvement of the caveolae dependent cell entry. In a similar control experiment we studied the uptake of fluroscent labelled cholera toxin B (CTX-B) which is a multivalent ligand protein known to be internalised by caveolae depenent endocytic pathway (Figure 3E). The CTX-B showed a significant inhibition in cell entry in the presence of filipin and nystatin. Taken together, the results verify that cellular internalization of PABA conjugates is mediated through the clathrin-dependent endocytosis pathway.

8. Oral uptake of variable PABA nanomaterials in Drosophila

Organic nano-assemblies have negligible adverse effect on cellular physiology, behaviour, sensitivity to adult sex and other pharmacokinetics parameters of Drosophila. We have screened nanoparticles conjugated with variable side chains for organ specific targeting in

Drosophila. Different sets of larvae, pupae and adult flies were grown with sole feeding of nanoparticle containing media. The accumulation to various tissues, selective organ uptake and their clearance was also monitored by imaging the fluorescence signals during the stages of development in Drosophila. In live insects, oral feeding of nanomaterials causes systemic spreading of signals through the gut by peristaltic movement tocross the cell membrane barrier. In general, majority of the nanoparticles carrying unsaturated side chains (C-11U, C-18U) showed a low level of incorporation in all stages of Drosophila life cycle although C-18U showed a comparatively high level of incorporation in two different life stages, larvae and pupae (Additional File 2 Figure S16A-B). We further investigated the efficacy of in vivo targeted delivery among nanoparticles that emits intrinsic green and nanoparticles with intercalated rhodamine B in the wall. Intrinsic green nanostructures carrying C-16 side chain showed a maximum amount of incorporation through cell membranes, compared to C-18, and C-11 that showed a variable amount of incorporation in different developmental stages. Animals fed with C-18 selfassembled particles exhibit a maximum incorporation during larval stage as compared to other tested stages. Animals fed with C11 showed an overall low level of entry in all the stages of development.

Delivery of rhodamine B embedded nanoparticlesC-12 showed an equal and maximal level of incorporation in all stages of development. The intensity was conspicuously greater than the nano-structure carrying C-14 chain. Taken together, specific carbon chains and associated morphologies of nanostructures brought a potential difference in entry through gut cell walls. These results suggest the possibility that the physiology of gut cells in different stages of the life cycle might influence nanoparticles uptake.

For in vivo tracking, fluorescence dyes attached to nanoparticles suffer with multiple problems including photo-bleaching and ability to interrogate multiple targets etc. The aftermath effect of such limitations of fluorescence imaging in live objects was described earlier. In all cases, during in vivo delivery there was no photobleaching of the nanomaterials through all stages of development providing a better advantage in tracking in live systems. But the fluorescence intensity was reduced conspicuously after extending the culture on an average of 18-25 days and nearly eliminated within 40-45 days allowing a total clearance of fluores-cence from the live tissues. We further screened the efficacy of nano-particles inheritance through germ cells. The adult flies emerging from sole feeding of nanoparticle containing media were cultured in normal food media for another 7 days. The fertilized eggs from different batches of flies after nanoparticle feeding emits only trace amount of fluorescent as a background effect. Therefore, this ineffective route of gem cell based heritable transmission prevents nanomaterials con-tamination in the environment and their natural entry into the food chain via eco-consumers.

9. Efficiency of organ specific delivery of PABA nanomaterials by side chain variation in Drosophila

To categorize the intensity of fluorescent molecules as an absolute reflection on efficacy of nanoparticle delivery, different internal body parts of the larvae were dissected and visualized

under fluorescence microscope. A wide range of variation in fluorescence intensity was observed in different larval body parts, for example mouth, brain, larval neural ganglia, salivary glands, alimentary canal and malpighian tubules etc (Figure 4; Additional File 2 Figure S16). A clear contrast was observed in the delivery of nanomaterials in the salivary glands. C-14 and C-18 containing nanostructures incorporated at a massive level in the glands but shows an intermediate level of incorporation in both neural tissues and organism itself (Figure 4). Surprisingly, we observed that malphigian tubules absorbed more nano-particles that emit intrinsic fluorescence. Therefore nanoparticles with different side chains showed a distinct distribution in various internal tissues in the larvae. Nanoparticle entry showed a clear variation in rapidly dividing cells of mature larval imaginal discs (the precursor organs of adult wings, eyes and legs). PABA conjugated with C-16 side chain showed a higher intensity of uptake in all three discs tested. However the intensity of fluorescence is moderate in C-11U, C-12 and C-18 particles (Figure 4). It suggests that the structure and surface texture of C-16 side chain is the most effective cargo for delivery in precursor and rapid dividing cells, though we cannot rule out other unmet criteria in the tracking process (Figure 4). As described above, the delivery of C-12 structure in all the stages of development is ideal compared to C-14 and nanomaterials with unsaturated side chains in C-11U and C-18U. Surprisingly a differential uptake of nanomaterials produced by C18 and C18U specially in leg discs that possess same number of carbon bonds interprets that length of the side chain is not an only criteria for nano-particles based delivery in imaginal discs.

The conjugated side chains of PABA nanostructures were also screened for delivery to complex adult body parts derived from same sets of larval imaginal discs. Entry of nanomaterials was analysed in adult eyes, halters and legs. Incorporation in adult eyes is complicated and novel from other body parts. Two different fluorescent tags showed distinct uptake through eye ommatidia (Figure 4) raising the possibility that difference in fluorophore emission and structure make their entry visible and distinct in adult eyes. The intrinsic green showed a poor emission through ommatidia. Only a trace amount of green colour was visualized whereas rhodamine B showed a greater intensity with a maximum incorporation of C-16 in the eyes. However, the incorporation pattern of nanomaterials conjugated with variable side chains in halters and legs is distinct from their distribution in eyes. Among all possible nanostructure, C-11U and C-18U were targeted orally at a maximal level to the legs while C-11, C-12 and C-16 in the halters showed an equal but greater amount of incorporation, which suggests that the unsaturated carbon chains have advantage for selective entry in the accessory organs of Drosophila. Taken together, the delivery of nanoparticles associated with variable side chains in the culture cells and in vivo uptake by oral delivery in different body parts is different.

Furthermore distribution of numerous neurons and other cells make brain more compact and the delivery of therapeutic agents in the neuronal tissues is the most challenging task. In spite of complicated entry in brain, two nanoparticles, C-11 and C-16 containing particles show a considerable amount of entry when incorporation of other particles is nominal in the brain (Figure 4). Truly, greater dissemination of nanostructures in adults, larvae and different body

parts including brains suggests that physio-chemical properties including shapes, surface texture of the C-11 and C-16 particles are the best-fitted materials (Figure 4).

10. Perspective

The key parameters of nanomaterials for easy and efficient delivery are shape, size and flexibility to enter and exit cell barrier. Our results clearly demonstrate that the properties of each acid side chain together with common PABA moiety influences size, shape and surface texture of nanomaterials that lead to differential uptakeand specificity in live cell delivery. The physio-chemical modifications of organic nano carriers also affect cell internalization mechanisms in sub-cellular organelles as found by distinct accumulation pattern of each nanomaterials following same energy dependent endocytosis. In vivo screening also showed that only C 11 and C-16 produce compatible shape and size of nanomaterials that are best fitted for easy delivery of PABA nanomterials. These results suggest that physical structure of nanomaterials and chemical properties of acid side chain required for self assemble procedure and size variation could be the initial step for cellular uptake.

In addition to cultured cells, tissue specific distribution specifically in adult eyes, imaginal discs, alimentary tracks and neuronal tissues was complex and needs more parameter to consider. Our data revealed that a complex interrelationship of PABA conjugates and cell physiological environment is important in live materials delivery. The internal tissue environment might provide additional barriers for nanomaterial entry as depicted by comparing variable accumulation of same nanomaterials in cross species; Drosophila and human cell lines. A similar difference was also noticed when C-11 or C16 accumulation was compared in multiple complex organ of Drosophila. However, nanomaterials compatible for oral delivery do not show any short-term toxicity, impaired growth of Drosophila larvae and adults. We hypothesize that two distinct parameter nano-skeleton frame with conjugated acid chains and live cell physiology are best suited for cell uptake and delivery to internal organs after oral consumption. Our results also differ from the hypothesis that nano-particle uptake in live cells occurred through energy independent non-endocytotic pathway involving insertions and diffusion across the cell membrane. Sub cellular internalization of PABA nanomaterials predominantly takes place by energy dependent endocytosis. Earlier we have found that PABA nanomaterials can penetrate plasma membrane in the human cells and enter into cytoplasm. The variable amount of different nanomaterial accumulation by energy dependent endo-cytosis in same cell type ruled out the possibility that a single internalization mechanism, endocytosis is exclusively required for uptake. However, a marked reduction of different nanomaterials under endocytosis inhibitory conditions believed that such discrepancies are due to sharp differences in size and shape of the self assembled structures. In addition as organic nanomaterials suffer from uncontrolled aggregation to form micron sized particles after prolong storage; thereby ruling out the possibility of insertion, diffusion and penetration mechanisms [22]. PABA nanoparticles have a high tendency to associate with cell membrane (Figure 2, 3).

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Such accumulation might give rise to artefact in cellular uptake of micro-sized aggregates as found in artifactual intake of HIV TAT peptide at 4°C [23]. Therefore, cellular entry of PABA might depend on the size of the nanoparticles which is mainly guided by the acid side chain.

Finally, a systematic screening of PABA conjugated library provides sufficient evidences to support the following statements: 1) Two nanomaterials carrying C-11 and C16 acid side chains are best suited for optimal entry in cells and multiple organs. 2) In live tissues, an internal environment might be a useful barrier for improving nanoparticles delivery in multiple organs. 3) Cellular internalization or uptake mechanism of nanomaterials might unravel the clues for smooth entry in human cells and efficient delivery and 4) finally screening of PABA conjugates determine a functional relationship between energy dependent endocytosis and nanomaterial structure for each organ specific targeting.

11. Conclusions

We have shown that two carbon linker group C-11 and C-16 forms tubular nanomaterials that are best fitted for mass oral delivery in complex multiorgans. The cellular uptake mechanism is energy dependent endocytosis. The detailed endocytosis pathways for nano PABA structure is operated thorough clathrin-coated pits rather than caveolae or lipid rafts. In vivo screening of PABA nanomaterials produced by different acid side chain select the compatible nano structure ideal for oral delivery and establishes energy dependent entry mechanism is of fundamental importance that will facilitate future developments of PABA nanoparticle transporters for biological delivery application

Author details

Utpal Bhadra[1], Manika Pal Bhadra[2], Jagannadh Bulusu[3] and J.S. Yadav[4]

1 Functional Genomics & Gene Silencing Group, CSIR-Centre for Cellular & Molecular Biology, Hyderabad, India

2 Centre for Chemical Biology, CSIR-Indian Institute of Chemical Technology, Hyderabad, India

3 Division of Organic Chemistry-I, Indian Institute of Chemical Technology, Hyderabad, India

4 Ex-Director & CSIR Bhatnagar Fellow, Indian Institute of Chemical Technology, Hyderabad, India

We are highly sympathetic due to sudden demise of Dr Jagannath Bulusu and we hope his soul lies in peace

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Chapter 14

Pharmacokinetic Properties and Safety of Cadmium-Containing Quantum Dots as Drug Delivery Systems

Lourdes Rodriguez-Fragoso,
Ivonne Gutiérrez-Sancha,
Patricia Rodríguez-Fragoso,
Anahí Rodríguez-López and Jorge Reyes-Esparza

Additional information is available at the end of the chapter

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1. Introduction

The pharmaceutical industry's current challenge to serve public health needs by has become increasingly difficult due to obstacles that slow down the process of identifying and developing new treatments of unmet medical diseases. There are many auspicious new therapies that have progressed into clinical trials in recent years; they include treatments for cancer, inflammation, neurodegenerative and psychiatric disorders, anti-infective respiratory and metabolic disorders, but their development has failed for a number of reasons. Overcoming these obstacles incurs tremendous costs and takes a lot of time; new therapies must then be identified get successfully issued into the marketplace. In response, government, academic and pharmaceutical industry researchers are looking for new ways to approach the discovery of new medicines and technologies that will not only combat illness but also improve the quality of life, a most important outcome of expensive new treatments. Counterbalancing this important goal are efforts to innovate in the face of increasing public pressure to control costs and increase the speed with which new medicines arrive on the marketplace.

For a few years now, nanotechnology has emerged as an area of science and technology that is leading us to a new industrial revolution. Nanotechnology is defined as scientific and technological development at the atomic and molecular levels, in the range of about 1-100 nm, to obtain a fundamental understanding of phenomena and materials on a nanoscale and to create and use structures, devices and systems that have novel properties and functions due

to their size. The most interesting aspect of nanotechnology is its ability to work with materials of small size that, however, can change radically on a physical and chemical level at this scale: electrical conductivity, color, resistance or elasticity, among other properties, behave differently than they do in volumetric material. The emergence of nanotechnology in the health sciences has led to a new discipline called nanomedicine, the main objective of which is the development of tools to diagnose, prevent and treat diseases when they are still not very advanced or incipient [1].

Nanomedicine includes three main areas: nanodiagnosis, nanotherapy and regenerative medicine [2]. Their main goals are explained in the following paragraphs:

a) The purpose of nanodiagnosis is to identify diseases in their initial stages at the cellular or molecular level and, ideally, down to the level of a single cell, using contrast nanodevices and systems [3]. Early identification would lead to immediate application of appropriate treatment, increasing the probability of healing. Nanosystem diagnostics can be used *in vitro* or *in vivo*. *In vivo* diagnosis normally requires that devices penetrate the human body to identify and (ideally) quantify the presence of specific pathogen or cancer cells, for example. This entails a number of problems associated with the biocompatibility of the material of the device, as well as sophisticated design to ensure effectiveness and minimize side effects. Meanwhile, the *in vitro* diagnosis provides greater design flexibility of design because it can be applied to very small samples of body fluids or tissue from which specific detection can be performed (pathogens or genetic defects, for example) in a very short time with high precision and sensitivity [4]. Because of these fundamental differences, *in vitro* detection using nanoscale devices is expected to reach the market faster and consolidate more easily than *in vivo* methods. There are two main areas of work: images and nanosystems and biosensors. These systems rely on the use of nanoparticles, semiconductors, or magnetic metals, such as contrast agents for *in vivo* labeling. These new systems can increase sensitivity and give better contrast in imaging techniques. One of the first proposed nanoparticle systems was the identification of tumor cells. In the case of nanodiagnostics, the main testing devices being developed are nanobiosensors, devices capable of detecting in real time without the need for fluorescent or radioactive markers and with high sensitivity and selectivity all kinds of chemical and biological substances [5].

b) The aim of nanotherapy is to drive nanosystems containing recognition elements to act or transport and release drugs exclusively in cells or affected areas in order to achieve a more effective treatment, minimizing side effects [6]. Approximately 40% of the novel new molecules (NNMs) selected for full-scale development based on their safety and efficacy data fail to reach the clinical development phase due to poor biopharmaceutical properties, which translate into poor bioavailability and undesirable pharmacokinetic properties. Several nanotechnology-based products, including Doxil® (doxorubicin HCl liposome injection) and Abraxane® (paclitaxel protein-bound particles for injectable suspension) are already on the market [7, 8]. In addition, Baxter and Elan are promoting Nanoedge® dispersion technology and Nano-Crystal® technologies respectively, so as to improve the biopharmaceutical properties of orally

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administered therapeutic agents [9]. Nanotechnology can play an important role in the development of proper formulations that address the drug delivery issues related to NNMs with poor biopharmaceutical properties, such as poor solubility, poor permeability across the intestinal epithelium, enzymatic or nonenzymatic degradation/metabolism, complexation with chelating ligands or metal cations, intestinal efflux, and poor transport properties. Additionally, nanotechnology can also achieve desirable pharmacokinetic and toxicological properties that aid in the accelerated development of the NNM. Nanoparticulate drug delivery systems are being used to alter the drug's biopharmaceutics and pharmacokinetics such as drug absorption, distribution, metabolism, and elimination [10]. Examples of nanoscale delivery systems include polymeric nanoparticles, liposomes, nanoemulsions, micelles, and dendrimers.

A number of nano-delivery systems are designed to encapsulate the drug in carriers (e.g., liposomes, micelles, polymeric nanoparticles, and dendrimers), which masks the unfavorable biopharmaceutical properties of the molecule and replaces them with the properties of the materials used to make the nano-delivery system. These approaches were used for a number of poorly soluble NNMs in aqueous phase or easily degraded and metabolized NNMs. Another approach involves the covalent conjugation of the molecules with carrier and targeting moieties (e.g., polymer-drug conjugate, antibody-drug conjugates, solubilizers-drug conjugates, etc.) that override the drug's poor biopharmaceutical properties and improve the pharmacokinetics and biodistribution. This approach was used for site-specific or targeted delivery to alter the pharmacokinetics of the drug by increasing the plasma elimination half-life, preventing degradation or metabolism of the drug in the systemic circulation, and possibly altering the organ and subcellular distribution of the drug, thus alleviating unwanted toxicity due to nonspecific distribution, improving patient compliance and providing favorable clinical outcomes [11]. Advances in nanomedicine are also applied for site-specific drug and gene delivery strategies, especially for the treatment of cancer and other life-threatening diseases [12, 13]. The nanotechnology approach, although expensive and time consuming, can significantly assist in the accelerated development of NNMs with adequate druglike properties and can assist the pharmaceutical companies in adding more lead molecules to their pipeline. One of the major challenges in this process is the development of "nanotherapies", specifically those targeting diseased tissues and organs while avoiding damage to surrounding healthy cells and, thus, the dreaded side effects of current treatments.

c) Regenerative medicine aims to repair or replace damaged tissues and organs using nanotechnology tools [14]. Regenerative nanomedicine deals with the repair or replacement of damaged or diseased tissues and organs by applying methods derived from gene therapy, cell therapy, chemical dosage and bio-regenerative tissue engineering, stimulating the human body's very own repair mechanisms [15]. The main contributions of nanotechnology to regenerative medicine are related to the production of new materials and support systems, the use of embryonic and adult stem cells, and the production of bioactive molecules that serve as signals for cell differentiation [16]. Nanotechnology can play a dominant role in tissue engineering by facilitating new materials and techniques that allow fro more efficient tissue

integration and the ability to generate microenvironment parts that are particularly conducive to tissue regeneration. The main difficulty lies in finding suitable materials that allow for the fabrication of structures that remain active while the affected organ regenerates the damaged area [17]. Some of the materials that are being used include carbon nanotubes, the nanoparticles as hydroxyapatite or zirconia particles, biodegradable polymer nanofibers, nanocomposites, etc. One of the greatest achievements is the development of biomaterials with the ability to mimic the extracellular matrix, forming a real support identical to what appears naturally in cells and on which stem cells can be grown for subsequent implant in patients to repair or replace damaged organs.

The enormous advances in nanotechnology during the past decades have allowed for substantial developments in the field of health sciences. The systems and methods described are only selected examples of the enormous activity that is taking place in thousands of laboratories around the world to improve health and quality of life across the whole of society. In the present chapter, we discuss the pharmacokinetic properties and safety of cadmium-containing quantum dots as tools for diagnosis and drug delivery systems.

2. Quantum dots in medical science

Quantum dots (QDs) have aroused much interest in recent years, especially in view of their potential applications in biology and medicine [18]. This heterogeneous class of engineered nanoparticles that are both semiconductors and fluorophores is rapidly emerging as an important type of nanoparticles with numerous potential applications in medicine [19]. QDs are semiconductor inorganic nanomaterials ranging from 1–10 nm. They contain elements found in groups II–IV (e.g., CdSe, CdTe, CdS, and ZnSe) or III–V (eg, InP and InAs) of the periodic table. QDs have fluorescent properties that offer superior features to conventional organic dyes, including high quantum yield, broad absorption, and narrow emission spectra (Figure 1). QDs are more photostable than conventional fluorophores; e.g., it has been reported that, under the same excitation conditions, 90% of the fluorescence of a normal organic dye fades within 1 minute, whereas the fluorescence of QDs remains intact even after 30 minute or more [20].

In terms of their basic structure, QDs consist of an inorganic core, an inorganic shell and aqueous organic coating. The size of the inorganic core determines the wavelength (color) of light emitted following excitation (Figure 1). The inorganic shell is responsible for increasing the photostability and luminescent properties of the QDs [21]. The photo stability of the inorganic shell has allowed QDs to be used as probes for imaging cells and tissues over long spans of time. While there are many useful features to QDs, there are also a number of issues related to their structure and function [22]. One of the most problematic is a phenomenon known as “blinking”. This is the term used to describe the alternation between the light-emitting and –non-emitting state of the QD. This factor limits the number of photons that can be detected in a given time period and it also contributes to unpredictable photon arrival

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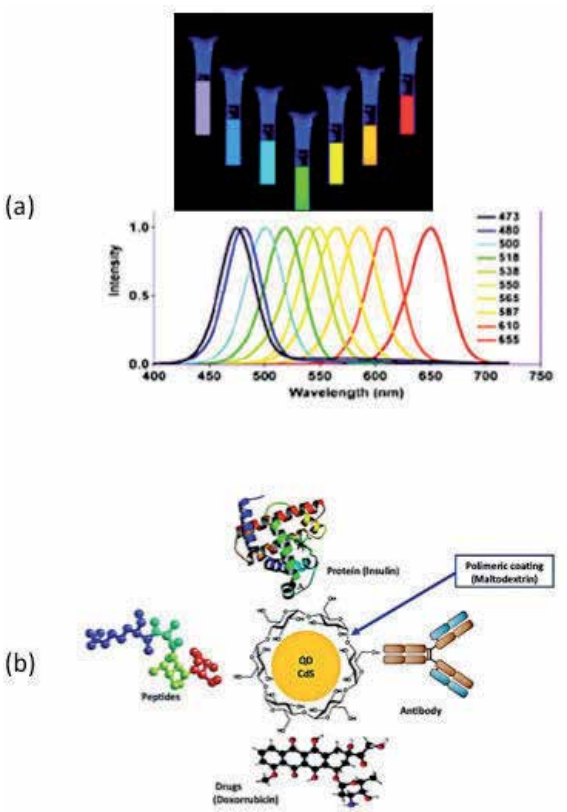

Figure 1. Spectrum and basic structure of quantum dots. (a) Emission spectra of quantum dots; (b) Schematic structure of quantum dots and conjugation to biomolecules.

times. It has been proposed that this feature of QDs could be suppressed by "passivating" the QDs' surface with thiol moieties, polymers, or by using the QDs in free suspension. A standard nomenclature is generally utilized to describe the component parts of various QDs: Core/Shell or Core/Shell-Conjugate. For example, a QD with a cadmium-sulfide core and a maltodextrin shell which has been protein conjugated would be designated as CdS/protein [23]. As fluorescent particles, quantum dots can be detected and tracked with the same approach developed for organic fluorophores. All the technical development has been directly transposed to QD imaging and tracking. Biomedical applications exploit the fluorescent properties of QDs, particularly their advantage over traditional organic dyes for both diagnostic and clinical applications. The *in vitro* biomedical and diagnostic applications of QDs include such techniques as the multicolor fluorescent labeling of cell surface molecules and cellular proteins in microscopy and other applications, detection of pathogens and toxins, DNA and RNA technologies, and fluorescence resonance energy transfer. QDs are also being explored for use in whole-body *in vivo* imaging of normal and tumor tissues. QDs may also find use in therapeutic applications such as targeted drug delivery, photodynamic therapy, and drug discovery [24].

3. Cadmium-containing quantum dots

Different results by various research groups indicate that cadmium (in group III–V) is extremely toxic if allowed to leach into the environment and this material also has DNA-damaging properties [25]. Other studies have shown that using cadmium in the cellular environment may lead to the formation of reactive oxygen species, resulting in cell death [26, 27]. The stability of groups III–V is known to be due to the presence of covalent rather than ionic bonding. The most optically suitable emitting 'core' materials have been cadmium-based materials. Cadmium selenide, Cadmium sulfide or Cadmium telluride particles provided bright emission across the visible and near infrared regions of the electromagnetic spectrum [28]. Questions have arisen regarding the suitability of cadmium-containing materials as biological labels. Other problematic factors include the suitability of the capping agents, the retention of particles over a certain size, biological magnification and, importantly, the breakdown and decomposition products of these inorganic materials. QDs are notoriously labile and the identity and ultimate destination of the inorganic decomposition products remains unclear. Despite this, cadmium-containing quantum dots provide a genuine advance in medical imaging and the numerous problems involving these particles are almost immediately dispelled if one wishes to image and explore fixed cells [29, 30].

Cadmium, which is the main component in the majority of quantum dots, is known to be acutely and chronically toxic to cells and organisms. In cells, it is taken into calcium membrane channels, where it accumulates [31]. Cadmium inhibits the synthesis of DNA, RNA and proteins, as well as breaking up DNA strands and mutating chromosomes [32]. On a cellular level, cadmium induces oxidative stress by depletion of endogenous antioxidants such as glutathione [33], as well as mitochondrial damage [34]. Cadmium nanoparticles exposure can lead to disturbances in cellular homeostatic mechanisms, resulting either in adaptive cellular responses or cell death. Cell death can occur either through an abrupt process named necrosis or a tightly regulated or programmed process (apoptosis and autophagy) [35]. Its toxicity is mainly associated with liver and kidney injury, osteoporosis and neurological dysfunctions at the level of living organisms. The toxic ions are commonly thought to be released from quantum dots when the surface of the nanoparticle is oxidized and early reports on the inclusion of simple quantum dots in bacteria support this [36]

Protecting the core can, to some degree, control toxicity related to cadmium leakage. However, the change in the physicochemical and structural properties of engineered quantum dots could be responsible for a number of material interactions that could also have toxicological effects [37, 38]. However, encapsulation is not simple and it has been reported that quantum dots have displayed toxicity even with well-protected cores. Recently, polymers that can act as coordination sites for cadmium ion aggregation have protected semiconductor nanoparticles. CdS nanoparticles protected with starch and, in particular, amylose, form a wide range of inclusion complexes for numerous guest molecules [39]. Soluble starch added during the synthesis has been used as a capping agent in the synthesis of CdS and CdSe nanoparticles, resulting in well-controlled and uniform particle sizes of cadmium-rich nanoparticles [40]. Early studies attempted to quantitatively determine values for the onset of cytotoxicity in CdSe

and CdSe/ZnS quantum dots, either coated with mercaptopropionic acid (MPA), embedded in a silica shell or embedded in an amphiphilic polymer shell [41]. They found that the majority of the nanoparticles were ingested into the cells and were stored in vesicles around the nucleus, irrespective of the surface coating. We have previously synthesized CdS nanoparticles coated with maltodextrin polymer, and our results revealed that CdS-MD nanoparticles produced distinct dose-dependent effects (Figure 2) [42]. It is clear from this and other studies that the surface coating is related to the toxicity experienced by cells, which affects the level of toxic material released from the nanoparticles. Other studies have shown that different cell types have varying thresholds for quantum dot-induced toxicity.

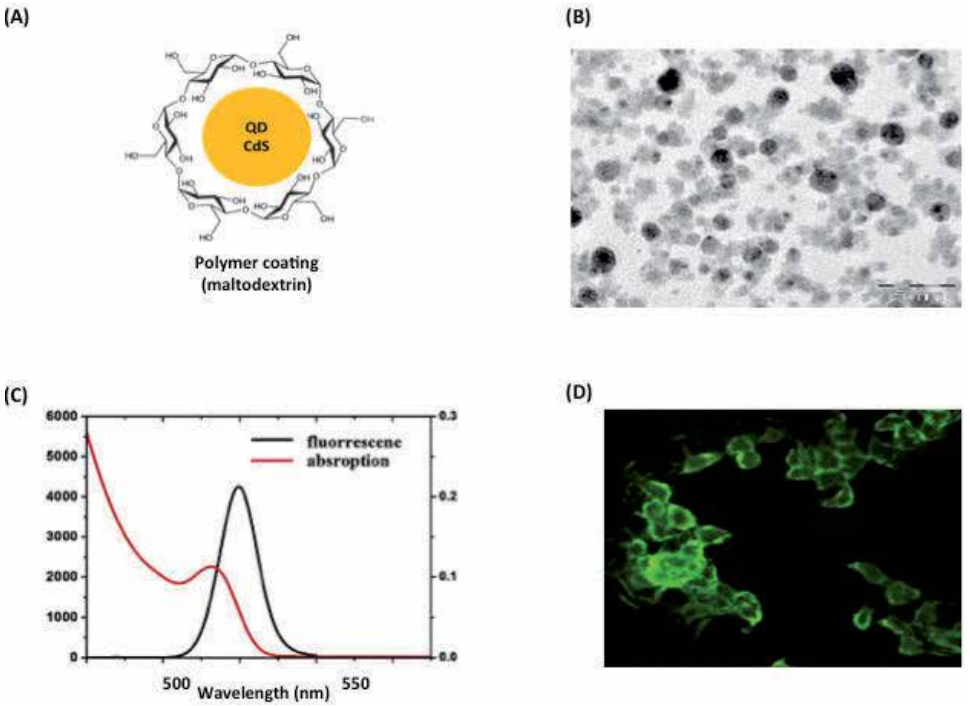

Figure 2. CdS/maltodextrin quantum dots. (a) Schematic structure of CdS/maltodextrin quantum dots; (b) Characterization of different sized CdS/MD quantum dots using TEM; (c) Spectra of CdS/MD quantum dots, the maximum luminescence of which is a wavelength of 520 nm (CdS/MD520); (d) HepG2 cells observed under fluorescent microscopy at (x40) magnification.

4. Biocompatibility and functionalization of cadmium-containing quantum dots

Biocompatibility is a word that is used extensively in biomaterials science. The incorporation of QDs into biological systems often requires strategies for the manipulation of the ligands bound to the surface of the QDs surface in order to make them water-soluble and biocompat-

ible, compatible with living tissue or a living system by not being toxic, injurious, or physiologically reactive [43]. QDs must be rendered water-soluble through the modification of their surface in preparation for biological applications. An ideal water-soluble ligand should meet the following requirements: (1) provide QDs with stability and solubility in biological buffers; (2) maintain a high resistance to photobleaching and other photophysical properties in aqueous media; (3) have functional groups which are able to conjugate to biomolecules; (4) minimize overall hydrodynamic size. The stability of QDs in water can be obtained through either a complete ligand exchange procedure, or through steric stabilization where the native hydrophobic surface is coated with amphiphilic molecules and/or polymers [44, 45].

QDs have been adapted to the desired application by conjugation to a recognition moiety, e.g., antibodies, peptides, oligonucleotides or aptamers, or by coating with streptavidin. Several functionalizations have been adapted to the shell layer coating the core, making the core/shell QDs most adaptable for biological applications [46]. In order to suitably functionalize the QDs, there are several methods that have been successfully used for conjugation of QDs to the desired biomolecules. These include electrostatic attraction, covalent linkage, adsorption, and mercapto (-SH) exchange [47]. The choice depends on the features of the biomolecule of interest; for example, thiol-containing biomolecules can be conjugated to QDs via mercapto exchange. In contrast, simple small molecules such as oligonucleotides and various serum albumins were found to readily adsorb non-specifically to the surface of water-soluble QDs. The factors affecting the adsorption are pH, ionic strength, temperature, and surface charge of the molecules [48]. There are three primary ways to target a biocompatible and functional quantum dot: with antibodies, with peptides, or with small molecules. The simplest labeling strategy uses antibodies; the most complicated is that of small molecules, as this approach usually requires more synthetic chemistry. Each approach has its advantages and disadvantages, and no approach is a universal solution.

Chemical conjugation of antibodies to semiconductor QDs is attractive because the proteins of interest can be visualized. For conjugation of QDs to antibodies, the orientation of the antibody on the QD is important given its functionality as a targeting moiety. The conjugation strategy contributes to the control of antibody orientation. For example, the use of biotinylated antibodies and streptavidin-coated QDs provides no control over the orientation of the antibody on the surface of the QD owing to the presence of multiple biotinylation sites on the antibody. Antibody-quantum dot conjugates have been used in a myriad of applications [49, 50]. An immunoassay for the detection of hepatitis B surface antigen [51] conjugated CdTe/CdS QDs to anti-hepatitis B surface antigen antibodies using protein G as a linking bridge, instead of covalently linking the QDs to the antibodies. Other studies developed a microplate immunoassay for detection of the cardiovascular marker C-reactive protein in 104 serum samples, with a limit of quantification of 0.19 μg/l within 1.5 h [52]. A multiplex immunoassay for the simultaneous detection of staphylococcal enterotoxin B and chicken IgY (IgG) in the same well of a 96-well microtiter plate was also undertaken [53]. A multiplex fluoroimmunoassay for the detection of lung cancer markers—neuron specific enolase (NSE) and carcinoembryonic antigen (CEA) in human serum—was recently developed [54]. A wide selection of antibody-quantum dot conjugates is also commercially available. Disadvantages

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to this approach include the availability of antibodies, their selectivity and affinity, and the increased hydrodynamic radius of the quantum dot conjugate. Nonetheless, antibody-QD conjugates are often the method of choice and make up much of the QDs in biology literature.

Peptides can also be conjugated to QDs in a direct approach by linking to thiol-rich domains. A direct binding approach was used to bioactivate and solubilize QDs with phytochelatin-related peptides [55]. Peptide-functionalized quantum dots have been successfully used for targeting cellular proteins such as growth factor receptors, G protein-coupled receptors, integrins, and ion channels [56, 57]. In particular, ~30-50 arginine-glycine-aspartatic acid (RGD) peptides have been conjugated to NIR quantum dots to specifically target $\alpha v\beta 3$ integrins in mouse tumor neovasculature *in vivo* [58], while other studies have relied on high-affinity peptide neurotoxin quantum dot nanoconjugates to image endogenous proteins in living cells and *ex vivo* tissue [59]. Overall, peptide-quantum dot nanoconjugates offer distinct advantages over antibody-mediated targeting, and their potential as biological probes is being actively explored. On the other hand, a single QD can be conjugated to multiple protein molecules, which can be similar or different depending on the intended application. Approximately 15-20 maltose binding proteins, a 44-kDa protein measuring 3 × 4 × 6.5 nm, can be attached to a single 6-nm QD [60]. Because of their brightness and photostability, water-stabilized QDs have been used to track many receptor-mediated endocytic trafficking events in live cells using fluorescence microscopy [61]. For example, QDs conjugated to EGF have been used to track the dimerization of the EGF receptor (EGFR) and its ability to elicit downstream signal transduction events [62]. Biotinylated α-bungarotoxin was bound to streptavidin-conjugated QDs to characterize the assembly dynamics of acetylcholine receptor clusters in postsynaptic membranes [63]. Recently, high-resolution imaging methods in the nanometer range have been developed to image the membrane transport and dynamics of tumor cell proteins during metastasis in living mice using antibody-conjugated QDs [64]. This technology can also be applied to detecting cancer cells in sentinel lymph nodes in whole animals using QDs conjugated to tumor-specific molecules [65]. One can envision several ligand-conjugated quantum dots, with each ligand conjugated to a different size (color) quantum dot, allowing a multiplexed fluorescent assay for drug discovery.

Different schemes have been developed to conjugate ssDNA and dsDNA to the surface of QDs. DNA-QD conjugates retain the selectivity of DNA and the photophysical properties of QDs, allowing detection of single or multiple DNA targets. DNA–QD conjugates require solubility in water, stability under physiological conditions and minimal nonspecific DNA binding to the QD surface. The thiol-modified oligonucleotide can be conjugated to QDs in a direct ligand exchange approach (native cap exchange) where the oligonucleotide displaces the surface-bound mercaptopropionic acid and yields aqueous stable and strongly fluorescent oligonucleotide bound QDs [66]. Applications of QDs include *in vitro* diagnostics, imaging and therapeutics. QDs are used as labels in immunoassays, immunohistochemical staining, cellular imaging and multiplex diagnostics.

5. Cadmium-containing quantum dots as a platform for nanoparticle drug delivery vehicle design

Recently, there has been an explosion in the development of nanoparticle-based drug delivery vehicles composed of lipids, polymers, carbon materials, and even hybrid combinations of those materials tailored not only for the dramatic improvement of the pharmacological properties of existing drugs, but also for enabling the delivery of new classes of potent anti-cancer drugs for gene therapy and immunotherapy [67, 68]. With QDs, a combination of unique physical, chemical, and optical properties facilitates in-depth study of nanocarrier interactions with biological systems through real-time monitoring of QDs biodistribution, intracellular uptake, drug release, and long-term nanocarrier fate. At the same time, compact size and compatibility with a variety of surface modification strategies enables substitution of virtually any QD core with a QD within single-nanoparticle drug delivery vehicles, or incorporation of QD tags within larger multicomponent vehicles. The combination of superior brightness and resistance to photo-degradation represents another set of QD properties that highly useful for long-term nanocarrier tracking.

The great interest in engineering NP-based drug delivery vehicles is driven by the powerful capability of nanocarriers to completely redefine the pharmacokinetic properties of virtually any drug, ranging from small-molecule therapeutics to large proteins and DNA plasmids. Encapsulation of the drug within the nanoparticles keeps it shielded from the biological environment until the moment of carrier degradation and drug release, thus minimizing non-specific and potentially adverse interactions en route to the target [69].

A few reports have appeared recently regarding this ambitious goal. It has previously conjugated captopril, an antihypertensive drug, to the QD surface and studied its pharmaco-dynamics and pharmacokinetics in stroke-prone spontaneously hypertensive rats [70]. The results show that the administered QD-captopril conjugates are capable of decreasing rat blood pressure to the same extent as the captopril alone in the first 30 min, but the therapeutic effect of QD-captopril disappears after 60 min. It is unclear whether the therapeutic effect results from the QD-captopril conjugates or captopril molecules detached from the QD surface. Another piece of interesting work was previously reported, wherein a targeting functionality was added to QDs by linking them with RNA aptamers (A10) that specifically bind to prostate specific membrane antigen (PSMA) [71]. Doxorubicin, a DNA-interacting drug widely used in chemotherapy, was immobilized onto QDs by intercalation within the A10 RNA aptamer [72]. Another study reported the ability of nanoconjugates of CdSe/CdS/ZnS and doxorubicin (Dox) to target alveolar macrophages, cells that play a critical role in the pathogenesis of inflammatory lung injuries. The results demonstrated that nanoparticle platforms can provide targeted macrophage-selective therapy for the treatment of pulmonary disease [73]. This was previously reported regarding siRNA delivery using QDs as delivery vehicles [74].

QDs already play an important role in fundamental biology and *in vitro* disease diagnostics and prognostics. Their unique structural and surface properties, such as their tunable and uniform size, flexible drug-linking and doping mechanisms, large surface-to-volume ratio and wide spectrum of surface reactive groups have enabled a new avenue of research: targeted

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and traceable drug delivery. However, high-quality QDs are mainly made with heavy metals, like cadmium, whose long-term toxicity is currently largely unknown. Despite this limitation, QDs have been applied to cells and small animals as drug carriers, serving as an outstanding discovery tool for drug screening and validation, and as prototype materials for drug carrier engineering [75]. One primary challenge of drug delivery is maintaining a useful concentration of the drug in the targeted tissue while preventing toxicity.

6. Pharmacokinetics of cadmium-containing quantum dots

Nanoparticle-based drug delivery is on its way to overcoming the fundamental limitations of simple free drug formulations, providing means to change their pharmacological properties and also understand their biological fate in great detail. Among many contrast agents for studying nanoparticle-based drug delivery vehicles, QDs are particularly suitable. Their unique amalgamation of useful features, such as small size, versatile surface chemistry, and exquisite optical properties make them an ideal platform for the comprehensive characterization of nanoparticle-based drug delivery vehicle behavior across single-cell to whole organism levels. In this new field, QDs have already made substantial contributions, enabling dynamic monitoring of nanocarrier cell uptake, intracellular distribution, circulation half-times, and biodistribution. Early on, the design of QDs drug delivery vehicles was governed by the intrinsically poor pharmacokinetic (PK) properties of conventional drugs. Low drug solubility, rapid metabolism and clearance and, most importantly, a lack of selectivity, regularly lead to therapeutic failure by causing severe systemic toxicity in healthy tissues, thus prohibiting the dose escalation necessary to eliminate tumor cells. Incorporating these drugs into nanocarriers offers an exciting opportunity to redefine the PK properties, improving therapeutic efficacy and reducing side effects.

When we utilize a nanopharmaceutical, it is important to realize that, in contrast to delivering a drug that is an organic molecule, we are delivering something of a discrete entity in a nanoparticle-comprised of atomic scale parts. Due to the quantum effects and electronic interactions that predominate at the nanoscale, we need to alter the way in which we think about pharmacological parameters in order to adapt to nanoscience. Nanopharmacology is further complicated by the need to establish the behavior of nanoparticles such as QDs within the traditional pharmacological parameters of absorption, distribution, metabolism and excretion (ADME). Nanoconstructs, in many cases, have limited metabolism and excretion and persist in biological systems; this becomes particularly important when toxic atoms such as cadmium are involved. The pharmacological parameters of the behavior of cadmium-containing quantum dots in biological systems is currently still under investigation. Dosing parameters, absorption, distribution, metabolism and excretion require considerable further study, since we have little information on these parameters at this point.

The first factor we need to establish before we study the pharmacokinetics parameter is the dose. Adequate estimates of QDs exposure during treatments such as cancer therapy must be in place so that both pharmacological and toxicological studies can be conducted within a

physiologically relevant dose range. Importantly, how does cadmium content relate to dose? To date, these parameters have not been rigorously addressed. Dose metrics of QDs have been reported in terms of mg/kg (or mg/ml for *in vitro* studies) or on a molar basis, and this will no doubt need refinement. Given that a QD, or any other nanoparticle, is an engineered entity or "mini-reactor", dose via mass or molar number may be an inappropriate descriptor. The length and extent of QDs tissue retention is an important parameter for dosing considerations, since repeated doses may induce systemic accumulation, contributing to potential toxicity. Furthermore, persistence of QDs in tissues demands long-term studies to accurately assess risk. Therefore, the dose is another parameter that requires consideration.

The second factor to establish is the route of administration. This, along with the risks for exposure and possible toxicity should be addressed cautiously to ensure experimental and clinical safety. Moreover, an understanding of possible interactions of QDs within human tissues may shed light on the prevention of health risks in the laboratory and daily exposure to QDs. To date, research has established that various nanoparticles can enter an organism through skin, inhalation, oral delivery, and parenteral administration. Intravenous injection is a major route for drug administration. It has been reported that intravenously injected QDs may accumulate in unintended tissues, which implies that the potential toxicity might result from a failure to clear them from the body [76]. The possibility of tissue uptake of QDs via ingestion following exposure has also been proposed in animal and cell culture models. Skin is another important portal of entry for nanoparticles as potential route of systemic drug administration.

The abilities of nanoagents to penetrate the skin barrier are debatable. Some studies indicate that NP penetrate skin barrier under skin lesions, UV sunburn or ultrasound [77]. The potential beneficial and/or side effects of intradermally accumulated NP primarily depend on their localization in the tissues. It is also important to elucidate further migration pathways of particles, as this would determine their pharmacokinetic properties and systemic distribution in the organism. Topically applied QDs were reported to accumulate in the dermis and muscles [78]; however, a more detailed localization of particles has not been described. Other studies reported that the majority of the intradermally injected QDs remained at the injection site and a minority of QDs migrated into lymph nodes, highlighting QDs advantages for lym-phatic imaging [79, 80]. However, high retention of QDs at the injection site raises concerns about long-term physiological effects. There is a lack of in-depth analyses of QDs localization and migration pathways in the dermis. It was recently reported that the subcutaneous injection of CdSe/ZnS coated with mPEG-5000 polymer into the CDF1 of mice showed that basement membrane and dense connective tissue fibers limited the diffusion of QDs in the dermis [81]. Negligible QDs penetration into the epidermis, hair follicles, sebaceous and sweat glands, nerves and blood vessels was also observed. However, low permeation of QDs through the tissues could result in slow clearance and raises the risks of potential immune, inflammatory and cytotoxic responses.

The cornea is another common route of drug administration and also an important route of nanoparticle exposure in occupational scenarios and daily life. However, the possibility of QDs penetration into the cornea has not been discussed before. It has been previously demonstrated

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that specific QDs influence corneal stromal cell viability up to a significant magnitude of 50% in a relatively low concentration (5-20 nM) and under a short exposure period (24-48 h) [82]. QDs can also be retained in the cornea up to 26 days in an *in vivo* mouse model. Therefore, since corneal stromal cells are crucial for the maintenance of the health and transparency of the cornea, potential QDs cytotoxicity due to bioaccumulation is a major concern given the potential threat to corneal health.

Absorption via tissues and biological fluids is generally the first hurdle to be met and this is, of course, dependent on route of delivery. Currently, the most important route of delivery for QDs appears to be systemic distribution through parenteral delivery [83]. Nanoparticles are considered to absorb more effectively into the respiratory, skin, and gastrointestinal systems than micron-sized particles because of their unique physicochemical properties, such as their size and surface modifications [84]. For example, instillation of 5 nm QDs shows faster translocation to other organs than in the case of 27 nm QDs [85]. In addition, a recent study showed that nanoparticles were only translocated into the circulation system when administered into the lung; their absorption rate varied, depending on their surface properties [86]. When different sizes of polystyrene QDs were administered orally to rats, the absorption of 50–100 nm polystyrene QDs was about 250-fold higher than the absorption of larger microparticles (500 nm, 1, and 10 μm) [87]. Oral administration of different sizes of colloidal gold particles to mice showed size-dependent absorption [88]. Regarding surface modification, positively charged particles show better absorption than neutral or negatively charged particles [89]. However, in a recent study using gold nanoparticles, negatively charged particles showed higher absorption rates than positively charged particles [90]. Therefore, no general rule can be made about absorption from these results and further studies on the impact of surface modification on gastrointestinal absorption are required. Studying the kinetics of nanoparticles is an important issue in nanotechnology.

Regarding distribution, one of the first elements that parenteral delivered QD will encounter is the environment of the blood. Here, we have little to no information about blood/QD interactions. Plasma half-life is no doubt related to surface coating and addition of biological targeting ligands, if any. For example, PEG coating was reported to increase plasma half-life [91]. In the case of oxide nanoparticles, many are coated with endogenous plasma proteins immediately upon entry into the circulatory system and this appears to enhance tissue delivery. However, the interactions between QDs and plasma proteins are unknown. Immune responses may also be triggered at this level, as reviewed by [92], but the precise interactions of QDs at this level have not been examined. Recently, some studies have investigated the *in vivo* biodistribution of QDs [93-95]. All of the reports in the literature unanimously conclude that QDs show a preference for deposition in organs and tissues and that they do not remain circulating in the bloodstream [96]. Real-time imaging of live animals treated with the hyaluronic acid (HA)-conjugated QDs showed that the luminescence of NIR QDs could be detected for up to 2 months [97]. Others reported that QDs coated using an amphiphilic poly(acrylic acid) polymer remain fluorescent after at least four months *in vivo* [98].

Organ-selective biodistribution and elimination routes of synthesized QDs coated with 3-mercaptopropionic acid (QD MPA) and commercially available Qtracker 705 nontargeted

quantum dots with poly(ethylene glycol) coating (QD PEG) were recently compared after intravenous injection to mice. QDs were deposited mainly in liver, spleen, kidney and lymph nodes [99]. Another study observed that, in the murine model, quantum dots (CdSe 100 pmol/kg) administered by intravenous injection mainly accumulated in the red pulps of the spleen, portal areas of the liver, and adrenal glands of the kidney within 1 h. It is believed that quantum dots do not degrade, as evidenced by the intensity of the fluorescence in the fluorescence spectrum image and comparable intensity profile of Cd [100]. One consistent theme in the existing literature is that QDs are eventually taken up by the reticuloendothelial system, including the liver, spleen, and lymphatic system [101]. On the other hand, it has been found that aqQDs are initially accumulated in liver after short-time (0.5- 4h) post-injection, and then are increasingly absorbed by kidney during long-time (15 – 80 days) blood circulation. Moreover, size-dependent biodistribution is obviously observed: aqQDs with larger sizes are more quickly accumulated in the spleen [102]. We have recently found that after i.p. injection, the CdS/Maltodextrin quantum dots mainly accumulated in the liver, kidney, spleen, thymus, intestine, lung and brain (Figure 3). The pharmacokinetics studies showed the presence of CdS/Maltodextrin in kidney 30 min after administration; at 18 h, the presence of quantum dots in kidney was more abundant. However, the amount of CdS/Maltodextrin quantum dots in liver was lower than in kidney, and the maximal presence was at 3 h. Our results suggest that the transition of CdS/Maltodextrin quantum dots via the liver is very fast, and the main route of elimination is renal (Figure 4).

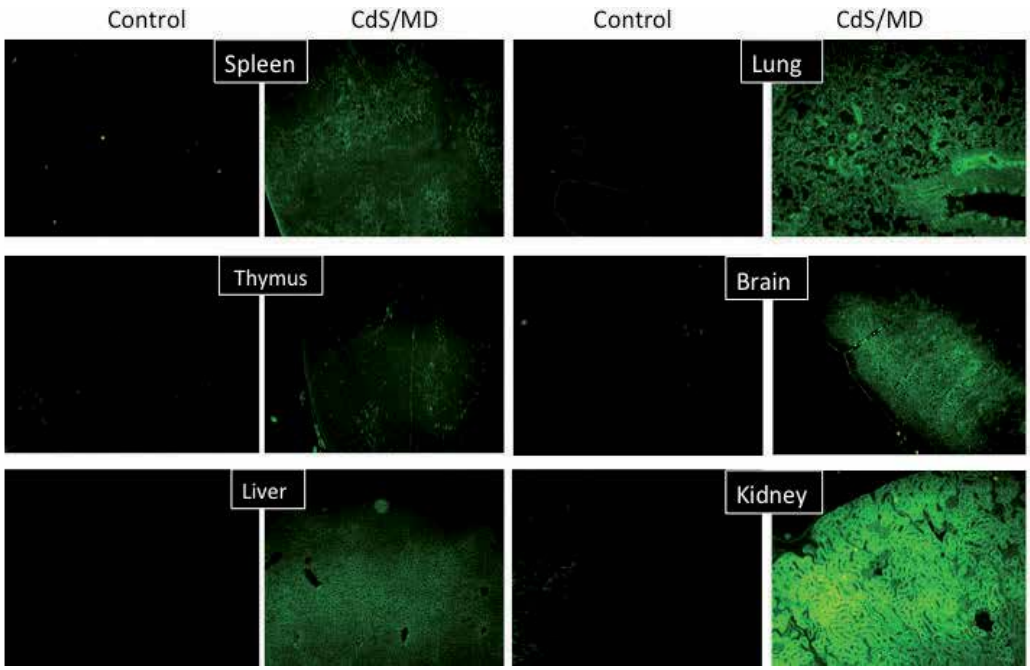

Figure 3. Biodistribution of CdS/Maltodextrin in Wistar rats. Tissue distribution of CdS/maltodextrin quantum dots after i.p. administration of 10 µg during 8 days. Quantum dots were observed in all analyzed tissues, including brain. The maximal observed presence of quantum dots was in lung and kidney.

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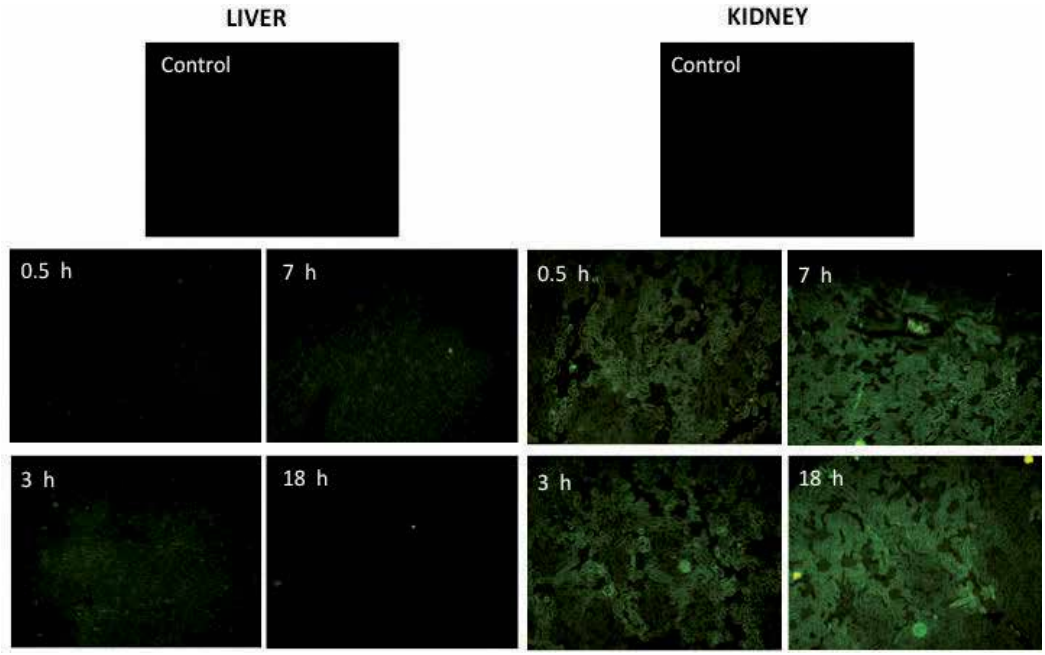

Figure 4. Biokinetics of excretion of CdS/maltodextrin quantum dots after a single i.p. administration of 10 μg in kidney and liver.

The blood–brain barrier (BBB), is a critical interface and acts as a physical and metabolic barrier between the CNS and the peripheral circulation that serves to regulate and protect the microenvironment of the brain. The primary function of the normal BBB is to establish and maintain homeostasis in the CNS [103]. Under normal physiological conditions, BBB prevents transport of bacteria, large molecules, and most small molecules into the brain. To be BBB permeable, molecules need to be lipid soluble and less than 400 Da in size. There are more than 7000 drugs in the comprehensive medicinal chemistry database, and only 5% of these drugs can be used to treat the CNS diseases. In a related study, 12% of all drugs are shown to be active in the CNS, but only 1% of all drugs are active in the brain for diseases other than affective disorders [104]. The importance of developing new approaches to brain drug development is illustrated by considering the limitations of the existing brain drug delivery strategies. Therefore, there is great interest in nanomedicine to develop optimized traceable drug-loaded nanoparticle formulations that can be safely used to facilitate drugs across the BBB for treating brain diseases and, at the same time, visualizing the distribution profile of the nanoparticles in the brain. The use of QDs bioconjugates as efficient targeted probes for transmigration across the BBB has been previously demonstrated [105]. Since the transferrin receptor protein is highly localized on the endothelial surface of the brain, transferrin (Tf) was selected to trigger receptor-mediated transport across the BBB. It was discovered that the migration rate of Tf-conjugated QDs crossing the *in vitro* BBB is both concentration-and time-dependent [106]. It was recently demonstrated that the QDs-Tf-Saquinavir nanoformulation increases the drug solubility, enhancing systemic bioavailability, while the excellent optical properties of QDs

also visualize the distribution and accumulation of nanoplexes in brain [107]. Such primary results offer a basis for the development of novel QDs with targeting molecules and drugs, which could enhance drug delivery efficacy across the BBB and facilitate the uptake of the QD-drugs in the brain. Despite the encouraging results on the use of QDs for drug delivery across the BBB, there is still serious concern regarding QDs toxicity *in vivo,* which mainly originates from the intrinsic, potentially toxic nature of the semiconductor materials themselves.

On the other hand, the placental barrier protects the embryo from various chemical agents and other foreign substances in the body. However, the passage of xenobiotic molecules through the placental barrier is not completely prevented, and drugs may affect fetal cell proliferation, embryonic growth, and organ formation [108]. Knowledge regarding embryotoxicity is of great importance because it is a necessary part of the toxicological profile that must be established for any new biologically active substance relevant to human safety. There are many studies in this field [109-111], but the detailed effects of NPs still pose many questions. A decrease in embryonic weight after QDs injection on the sixth day of embryogenesis (CdSe/ZnS QDs, 9.6%; CdT QDs, 6.2%) has been reported, and no embryotoxic or teratogenic effects were observed at any stage of embryogenesis [112]. Others have shown that NPs (nSP and TiO2s 70 nm and 35 nm in diameter, respectively) can cross the placental barrier in pregnant mice and cause neurotoxicity in their offspring [113]. They showed that the NPs were found in the placenta, fetal liver, and fetal brain. Other authors argue that some NPs (CdSe and CdTe/CdS) in different sizes, at different dosages, and with different outer capping materials can increase the rate of early-stage blastocyst death in mice and can be potentially transferred across the placenta to the fetus [109, 114]. These studies show that NPs can enter the embryo through the placenta, which is a natural barrier for a large variety of organic substances with diverse molecular structures. The NPs, which appear in the maternal body during pregnancy, can cross the placental barrier and may even cause developmental deformities. Recently, we demonstrated that CdS-MD nanoparticles were embryotoxics in a chicken embryo model (Figure 5) [42]. The nature of the observed abnormalities suggests that these effects could be directly associated with concentration. Therefore, according to the observed effects, the prolonged accumulation of quantum dots in the maternal organism may increase the risk of adverse effects on embryo development.

The liver is know to have a high capacity to metabolize and degenerate a multitude of xenobiotics. The binding of proteins plays a key role in delivering xenobiotics from plasma into the liver. In addition, the reticuloendothelial system (RES) can phagocytose particles larger than 100 nm and then transport the particles into the liver in vivo. Metabolism of QDs is yet another understudied aspect of cadmium QDs. The QD cores do not appear to be subject to extensive enzymatic metabolism, but shells and coatings are. The extent of metabolism of the shell and coating becomes critical for toxicity, since they shield the more toxic CdSe or CdTe cores from the intracellular environment. Rather than metabolism per se, degradation of the shell and coatings within the biological environment appear to be more important. QD shells and coatings appear to degrade under photolytic and oxidative conditions, yet we know little about the degradation products or their biological effects, which may regulate release of toxic cadmium cores. The behavior of nanoparticles *in vivo* depend on properties such as the

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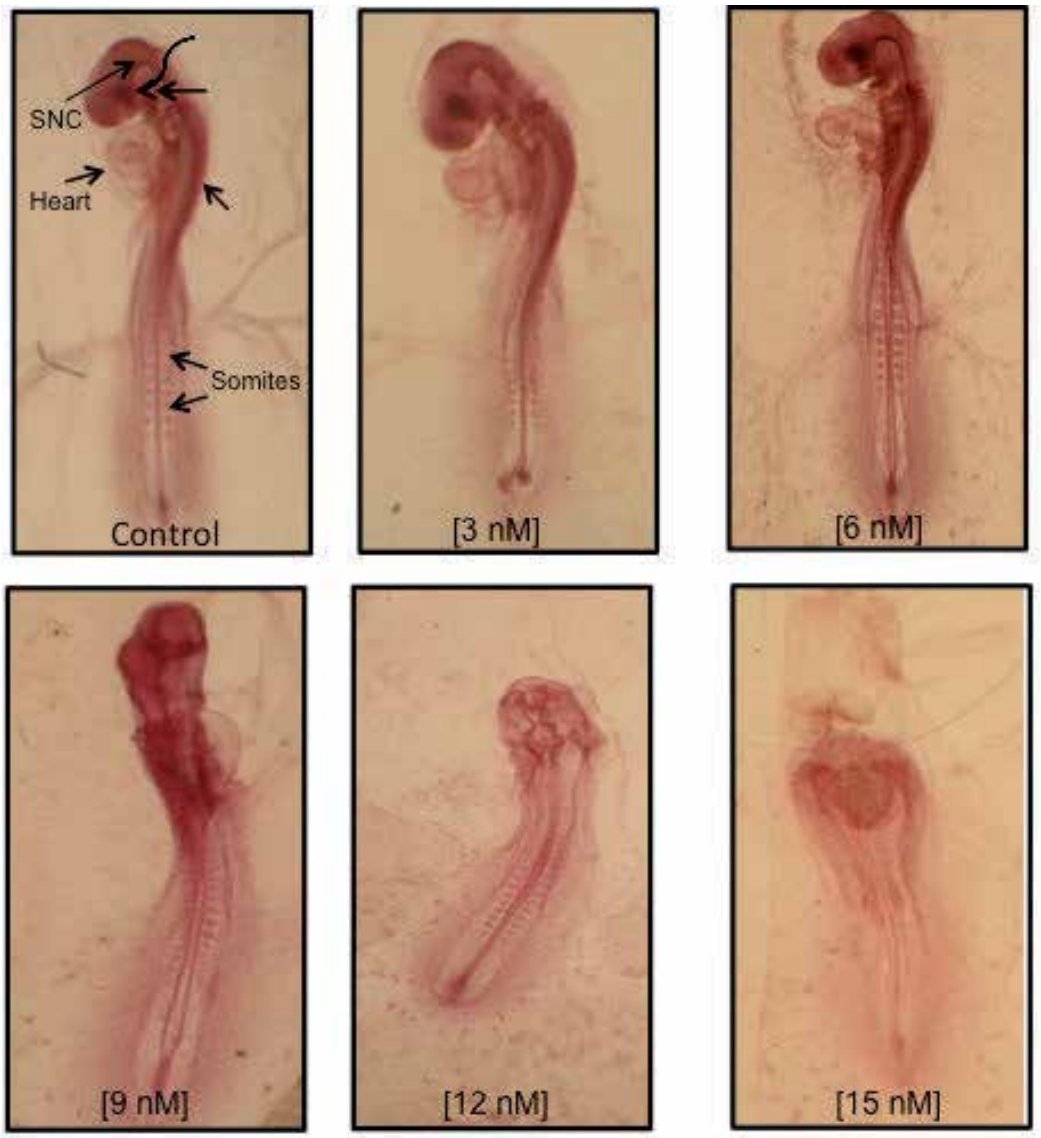

Figure 5. Photographs of 72 h-old chick embryos treated with CdS/Maltodextrin. Observed alterations were dose dependent.

nanosize (surface area and size distribution), chemical composition (purity, crystallinity, electronic property, etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings, etc.), solubility, shape, and aggregation [115]. It has been suggested that the metabolic paths of QD nanoparticles are closely correlated to their aggregation states, and three metabolic paths were disclosed after intravenous injection: 1) the QDs that maintained their original nanosize without binding *in vivo* were rapidly excreted via the kidney; 2) some QDs binding to proteins were translocated to the liver and were excreted with feces; 3) a small

fraction of the QDs aggregated to larger particles and were retained in liver tissue for a long time [116].

Several studies suggest that the kidneys can remove QDs that are less than 5 nm. It has been observed that, after i.v. administration of CdSe/ZnS-QDs, only 10% and 40% of the injected dose was found in the kidney and liver respectively, suggesting that only a fraction of the total QDs dose passed through this route [117]. Another study quantitatively detected the excretion of QDs in mice feces and urine after i.v. injection of CDSe QDs. The speed of excretion was quicker via feces, and the peak (0.214 ng of Cd) occurred 6–12 h postexposure. The excretion from urine was relatively delayed, and the peak (0.174 ng of Cd) was 24–36 h post-exposure [118]. The elimination of silica-coated CdSe QDs (~5.5 nm) from the body via feces and urine occurred in its totality after 5 days. Yet many reports propose that a portion of the administered QD dose may not be excreted and remains in the tissues. The extent of excretion and the extent of persistence in tissues take on added importance when one considers the potential delivery of QD as a cancer-targeting drug. More comprehensive studies of potential excretion will therefore be critical to QD development as a nanopharmaceutical.

Numerous studies have addressed the cellular level, but these are often difficult to compare due to varied dosing parameters and lack of physicochemical particle characterization. In general, it appears that most QDs examined found ready uptake at the cellular level, primarily via endocytic mechanisms, which depends upon the surface ligands coated over the QD surface [119, 23]. It has been reported that CdSe QDs and CdTe QDs that enter the cell, are visible at the cell surface and in the cytoplasm after a short time. QDs are likely to bind to the cell surface due to their interaction with cell surface glycoproteins and glycolipids [66]. Employing red-and green-emitting cationic QDs, it was found that the CdTe QD distribution was in part dependent on nanoparticle size. In the murine microglial N9 cell line, red cationic QDs (5 nm) were distributed throughout the cytoplasm. In contrast, green and also positively charged QDs (2 nm) were often found in the nucleus of N9 cells upon 1 h of QD exposure [120]. It has been shown that green QDs coated with tri-metoxysilylprolyl urea and acetate groups bind with high affinity in the cell nucleus of mouse 3T3 fibroblasts [121]. In human mammary epithelial tumor cells (MDA-MB-231) green-emitting CdSe/ZnS/SiO2 QDs were packaged in large vesicles found in the perinuclear region [23]. Confocal images have showed that MPA-coated QDs were distributed inside the cytoplasmic region of cells. In contrast, GA/TOPO-coated QDs were not found inside cells. These results indicate that cellular uptake of QDs depends upon the hydrodynamic size of the QDs as well as surface coating material [122]. These results strongly suggest that surface coatings can improve cytocompatibility and, consequently, decrease toxicity. Previous studies have shown that long-term exposure of surface coated QDs to their bioenvironment can destabilize the binding strength of the surface molecules, which in turn can yield unprotected QDs inside or outside the cells [123]. Therefore, the stability and binding strength of surface molecules over the QD surface define the cyto-compatibility of the QDs and hence their cytotoxicity. Thus, according to previous studies: (1) the surface coating strategy could improve the cytocompatibility of QDs, (2) surface molecules could determine the intracellular uptake and consequent cytotoxicity of QDs, and (3) intra-cellular uptake of QDs could depend on their hydrodynamic size.

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7. Strategies for safe drug delivery using cadmium-containing quantum dots

Discussion regarding the toxicity of cadmium-containing QDs can be somewhat confusing because of the diversity QDs being synthesized. Besides and as we have mentioned before, we have to consider that not all QDs are alike. Each individual type of QD possesses its own unique physicochemical properties, which in turn determine its potential toxicity or lack thereof [124]. In general, there are discrepancies in the current literature regarding the toxicity of QDs and these can be attributed to several factors: the lack of toxicology-based studies, the variety of QD dosage/exposure concentrations reported in the literature, and the widely varying physicochemical properties of individual QDs.

Up to date, toxicity studies have been conducted on a variety of both human and non-human cells and cell lines; research has been focused on *in vitro* assays of cytotoxicity [125-131]. *In vitro* studies are very important and can serve as background data to inform the design of *in vivo* studies but, on their own, they provide an insufficient basis for a complete risk assessment. Administration of QDs in animal models has revealed that QDs induce: (1) accumulation of QDs in specific organs [23, 132, 133], (2) excretion in urine, bilis and feces [134], (3) toxicity in selective organs [135], (4) embryotoxicity [109, 111], and (5) oxidative damage [136-138]. Importantly, and a potential source of confusion in assessing QD toxicity, the latter depends on multiple factors derived from both individual QD physicochemical properties and environmental conditions: QD size, charge, concentration, outer coating bioactivity (capping material, functional groups), and oxidative, photolytic, and mechanical stability have each been shown to be determining toxicity factors. Therefore, all these aspects should be extended to examine alternate QD formulations, compositions, and shapes to help facilitate any future generalizations regarding size thresholds in the regulatory context. There are only a few studies specifically designed for toxicological assessment (e.g., dose, duration, frequency of exposure, mechanisms of action). Many of the studies, from which QD toxicity information is derived, it has been cited in reference to it were performed by nanotechnology researchers rather than toxicologists or health scientists. It is therefore difficult to extrapolate the results of such studies in order to reach any conclusions regarding the health and safety of QDs. Nonetheless, these studies may provide important insights that will be useful in guiding the eventual design of standardized toxicity tests and protocols.

The wealth of data accumulated from QD toxicity studies is an invaluable asset that should be exploited to design appropriate methodologies to further assess the toxicity of novel QDs. Researchers often neglect to carry out a comprehensive characterization of QDs prior to using them. In our opinion, this step is absolutely necessary, especially before any toxicity screening is started, precisely because the exact property or properties of QDs responsible for said toxicity are still poorly understood. This omission is one of the reasons behind the current state of confusion surrounding this issue. As epigenetic changes may lead to long-term reprogramming of gene expression long after the initial insult has been removed, results from "nanoepigenetic" assessments may have important implications on the future use of new

nanomaterials in bioimaging and therapeutic applications. They should be evaluated early in the development of new QDs as well as QD-based devices and clinical tools. Future QD toxicity studies should be standardized and systematized because methodological variability in the current body of literature makes it difficult to compare and contrast results. We advocate the following steps for consistent, comparable toxicology data: (a) standardize dose metrics, (b) characterize QD uptake concentration, (c) identify *in vitro* models that reflect how the QDs QDs interact with cells *in vivo*, and (d) use multiple assays to determine sublethal toxicity and biocompatibility. Proceeding without careful evaluation of these critical areas will blunt the progress of nanomedicine and place human health at risk. However, judicious further research into these areas will undoubtedly contribute to development of nanopharmaceuticals for cancer treatment and drug delivery that have minimal to low risk and can highly benefit public health.

8. Conclusion

Cadmium-containing QDs are leading the way toward new preparations that can overcome the fundamental limitations of simple free drug formulations, providing the means to change their pharmacological properties and also understand their biological fate in great detail. However, ADME properties depend on multiple factors derived from both inherent physico-chemical properties and environmental conditions. The findings also suggest that, under certain conditions, QDs may pose risks to human health, as determined by rodent animal models and *in vitro* cell cultures. This review outlined the unique features that make QDs an ideal platform for nanocarrier design and discussed how this model has been applied to study vehicle behavior for diverse drug delivery applications. However, it is clear that to make such a goal feasible and relatively risk-free for human beings, more extensive pharmacological and toxicological research of QDs are needed.

Author details

Lourdes Rodriguez-Fragoso[1*], Ivonne Gutiérrez-Sancha[1], Patricia Rodríguez-Fragoso[2], Anahí Rodríguez-López[1] and Jorge Reyes-Esparza[1]

*Address all correspondence to: mrodriguezf@uaem.mx

1 Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Morelos, Mexico

2 Departamento de Física, CINVESTAV-I.P.N Apartado, México

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Chapter 15

Anticipating Market Introduction of Nanotechnology-Enabled Drug Delivery Systems

Haico Te Kulve

Additional information is available at the end of the chapter

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1. Introduction

Nanotechnology-enabled drug delivery systems (NDDS) are associated with high expectations regarding their economic and societal value. NDDS are expected to contribute to important issues in healthcare such as enabling novel pharmaceutical therapies which only target the site of the disease and help to reduce costs of healthcare. To date, more than two dozen NDDS have been developed into marketed products and many more are under development [1]. Market forecasts have estimated that the market for these technologies will grow from US$ 1 billion in 2010 to US$ 136 billion in 2021 [2, 3].

The pronounced expectations surrounding nanotechnology-enabled drug delivery applications and its claimed market potential suggest that the path toward market introduction is clear. This is however not the case, as is often with newly emerging technologies. While for instance claims have been made about NDDS contributing to a reduction of undesirable side effects of drugs (compared to conventional delivery systems), to date limited clinical data is available to actually support such claims. Uncertainties about the application of regulatory regimes and which methodologies to apply in order to assess novel nanotechnology-enabled drug carriers have created further challenges for firms to introduce new products on the market.

Making promises is almost inevitable in order to attract attention and mobilize resources. However, too broad promises may make sponsors such as large pharmaceutical companies and venture capitalists reluctant. Uncertainties about the performance of specific future drug delivery systems, the demand for such technologies and how they will be evaluated by

regulatory authorities have contributed to impasses, 'waiting games' as we have called them [1, 4] which constrain development and potential market introduction of NDDS.

In such uncertain and ambiguous situations actors need to make sense of what is happening already and what might happen [5], before taking a specific course of action. There are two main strategies available to developers of NDDS to handle this challenging situation. Academic entrepreneurs and firms interested in developing and introducing NDDS on the market may view uncertainties regarding performance and value inevitable and/or postpone such discussions until a later stage and wait for the 'invisible hands' of the market to do its proverbial work. Or, they can anticipate reception of new products and interact with the broader environment to work towards the market introduction of what they consider to be desirable products.

Pro-active interactions will have to go beyond the promotion of promises of nanotechnology-enabled drug delivery systems in anticipation of the opening up of new markets. Market success of newly introduced NDDS products involves more than attractive sales figures. Deuten et al. [6] argued for broadening the notion of market success by thinking in terms of what they call 'societal embedding'. They characterized societal embedding by three dimensions (p. 132): (1) Integration: new technologies need to be integrated in industries and markets; that is, within business practices and repertoires of users, in this case clinicians and patients. (2) Admissibility: new products need to be acceptable according to rules and standards within the sector or set by the government. Think for instance of good manufacturing practices, pre-clinical testing and clinical trials. (3) Acceptance: new products have to be accepted by the public. That is, societal concerns should not be too strong, there should be sufficient articulation in order to make well-informed choices by clinicians and patients, and the product should actually be used.

Pro-active action then requires taking a broader perspective than that of a single actor, saying an academic researcher or a start-up firm with a patent in the field of NDDS. Societal embedding involves a variety of issues which create openings for different actors - who have different interests in, and perspectives on, emerging NDDS - to engage in strategic actions and interactions. In the drug delivery sector, firms, governmental bodies, health insurers, scientists, clinicians and patient organizations are all more or less involved in interactions with respect to one or more dimensions of societal embedding. Thus, important dynamics related to the market introduction of NDDS exist at the level of the drug delivery sector rather than at the level of individual academic or business entrepreneurs. Put differently, for the market introduction of NDDS entrepreneurial individuals and organizations are dependent on interactions with other players in the sector which is beyond their full control. Pro-active action then requires understanding of what happens at the level of the domain and feeding back such insights into individual or collective strategies to further development and market introduction of NDDS.

Anticipation on future market introduction and embedding may seem the wiser option compared to trial-and-error strategies, but is also difficult and precarious. Anticipation on how

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new technologies become embedded is difficult in itself, due to uncertainties regarding how new products will eventually look like and their impacts. Anticipation of how other involved actors perceive and cope with parts of the embedding process and what this means for individual actors' strategies then introduces further complexities. Yet, taking into account these perspectives and strategies is exactly what is important in the case of the drug delivery sector, which consists of an intersection of different value chains with a large number of mutually dependent actors. Then how to anticipate future market introduction and embedding of nanotechnology-enabled drug delivery systems? How to support articulation of anticipatory strategies and decision making of, say academic and business entrepreneurs, taking into account other actors' perspectives in the domain of drug delivery systems?

The question of anticipation of future introduction and embedding of NDDS is a common challenge of emerging technologies. Within the field of technology assessment a number of approaches have been developed to deal with uncertainties of emerging technologies emphasizing interactive anticipatory approaches such as real-time technology assessment [7], anticipatory governance [8], interaction research in lab-settings [9] and constructive technology assessment [10]. Such approaches are devised to support stakeholders in their anticipatory competences and to support strategies and decision making. These approaches differ in terms of scope, i.e. which dynamics and actors are taken into account, and in their main target audience, i.e. whose strategy articulation and assessment is actually supported.

In this chapter I will focus on the approach of Constructive Technology Assessment (CTA) which has a particular emphasis on exploring future developments with stakeholders in a domain and feeding insights back to researchers and technology developers. This approach is by now well established and has been applied for different nanotechnologies and their applications. I will describe the methodology of CTA scenario workshops and demonstrate the approach by offering the results of a study [11] where this approach was used to map and support anticipation of opportunities and challenges of nanotechnology-enabled drug delivery technologies.

In section 2 I will start with offering a general perspective on how different types of actors perceive and assess emerging technologies. This is important to recognize when interacting with a variety of involved actors and forms the backdrop against which I position the methodology and approach of Constructive Technology Assessment and its scenario workshops. I will describe the CTA methodology and discuss how to organize and prepare for such interactive workshops. In section 3 I will set the scene for the workshops by briefly describing the main actors involved in the drug delivery sector and the promises of NDDS. In section 4 I will report on the preparation of the workshops and discuss in detail the main lines of discussion in the workshop and participants' assessments of the situation in which the drug delivery sector finds itself regarding emerging nanotechnologies. In section 5 I will conclude by summarizing main findings and reflecting on the merits of the CTA approach for anticipating, and supporting anticipation of, market introduction of nanotechnology-enabled drug delivery systems.

2. Constructive technology assessment & scenario workshops

2.1. Perspectives on emerging technologies

There are structural differences in how actors perceive and interact with novel technologies. In our modern societies there exists an asymmetry between those who develop new science and technology and those who are impacted by these developments. For one this is related to a difference in timing between the development and actual introduction of new technologies; for another it is related to differences in involvement and perspective of a variety of actors which to some extent is institutionalized in a historically grown division of labour regarding novel technologies. That is, there exists a separation between individuals and organizations involved in either the generation or the uptake – 'selection' - of technologies. For instance, technology actors have had 'a mandate' to develop new technologies and could confront society with new technologies when linked with ideals of progress. Even if this mandate is not taken for granted anymore, it has led to institutions and divisions of labour with respect to the promotion and selection or regulation - 'control' - of new technologies which cannot easily be undone [12].

For understanding actors' perspectives and interactions regarding (emerging) technologies the actor typology developed by Garud and Ahlstrom [13] is helpful. Garud and Ahlstrom emphasize the structural difference in the ways actors assess technologies. They relate differences in views and action perspectives to two different positions: insiders and outsiders with respect to technologies. To emphasize the difference in position and style, rather than inside/outside boundaries, the terms enactors and comparative selectors have been proposed [14].

As we formulated in the yearbook Nanotechnology in Society [15], enactors, i.e. those who promote and aim to realize novel technologies "construct scenarios of progress, and identify obstacles to be overcome. They thus work and think in 'enactment cycles' which emphasize positive aspects. This includes a tendency to disqualify opposition as irrational or misguided, or following their own agendas. For nanotechnology, enactors now also anticipate obstacles similar to the ones which occurred for GMO (Genetically Modified Organisms) in agriculture and food, cf. Colvin [16]."

Enactors will identify with a novel technology and its applications such as nanotechnology-enabled drug delivery systems and may believe that the world is waiting for these products, e.g. because of its attractive performance characteristics and 'a good product sells itself'. However, for 'the world' the product is just one of many options and it may see alternatives. This group takes a position of comparing and selecting different technological options, thus act as 'comparative selectors'. While enactors may downplay considerations regarding costs and risks, selectors will often take broader evaluation frames where these considerations are put upfront [13: 40].

There can be different types of such so called 'comparative selectors'. This act of 'comparative selecting' can be done on a professional basis, such as health insurance authorities deciding which novel drugs are worth reimbursing and to be included in insurance packages on the

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basis of cost-benefit analysis, or the Food and Drug Administration deciding whether or not to grant market access to novel drugs. It can also be done by actors who are less tied to certain methods such as patients and their individual experience with specific medication. Public interest groups may act as selectors, for instance organizations which have opposed the introduction of GMOs or those who have called for more control and regulation of nanotechnologies such as the ETC Group [17].

These different positions may, and sometimes have to, interact with each other. For instance in the case of drug delivery, firms aiming to introduce novel drugs will at some stage have to interact with the US Food and Drug Administration and/or the European Medicines Agency or other regulatory body. Firms acting as enactors of drug delivery systems will have to act with pharmaceutical companies who (then) may act as selectors, choosing between different delivery devices to be used for their pharmaceutical agent. They may also meet each other at conferences or in dedicated discussion platforms which may be set-up to foster mutual understanding.

Actors pursuing promotion activities and actors pursuing selection activities will interfere anyway, eventually. The next step then is to identify where and when these activities interfere and what happens there. That is, what do these different actors learn at these occasions from each other. How does that shape how they view the novel technology under consideration and what does this imply for their strategies regarding development or uptake of novel technologies. Garud & Ahlstrom (1997) call such occasions 'bridging events' and discuss some examples.

The important point here is that such bridging events can be created on purpose. This can be done by technology enactors themselves who for instance engage in market research, or by selectors such as regulatory authorities who invite sponsors of new pharmaceutical therapies to discuss their future products and how to evaluate them. It can also be done by academic researchers or more disinterested actors who are working with the perspective of Constructive Technology Assessment (CTA).

2.2. Constructive technology assessment

The approach of Constructive Technology Assessment (CTA) has been developed since the 1980's and has become a key methodology within the field of technology assessment. It aims to broaden design, development and implementation processes rather than only assess impacts on novel technologies [18]. In CTA, technologies and their impacts are not seen as given. "For CTA, the dynamics of the process are central, and impacts are viewed as being built up, and co-produced, during the process of technical change. Many technology studies have shown that impacts are not just passive effects of a given technology on its environment, but are actively sought (or avoided) by technology producers, users, and third actors such as governments, unions, and pressure groups alike" [18, p. 257]. Technologies and their impacts co-evolve, and actors involved try to shape this process and make assessments of what is happening or could happen.

CTA does not aim to introduce assessment – as enactors/selectors are making assessments the entire time- but rather to modulate ongoing processes of assessment and feedback into actor decisions and strategies with respect to technology development and introduction. In particular it aims to broaden actors perspectives by offering an overview of actors and aspects involved in development and embedding of emerging technologies [19]. Second, it aims to enrich actors understanding of the dynamics of such processes, for instance the role of reimbursement in health care innovations. By broadening and enriching perspectives of actors, CTA interventions aim to support individuals and organizations in identifying their role and impacts in the overall innovation processes. This helps actors to evaluate effects of their strategies and consider what they may need to change in their activities in the present and near future in order to work towards desirable outcomes (for instance to improve chance of market success of new NDDS).

While CTA events are an intervention, they are also a tool to understand what is happening in a particular domain of technology. They provide an entrance point to elicit perceptions of enactors and comparative selectors in an interactive setting. As we formulated it [15], it is creating and orchestrating spaces where interactions occur, even if the interactions between citizens/consumers and technology developers and promoters will always be partial (because of their difference in perspective). There will be "probing of each other's realities" (as Garud and Ahlstrom (1997) called it), with more or less contestation.

The CTA workshop which convenes stakeholders in a particular domain, is a micro cosmos which reflects parts of the macro cosmos, in this case the drug delivery sector, through participants' interactions and their assessments of the force fields in which they find themselves. The workshops provide a space in which actors with different socio-cognitive positions, which I summarized as enactors and comparative selectors, can interact. Thus, the temporary space is a bridging event, and is designed as a bridging event.

Within this general framing, CTA workshops are tailored towards stimulating actors' anticipation of embedding through broadening and enriching actors' assessments of ongoing dynamics, and actors' articulation of possible embedding strategies. Facilitating interactions, especially mutual 'probing', between enactors and selectors is one of the mechanisms. At the same time, interactions between enactors and selectors offer insights into what is happening in a domain. Supported by careful preparation – 'pre-engagement' [20] – CTA workshops then provide a 'window on the world' to the participants; their world as it is, and might be in the future. The articulations in this micro cosmos then will offer a view of potential developments in the domain. On the other hand, the temporary (and protected) space of the workshop will not fully reflect the force fields in the macro cosmos. Still, the patterns that are found in actors' articulations and their assessments of force fields affording actions, offer good indications. One reason is that participants probe into or comment on each other's positions and considerations, introducing checks on what happens in the drug delivery sector.

In interactive workshops, probing and commenting can be supported by socio-technical scenarios. In the case of nanotechnologies, socio-technical scenarios are necessary to address their doubly fictional character [15]. Many of the expected applications enabled by nanotechnologies (and nanosciences) are still envisioned, part of 'science fiction'. The eventual impacts

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of such applications are unclear, and attempts to find out about impacts amount to social science fiction. Socio-technical scenarios capture ongoing dynamics and develop assessments of future developments. They show the effects of interactions between enactors and selectors which provides more substance to interactions in workshops as actors can draw upon the scenarios for inspiration.

The use of scenarios and interactive workshops has further effects. They provide participants in workshops with competences to support anticipation and strategy articulation. Tools such as scenarios, which are based on insights in ongoing dynamics and debates during interactive workshops, provide actors with understanding of the overall situation and clues for how to take into account ongoing developments and future impacts. So, while actors will likely value anticipation of embedding as a prudent strategy relevant for their own activities, they now are also provided with some skills to fill in such strategies.

2.3. Workshop design and preparation

To prepare for a CTA workshop, the actual 'engagement' between stakeholders, the organizers of this event need to prepare themselves, or 'pre-engage' with the technologies and domains under consideration, in this case NDDS in the drug delivery sector. Preparing for the workshops clearly includes an organizational component, such as identifying possible collaborators, preparing input documents for the workshops themselves, and interactions with participants and actors potentially interested in participating in the workshop. Preparing also requires analysis to support anticipation in a situation filled with uncertainties. This helps to focus the discussion on key issues and be more productive while at the same time the organizers should remain open for other themes and questions.

The organizers of a CTA event need to have a thorough understanding of the emerging science & technology. What are the dynamics in its development, to what extent is there still room to change the course of technology developments and how these technologies can be integrated in business practices, and how they are perceived by regulatory authorities and further individuals and organizations in society. A second requirement is that organizers need to have a sense of various actors' willingness to anticipate future developments and tune their activities with other actors in the domain. For instance some companies may not be willing at all to engage in co-ordination activities with other companies or societal actors. A third requirement is to identify, select and position potential participants which is related to their role in the overall technology development and embedding process. For instance large pharmaceutical companies are important in the overall innovation process and should ideally be included in such an exercise. For a productive discussion the workshops benefit from an appropriate mix of participants with an enactor or selector perspective toward the technologies under consideration [19]. Finally, the organizers should be aware of broader dynamics which may not always be immediately obvious to actors involved in developing novel technologies. In the case of the drug delivery sector, one may consider involving health insurers as they may be not directly involved in developing new options, but will definitely be important when new pharmaceutical options are introduced on the market.

The design of a CTA workshop can take different shapes, see also [19, 21], but will often be geared toward eliciting actors' perspectives on societal embedding of emerging technologies and to stimulate broadening and enriching of understanding of dynamics in development and future introduction of these technologies. To do so the workshop can be structured around two themes which will be recognizable for participants: (1) identification of challenges, opportunities and directions for development of emerging technologies in a specific domain; (2) identification of ways to cope with challenges and opportunities of these technologies. These are broad themes in order to simulate actors to articulate linkages between emerging technologies like NDDS and aspects of societal embedding and prevent too early lock-ins into particular options or strategies. Such open-ended character will often be unavoidable considering the emergent character of the application of technologies. It was intentionally open-ended in order to allow for open discussion

Some reduction of the open-ended character of these two discussion themes will be important in order to have a productive meeting and attract participants. In CTA workshops this is often done by means of a preparatory document which will be given to all participants, justifying and framing the meeting. To link up with interests of potential participants, such a document can identify key issues and dilemmas which will be recognizable to at least part of the participants. In addition the document will contain the scenarios about future developments. These scenarios depart from major challenges in the present situation and explore strategies to overcome them, including the possible responses of actors involved, for scenario methodology see also [15, 20, 22]. In this way the scenarios help to make anticipations of future developments concrete and can support actors in their formulation of strategies.

Finally, to stimulate an open discussion and overcome possible concerns regarding confidentiality, a CTA workshop can be held under the 'Chatham House rule'. This rule is as follows: "When a meeting, or part thereof, is held under the Chatham House Rule, participants are free to use the information received, but neither the identity nor the affiliation of the speaker(s), nor that of any other participant, may be revealed" [23]. By adopting this workshop rule, the organizers aim to create an informal atmosphere and stimulate an open discussion.

Before I will discuss the results of the workshop discussions I will briefly introduce the drug delivery sector and nanotechnology.

3. Setting the scene: Nanotechnology in the drug delivery sector

The drug delivery sector consists of different value chains related to the technology under consideration. A drug delivery system is a formulation or device "that delivers therapeutic agent(s) to desired body location(s) and/or provides timely release of therapeutic agent(s). The system, on its own, is not a therapy, but improves the efficacy and/or safety of the therapeutic agent(s) that it carries."[1] These delivery devices can not only be used as carriers for drugs but can also be applied for medical imaging purposes and as carriers for food ingredients. The

1 From www.drugdel.com/glossbot.htm

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drug delivery sector, then, is an intersection of two product value chains involving the 'primary manufacturing' of the active pharmaceutical ingredient (API) and the 'secondary manufacturing', i.e. the formulation (including drug delivery systems) and packaging. Both stages of manufacturing can occur within one (integrated) firm or be outsourced to contractors [24].

Dynamics in the sector then come from both chains and their intersection, but also from the broader health care environment in which these chains are embedded. For embedding new drug delivery systems, enactors, e.g. business entrepreneurs, not only need to deal with business dynamics in the world of pharma, but also with broader developments in health care such as overall pressures on cost reduction of treatments, debates on reimbursement. In addition to firms, there are knowledge institutes, clinicians, patients, governmental actors and health insurers. Figure 1 offers a (simplified) overview of actors in the drug delivery sector.

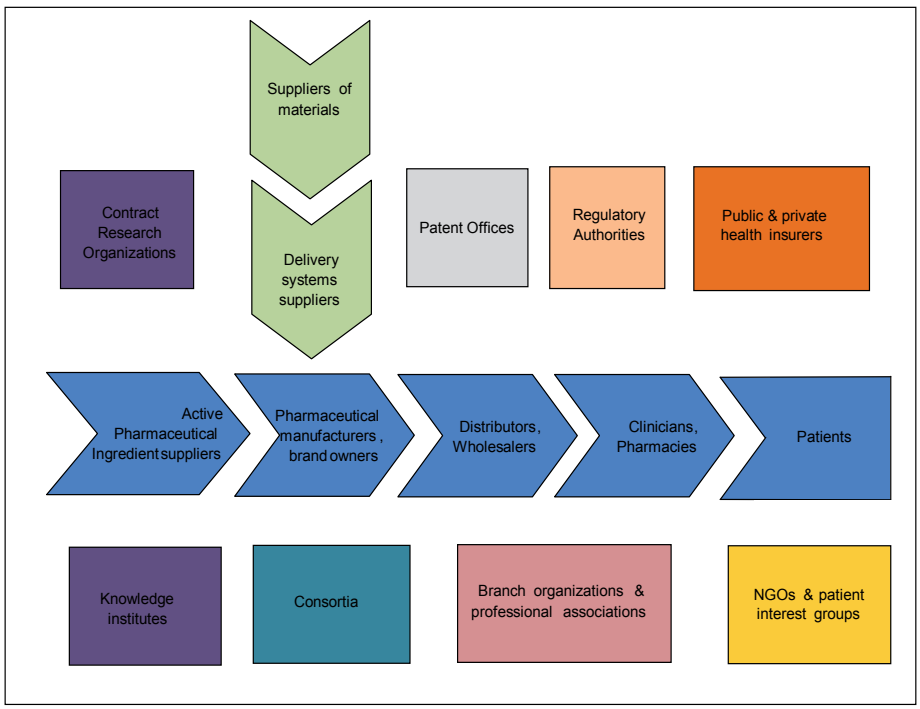

Figure 1. Organizations in the drug delivery sector

The development and introduction of nanotechnologies plays against a backdrop of increasing difficulties of pharmaceutical companies to develop and market new drugs [25, 26]. Nanotechnology-enabled drug delivery systems promise new solutions. The application of nanotechnologies which has attracted the most attention is the promise of releasing drugs at a particular target. While there are other targeting approaches, nanotechnology engineered delivery systems are considered to be particularly promising. In a conventional delivery system, the drug is distributed systemically across the body, but this may not always be

sufficiently (therapeutically) effective or have adverse toxic effects. For targeted drug delivery there are two general approaches. Drugs can be released near the desired location in the body or drugs can be designed for active or passive targeting purposes. In both cases the application of nanotechnologies (devices and molecules) promises to contribute to targeted delivery.

The promise of targeted delivery is not entirely new. The concept of drug targeting is linked with Paul Ehrlich's idea of 'Zauberkugeln', 'magic bullets' introduced over a century ago. The 'magic bullet' refers to the idea of homing in on the target and being effective - in this case affecting only the diseased tissue. Work on what are now considered to be nanotechnology enabled drug delivery systems has evolved since the 1960s [27-29] – although not exclusively related to targeting. Systems which are currently labeled as 'nanovehicles' have existed for some time, such as liposomes and polymer micelles (1960s), nanoparticles and dendrimers (1970s) [30]. The connection with the term 'nano' can thus be considered as a relabeling of what was already occuring.

Pharmaceutical challenges	**Expected solutions from NDDS**
Difficult or unacceptable pharmaceutical format due to poor solubility or toxicities linked to particular excipients.	Enhancing drug solubility, e.g. by micelles and liposomes providing hydrophilic and –phobic environments.
Undesirable side-effects caused by extravasation (e.g. by leakage) of drugs from diseased to surrounding tissues.	Regulated drug release can reduce or prevent tissue damage by extravasation.
Loss of activity of drugs due to rapid breakdown in the body.	NDDS protect drugs from premature degradation and may enable use of lower doses.
Loss of activity due to too rapid clearance of drugs.	NDDS can reduce clearance and may enable use of lower doses.
Undesirable side-effects due to too widespread distribution in the body affecting healthy tissues.	Particulate character of NDDS lowers distribution and helps to reduce side-effects.
Suboptimal therapeutic effects due to use of low concentration of drugs to reduce side-effects.	NDDS can increase drug concentrations by passive and active targeting (EPR-effect, targeting ligands).
Insufficient drug absorption and intracellular penetration.	NDDS can improve absorption through epithelium and improve intracellular penetration and distribution.
Difficult or unacceptable excipients to stimulate immune responses in case of vaccines.	NDDS can be engineered to stimulate immune response, e.g. virosomes and virus-like particles.

*Modified and expanded from Allen and Cullis [31] with items from Couvreur and Vauthier [32].

Table 1. Expectations of nanotechnology enabled drug delivery systems

Considering the history of drug delivery systems, promises of the application of nanotechnologies may not be very effective in mobilizing actors. According to Boyd [27] the claim that "advances in nanotechnology are stimulating a 'revolution' in colloidal drug delivery" should be reconsidered given evolutionary developments over the last decades. Available funding

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related to rhetorics of nanotechnology in general and for drug delivery in particular, as well as advances at the level of materials, have created new openings for pursuing targeted drug delivery.

The application of nanotechnology-engineered drug delivery systems (NDDS) is expected to be beneficial for the generation of novel pharmaceutical therapies and thereby appealing to current pressures on pharmaceutical companies to generate novel therapies. The idea of the magic bullet enabled by nanotechnologies is a powerful image. There are further expectations of the application of nanotechnologies which link up with issues in the drug delivery sector, in particular the challenge of sustaining pharmaceutical business: (1) creating new drugs or extension of patent life of existing drugs by providing new and improved formulations with respect to therapeutical effectiveness and safety; (2) enabling formulations for API's which are difficult to develop pharmaceutically, including promising new biopharmaceutical therapies such as those based on genes. In table 1 an overview of expectations of the applications of NDDS is presented.

4. Workshop results

4.1. Preparing the interactive workshop

To prepare for a CTA workshop on NDDS it is important to have a solid understanding of dynamics related to the development and introduction of these emerging technologies. To do so I addressed the pre-engagement requirements mentioned in section 2.[2] Table 2 summarizes major findings in the pre-engagement phase. For an in-depth discussion of dynamics in the drug delivery sector, nanomedicine and pharmaceutical developments more generally, see also [1, 33-35].

For the organization of the drug delivery workshop I co-operated with two (regional) branch organizations. One of them was an association of companies, including large pharmaceutical companies, who develop new pharmaceutical products. The other organization was an association of companies and organizations involved in biotechnology, including pharmaceutical applications. For the former, nanotechnology was not a central topic as it was not (yet) an important theme for its members. Its members are relatively little involved in R&D activities and therefore activities in this area are limited almost by definition as many nanotechnologies are still in a pre-clinical stage. For the other the situation is somewhat different. Biopharmaceutical companies are likely to be interested in nanotechnologies considering the promises for (difficult) delivery of macromolecules such as siRNA.

2 Mapping of dynamics linked to nanotechnologies and the drug delivery sector was completed by analyzing relevant reports, papers, conducting interviews and attending international conferences on nanomedicine. Interviews were conducted with experts in the field in order to map opportunities, challenges and dynamics. In addition, interviews were used to find out about existing activities to develop new framing conditions, rules and practices and attempts at co-ordination with respect to nanotechnologies and drug delivery systems [11].

Pre-engagement requirements	Findings
Understanding of dynamics in the domain	Focus on promises of targeting applications, but also on other promises such as longer circulation time of drugs. Long history of development and few products on the market yet. Nanotechnologies not high priority on drug delivery sectors' agenda.
Actors' propensity to co-ordinate development and embedding of NDDS	Waiting games between actors in the value chain. Emerging consortia and platforms for drug delivery researchers and other actors interested in drug delivery (often linked to nanomedicine). Uptake of notion of 'translational research'.
Selection and position of actors	Big pharma as gatekeepers regarding development of new options. Important to involve and link academia, industry and clinicians
Assessment of broader dynamics	Linkages between drug delivery and imaging sector; between drug delivery and food sector. Attention to regulatory and clinical aspects, less on broader issues such as patient involvement, reliability and liability. Overall developments: reimbursement pressures; mergers and job cuts at large pharmaceutical companies; perception of nanotechnology risks

Table 2. Summary of pre-engagement drug delivery workshop

I expected that attracting workshop participants would be difficult. Large pharmaceutical companies might not be interested due to waiting games and the low priority for nanotechnologies. Clinicians might not be interested, due to their limited involvement until now. It indeed proved difficult to attract participants from large pharmaceutical companies and, for that matter, biopharmaceutical companies, to attend the workshop on drug delivery - despite efforts by the co-organizing branche organizations. Nanotechnologies were not a high priority for potential participants and caution in discussing R&D developments were provided as important reasons for not attending the workshop. Attracting clinicians also proved to be difficult, albeit for different reasons. While some clinicians were interested in the phenomenon of nanotechnologies, but not able to attend due to busy schedules, others were sceptical about the value of nanotechnologies and not interested in participating in the workshop. These observations are relevant as they already give indications of possible difficulties in bringing about co-ordination in the field regarding emerging NDDS.

While eventually no clinicians or participants from large pharmaceutical companies attended, participants from different parts of the chain were present at the workshop, including some who had experiences with interactions with pharmaceutical companies and clinicians. Participants from knowledge institutes, suppliers of delivery systems, and a drug development firm were present. In addition, a firm involved with microsystem technologies and a governmental organization involved with nanotechnologies were present.

To prepare for the discussion and support participants thinking about current and future developments, three scenarios were crafted. One scenario explored two different (and

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contested) development paths. A consortium of researchers anticipated that demonstrating clinical value would convince investors in the added value of targeted drug delivery systems which would contribute to overcoming the current impasse. To do so the consortium focused on incremental improvements of carriers with which there was already a lot of experience with regarding safety and effectiveness. Another set of actors disagreed with this approach and anticipated that big steps were needed in order to fulfill the promises of nano-enabled drug delivery. This group of actors formed a more ambitious consortium working on theranostics. The scenario speculated that the eventual fate of the consortia was not so much determined by its technological achievements but due to contextual factors. Concerns about possible risks of nanotechnology enabled drug delivery systems and gaps between diagnostic and therapeutic possibilities co-determined the fate of the two development paths. In the end the ambitious consortium was disbanded.

The two other scenarios explored initiatives which more directly worked on improving co-ordination across actors in the drug delivery sector. One of these scenarios described the formation of a broad platform involving material and pharmaceutical researchers, clinicians and people from industry. Discussions and differences of opinion among the platform participants about how to co-ordinate technology developments and their future introduction forces the platform to abandon the initial broad scope and focus on specific carrier systems. While the platform is successful in attracting a broad variety of stakeholders, including interested actors from outside the nanomedicine world, attempts at co-ordination across the domain are met with criticisms. One of the co-ordination mechanisms proposed by the platform is a stage-gate model which articulates criteria for the development and clinical introduction of novel targeted medicines. According to critics the stage-gate model is too restrictive: commercially uncertain, but potentially interesting and promising technologies are too quickly shifted aside – effectively constraining opportunities for breakthrough technologies.

The third scenario explored attempts at stimulation and co-ordination from the demand-side. An alliance of patient organizations, knowledge institutes and firms is forged with the general aim of stimulating demand for cancer medicines with little side-effects. The alliance develops a broad research programme and actively involves itself in political circles and decision processes on the restructuration of the health care system and reimbursement policies. The broad focus on stimulating demand for reduction of side-effects of medicines attracts various alternative technology solutions. Only with help with the reference to cancer medicines, nano-enabled targeted drug delivery system remain on the agenda within the research programme which is eventually funded. First clinical evidence suggests that complexity of cancer requires different forms of drug delivery systems which may be commercially less attractive because of limited market volumes. Involved patient organizations are disappointed and press for applications fitting their specific diseases. The push of the alliance for medicines with less side-effects then starts to lose its momentum. The emphasis on cancers as the major disease area now appears to be less effective to mobilize resources.

To inform the participants a preparatory document was distributed one week before the workshop. This document contained: (1) a program of the meeting; (2) a short introduction

into and justification of the topic of the meeting; (3) a brief analysis of the current situation of development and embedding of nanotechnologies for drug delivery; (4) the presentation of scenarios; and (5) a list of identified dilemmas where strategic choices about development and societal embedding of nanotechnology-enabled drug delivery systems had to be made. The document aimed to create common ground for participants, and offer ideas for discussion. In particular the scenarios and dilemmas were offered as ways to think about future developments and strategies. It was emphasized that the scenarios were controlled speculations [22], i.e. imagined developments but based on what was happening in the drug delivery sector already. Participants were invited to modify and add to the scenarios during the workshop.

4.2. Discussions during the workshop

The workshop consisted of a half-day of intense discussions which took place in an informal atmosphere.[3] While the workshop discussions covered a variety of themes, there was a strong focus on the clinical value of nanotechnology-enabled drug delivery technologies. Sectoral issues of co-ordination between disciplines and across positions in the chain emerged as the most important challenges to be overcome. They were recognized and were actually highlighted by some participants in the workshops as being a key factor holding back embedding processes of nano drug delivery technologies. The lack of clinical evidence of (significant) therapeutical effectiveness was positioned as the reverse salient for furthering developments in the field. Strategies to stimulate and improve further developments in the field of nano-enabled drug delivery revolved around the challenge of demonstrating clinical value.

Interactions during the targeted drug delivery workshop are characterized as a series of exchanges on diagnosing the key challenges in furthering developments in the field of nanotechnologies and drug delivery, and on the best methods to cope with those challenges. While there was no explicit consensus on which strategies should be pursued in the future, the emphasis on problems of co-ordination and lack of clinical evidence effectively constituted a lock-in in the discussion. To show how participants in the workshops assessed current dynamics and anticipated future developments relevant for the commercialization of nano-enabled drug delivery systems I will report on salient items in the discussions.

4.2.1. The unique character of nanotechnology and the pharmaceutical sector

Puzzles about the unique character, if any, of nanotechnology engineered drug delivery technologies set the stage for a series of exchanges. The discussion was initiated by a participant wondering about specificities of the application of nanotechnologies and how these contributed to reluctance in uptake and development of nanotechnology-enabled drug delivery technologies. A participant from a drug development company replied by pointing out uncertainties about the unknown safety profile of nanoparticles. Whether this meant that there was a lack of testing methodologies and knowledge about distribution and effects of nano-

3 The quotations in this chapter are anonymized, and used with permission of the participants. The quotations were translated into English by the author.

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particles in the body – which would suggest the existence of specific nanotechnology related challenges - or required more efforts during testing was unclear.

This question regarding safety of nano drug delivery technologies prompted a participant, working for a company supplying drug delivery systems, to frame the question differently by asking about the status of knowledge and methodologies for assessing 'conventional' pharmaceutical materials. This participant considered questions regarding safety to be the responsibility of their customers and not a topic for his firm. However, by asking about evaluation criteria for their customers' products, his understanding of broader developments increased. [4] In that respect, this participant did consider broader developments rather than only customer-supplier exchanges.

Delivery systems supplier: May I ask a simple question? We discussed that we cannot observe where nanoparticles are travelling to, but this is also unknown for pharmaceutical substances, molecules. Also in these cases one doesn't analyze in detail whether particles travel to the liver, or to.

Knowledge institute 2: Well, well

Delivery systems supplier: They do?

Knowledge institute 2: There is pre-clinical pharmacokinetics, tissue distribution; this should all be done.

Governmental organization: But that is not different for what needs to be done already for pharmaceutical substances.

Delivery systems supplier: Hence, my question. If this is already being done for small molecules, why would this be problematic for nanoparticles?

Governmental organization: Because for non-nanoparticles, let's call them that way, for other chemical substances, not necessarily pharmaceutical compounds, already a number of patterns are known. [...] The case of nanoparticles is becoming a totally different story for us.

Even if questions concerning the unique character of nanotechnologies for drug delivery were unsolved, the link between general conceptualizations of the term nanotechnology and drug delivery was problematized. Participants from research institutes and a drug development firm pointed out that the associations of targeted drug delivery with the umbrella term 'nanotechnology' also, albeit incorrectly, implied connections with discussions of 'disadvantages or risks' linked to nanotechnologies in the public domain. According to these participants, such associations could provide nuisances for nano drug delivery technologies. This type of reasoning shows that these enactors take broader aspects into account, yet in a way which resembles other patterns which have been called by Rip [14] as 'folk theories': taken for granted patterns, which have not systematically been checked. In this case, the expectation that nanotechnologies may suffer from the same public backlash as what happened to GMOs.

4 During my post-workshop interviews this participant expressed that understanding in this area helped the firm to assess their business plan forecasts as uncertainties in this area might slow down introductions of their customers' products, and therefore the participants' sales volume.

A series of interactions followed in which a participant from a governmental organization questioned this implicit pattern. This participant pointed out that specificities of the drug delivery sector would limit possible risks of nanotechnologies. Exposure to nanotechnologies through pharmaceutical therapies would be well controlled and registration procedures would check, among other things, toxicity. In addition, access to consumers – patients – would be regulated through intervention of clinicians. Furthermore, authorities had already considerable experience with delivery systems such as liposomes, suggesting that registration procedures should not pose particular difficulties. However, the participant acknowledged, patients might think differently about risks than experts do.

The point about regulatory expertise was contested by one of the participants who had experience with regulatory authorities, puzzling over whether existing evaluations were sufficient – even for liposomal formulations. The participant speculated that more knowledge about risks of nanotechnologies might lead to re-evaluating existing registration procedures. This prompted a reflective comment from the participant of the governmental organization, noting that there were tendencies in society to reduce and solve all uncertainties and problems linked to nanomaterials. While such an objective might be laudable, the participant warned that one should not increase risk assessment criteria for nanotechnologies beyond what was presently accepted.

4.2.2. Challenges in co-ordination across the innovation chain

During the discussion the point was made that the development of linkages between research on drug delivery materials and specific diseases was difficult. A participant from a knowledge institute suggested that research programmes should stimulate the improvement of interfaces within a chain of activities involved in developing these linkages. At the same time, this participant observed that developing linkages would not be straightforward, for different reasons.

Knowledge institute 1: There are also groups that only focus on researching their own chemical entities and do not develop them further. While, clearly, further development of these substances should be considered. In which area do you want to have an application? Then you also need a partner to do this. We, as material developers, are all confronted with the problem that we have difficulties in reaching those people, particularly the industrial actors which are interested in these materials.

According to a participant from another knowledge institute, the difficulty in bringing the field of nanotechnology enabled targeted drug delivery further was rooted in the lack of clinical evidence. This would make it difficult for researchers and drug delivery firms to link up with large pharmaceutical companies. Later, the participant commented that big pharmaceutical companies were to some extent dependent on these new technologies. So, we see here a waiting game at work, considering that researchers and firms are to some extent dependent on large pharmaceutical companies for funding and further exploitation of nanotechnology enabled delivery systems.

Knowledge institute 2: There is still too little on the market that convinces large companies to put effort in this area. There is very little data on the clinical benefits. Real, concrete proof. And that is what the

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industry is waiting for [..] but big pharmaceutical companies are not in-active altogether. On the one hand there is a development which forces them to pay attention to these type of products, eventually. Because there are increasingly less blockbusters. [..] Big pharmaceutical companies do have interest in these [nano drug delivery] type of systems. Watch it carefully.

According to participants from research institutes, big pharmaceutical industries were reluctant. This led the participant from the governmental organization to probe into big pharma's considerations. While no participant from big pharma was present, participants replied by referring to big pharma's waiting strategy, which was considered to be independent of nanotechnologies. A participant from a drug development company pointed out that, among other commercial considerations, clinical proof established in Phase II studies was required to demonstrate the added value of a new pharmaceutical technology. The participant from the governmental organization challenged this claim. The participant probed whether clinical studies were really required in order to convince pharmaceutical companies to invest in nanotechnologies. This was confirmed by a number of participants and not questioned by others.

Focus on convincing large pharmaceutical companies by acquiring – hopefully – significant clinical data (for a specific drug – delivery systems – disease combination) was an important topic in the workshop. The consideration of evaluation criteria from pharmaceutical companies (acting as future selectors of concepts generated by research institutes) by participants from research institutes and firms implies that these actors did take into account broader aspects. Still, the discussion was focused on pushing forward nanotechnologies (from the world of research). The overall strategy itself is predicated on the assumption that convincing firms and health insurers that clinical evidence is 'out there' and that expected benefits only need to be harvested – after which new drug delivery technologies will enter into the clinics. This type of reasoning resembles a typical enactor perspective.

One of the participants pointed out further sectoral dynamics. The participant argued that big pharma had a strong focus on blockbuster drugs and that novel nanotechnology enabled drug delivery technologies would not likely fall under that class of drugs. This then led to a series of interactions regarding structural features in the drug delivery sector constraining development of new pharmaceutical technologies in general. During this set of interactions one participant, who emphasized clinical proof, suggested that if the clinical value would be convincing, actors (which were left unspecified) could not dismiss these technologies. The emphasis on benefits, which would overcome all barriers, is a typical enactor perspective. But this was not left unchallenged. One participant remarked that patients then probably needed to take action as health insurers might be reluctant to pay for new (costly) therapies. Here, we see a typical selector argument, pointing out that benefits alone might not be sufficient, as issues of costs were known to limit introduction of new pharmaceutical therapies.

Participants raised further points to open up the discussion, thereby moving away from the lock-in on clinical value of drug delivery technologies, which was pushed by a number of participants. One of the participants challenged the idea of initiating technology development trajectories from a disease oriented point of view.

Knowledge institute 3: I would like to react to your comment to take diseases as a starting point. There are of course many material research groups which start to think from their technology. [...] If you assert that one needs to start to think from the clinical picture, this means that you actually need to involve all groups in that discussion. [...] For each disease there are then several delivery systems. Whereas one could also say that one should start thinking from delivery systems and whether they are toxic or not.

Knowledge institute 2: Yes, but eventually we develop, we produce [...] not things that are safe. No, we produce things that have to work effectively and which have to help patients [...] Look, it is a bit like, disease searches for a device, or device searches for a disease.

Microtechnology firm: It is an interaction.

Knowledge institute 2: It is an interaction. And actually I am also in favor of broad academic research. But, if one takes the step to, let's call it, valorization, then one needs to make a small value chain and this should be done by spin-offs.

[..]

Knowledge institute 1: You need them both, of course. You need to have a lot of knowledge about particles in order to know how and for what you can use them. [..] So, there is a disease and there is a material, and these should be brought together. How would you like to improve this? Then one would say, for these connections, these points, there should be programs that support them.

The conclusion that interfaces between actors needed to be improved can be interpreted as a call for translational research, although the term itself was not employed. The conclusion shows a non-typical enactor perspective; enactment of new technologies is guided by a diagnosis of what happens at the level of a sector and what should be improved upon.

4.2.3. Anticipatory strategies: next steps in mobilizing R&D funds and research

The relatively focused discussion created time and space for discussion and articulation of strategies. Discussions focused on the question of how to further develop nanotechnologies in the drug delivery sector. Overcoming what was seen as the reverse salient in the overall development, the lack of clinical evidence, was a central theme in that part of the discussion. Participants explored possible strategies of co-ordinating developments in the sector, including the creation of a nano drug delivery exemplar which – if successful – might convince the field of drug delivery of the value of the application of nanotechnologies. Toward the end of the workshop participants expressed interest and enthusiasm in adopting the discussed strategy in order to try to actually implement them

Other strategies were explored well. One is particularly interesting as it appealed to the promise of reducing undesirable side-effects. In the interactions that followed, not only researchers, enactors, of drug delivery technologies were articulating this strategy, but also the participant of the governmental organization. One of the participants rebutted this strategy by referring to negative experiences with large pharmaceutical companies. According to this participant, the strategy of re-evaluating problematic drugs did not fit with big pharma's practices. By providing an account of those experiences, the participant also provided further insights into the world of large pharmaceutical companies:

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Knowledge institute 1: There is also an opportunity in which one could make up for some costs. There are of course many pharmaceuticals which in the end have not made it due to side-effects. Targeted delivery offers an opportunity to avoid such toxic side-effects. The therapeutic effect will probably already have been demonstrated very clearly, but in the end they have not made it due to the side-effects. In that respect one may skip some developments, or at least short-cut them and focus on whether one can reduce these side-effects through targeting.

Knowledge institute 2: Yes, yes, but you can also evoke them [side-effects] via targeting. That automatically appears, safety, you can not eliminate that, because through linking…

[...]

Knowledge institute 3: Big pharmaceutical companies have many pharmaceuticals on the shelves [which cannot be used due to drug delivery problems].

Governmental organization: Yes, [...] one should also have a look at the deleted products.

Knowledge institute 2: We have already tried that many times in the past. [...] And eventually it works, pre-clinically, and they [big pharmaceutical companies] do not do anything with it. Because it doesn't fit with their block buster model eventually, and it is too laborious, costs too much money and finally they pull out. We had spoken already with a number of big pharmaceutical firms in the past about creating a better life for interesting pharmaceuticals, problem medicine. And, that is, … well, yes, big pharma does not think that way.

During the discussion of strategies to further the field, also the question of mobilizing resources for such strategies was put forward for consideration. Toward the end of the meeting the moderator pointed to one of the scenarios in which patient foundations and organizations were involved and asked whether that would be a feasible option. Patient organizations can be involved for financial but also symbolic (moral) support. Participants from research institutes and the governmental organization were hesitant and argued that it might be too early to involve them for funding and moral support. Too-high expectations based on too little evidence and uncertainties over risks were mentioned as reasons (without making explicit the expected effects). Between the lines, the analyst can see a folk theory of a hype-disappointment cycle at work.

Interestingly, one of the participants from a firm not directly involved with drug delivery technologies responded to this discussion by pointing out that little involvement of patient organizations might induce a pattern reminiscent of the biotech discussions. A pattern, argued the participant, in which little information by enactors of new technologies is distributed, leaving civil society organizations to guess what is happening and perhaps leading to a rejection of new technologies. This was acknowledged by one of the participants from a research institute as something for which an answer should be developed, but not as something directly important for the question of furthering the field. This participant considered this theme as off topic and (again) emphasized the importance of clinical evaluation of new delivery technologies.

By convening participants at various positions in the chain and facilitating mutual understanding of each other's positions and perspectives, the workshop supported the participants

in getting a richer understanding of what happens at the level of the drug delivery sector. This was acknowledged by one of the participants after the workshop who remarked that the meeting discussed the big challenges at the level of the domain which was usually not done as people tend to be preoccupied with their day-to-day affairs.

5. Conclusions

The approach of Constructive Technology Assessment offers a useful methodology and set of tools such as scenario workshops to support researchers, firms, policy makers and other stakeholders in identifying dynamics in innovation processes and anticipating plausible future developments. In this chapter I have described this approach and showed how to actually do this in the case of nanotechnology-enabled drug delivery systems.

A key finding from the scenario workshop on NDDS is that participants' assessments of development dynamics and future market introduction of nanotechnology-enabled drug delivery systems often took into account what was happening at the level of the sector. That said, participants did discuss nanotechnology specific aspects, often in the context of uncertainties about performance, risk and demand for nanotechnology engineered products. Still, during interactions and positioning of actors, broader considerations about sectoral dynamics and circumstances came to the fore. Participants discussed patterns of interaction between actors in the chain and developments at the level of the sector that were independent of, but relevant for, nanotechnologies. In this way, participants drew from a general repertoire of embedding issues in their sector, independent of specific emerging technologies, as part of their anticipatory competences. Discussing dynamics at the level of the sector rather than Focussing on a specific NDDS technology was appreciated by participants as they usually did not look at NDDS from such a perspective.

Occasionally participants also discussed issues transcending sectoral aspects such as overall changes toward dealing with risks of (new) technologies in general and nanotechnology as an umbrella term. These broader discussions will offer further, though non-specific clues, such as general pressures to take into account risks of nanotechnologies and take into account ethical and societal aspects during the development of nanotechnology-enabled products.

Present uncertainties of performance of emerging NDDS will make concrete anticipation of societal embedding difficult. Then, considerations about sectoral conditions and patterns of interactions between actors in the sector are likely to be highlighted. This is relevant as a variety of actors and interests are involved during the development and market introduction of novel NDDS. Understanding of sector-level patterns linked to drug delivery technologies in general then offers clues as to what will be important to take into account when working on the development and introduction of specific combinations of drug delivery devices, pharmaceutical agents and diseases. Scenarios offer playgrounds to experiment with specific cases of NDDS which will anyway be embedded in dynamics of the intersecting supply chains of pharmaceuticals and delivery systems.

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By organizing an interactive discussion involving participants at different positions in the value chain, supported by well-prepared scenarios, analysts or practitioners adopting CTA methodologies can support articulation of anticipatory strategies and decision making. Whether the insights gained during such events actually make a difference is more difficult to determine, among others because this depends on how much opportunities and room to maneuver participants have after the workshop. The workshops will however contribute to an emerging shared understanding of dynamics and issues which cannot be easily ignored by the individual participants. This will be different than before the workshop and in that sense the workshop will already have effects on how actors will anticipate market introduction of nanotechnology-enabled drug delivery systems.

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Author details

Haico Te Kulve

Department of Science, Technology & Policy Studies, School of Management and Governance, University of Twente, The Netherlands

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Chapter 16

Current Status and Future Scope for Nanomaterials in Drug Delivery

Biswajit Mukherjee, Niladri Shekhar Dey, Ruma Maji, Priyanka Bhowmik, Pranab Jyoti Das and Paramita Paul

Additional information is available at the end of the chapter

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1. Introduction

Nanotechnology is a revolutionary field of micro manufacturing involving physical and chemical changes to produce nano-sized materials. The word "nano" is a Latin word meaning "dwarf". Mathematically a nanometer is equal to one thousand millionth of a meter [1]. A nanomaterial consists of aggregated as well as unbound particles. Nanotechnology in scientific terms is defined as the science which deals with processes that occur at molecular and atomic level or at nanolength size. It involves designing, synthesis and characterization of material structure by controlling the shapes and sizes at nano scale. The conversion of a particle to nano scale size changes the properties of the material such as increase in surface area, dominance of quantum effects often associated with minute sizes, higher surface area to volume ratio etc. and varies material's magnetic, thermal and electrical property. For example, copper which is opaque at macro scale becomes transparent at nano scale. Similarly the properties of gold at nanoscale causes change in melting point from 200°C to 1068°C and colour changes from yellow to blue to violet along with the change in its catalytic property [2]. Nanoparticles are persistent in nature as well. Functional proteins may be classified as nanoparticles. Some biological system consists of nanoparticles which are devoted to locomotory function. The colours on butterfly's wings are due to light being bounced off nanoscale layers in the structure of the wings. The red and yellow colours seen at sunset are also due to nanoparticles [3]. Super paramagnetic iron oxide less than 50 μm are used for imaging of organs. They can be even used for treating complicated brain disorder bio-imaging at nano scale size [9].

Indian craftsman and artisan used nanotechnology for designing weapons in early times. The first observation and size measurement of nanoparticle were carried out using an ultra microscope by Richard Zsigmondy in 1902. The term nanotechnology was first time used by a researcher named Norio Taniguchi in University of Tokyo in 1974. In 1980 the inventions of two atoms further advanced the field of Nanotechnology. In 1985 fullerene C_{60} was discovered by Kroto's and Smalley's research team. In 1991 carbon tubes were discovered by Saumio lijima and by 2000 National Nanotechnology Initiative (NNI), The United States was launched which paved the way for future development in nanotechnology [2].

Nanotechnology may be considered as one of the main propellants for technological, economical change as industrial competeition. Nanotechnology has integrated various disciplines including biomedicine, engineering and technology. Nanotechnology is being used for improving the existing products and to create new products. The strength can be varied accordingly with the requirements of engineering. It can be used to make the water cleaner by remediation to remove its pollutant. It has helped to clean the environment by removing pollutants and has generated cleaner and cheaper energy. It has improved the healthcare system by introducing new devices for diagnosis, monitoring, treatment of diseases and drug-delivery [1].

Nanomaterials have wide applications in pharmaceutical sciences and technology. Few other predominant areas of use of nanotechnology are in drug delivery, and as diagnostic imaging and biosensor. These devices of nanoscale size are popularly known as nanomedicine. Thus nanomedicines are sub-micron size materials (<1μm) which are used for treatment, monitoring and diagnostic purposses. In the present chapter we will discuss on the current status and future strategies of nanosize drug delivery systems.

2. Significance of nanomaterials in drug delivery

There are many reasons for which nanoscale size drug delivery systems are attractive to formulation scientists. The most important reason is that number of surface atoms or molecules to the total number of atoms or molecules increases in drug delivery systems. Thus the surface area increases. This helps to bind, adsorb and carry with other compounds such as drug, probes and proteins. The drug particles itself can be engineered to form nanoscale size materials too [4]. The nanosize device systems, sizes smaller than eukaryotic or prokaryotic cells, can eventually much more in amount reach in generally inaccessible areas such as cancer cells, inflamed tissues etc. due to their enhanced permeability and retention effect (EPR) and can impair lymphatic drainage thus that can be used for administration of genes, proteins through the peroral route of administration [5]. They can be used to target the reticuloendothelial cells, thereby facilitating passive targeting of drug to the macrophages of liver and spleen and thus enabling a natural system for treating intracellular infections [6]. The nanomaterials used for the purpose should be soluble, safe and biocompatible as well as bioavailable. They should not occlude blood vessel and less invasive and the toxicity associated with the nanomaterials for drug delivery should be

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very low so that they can be used to target the specific diseased tissue in a safe concentration [7]. They need protecting drug from enzymatic and hydrolytic degradation in the gastrointestinal tract and help in bypassing the "first-pass" metabolism in the liver. They generally remain in the circulation for longer time especially those coated with hydrophilic polymers and hence suitable for enhancing the efficacy of drugs with short half-lives and can be used to monitor drug as sustained release formulation as well as for delivering DNA [8]. The dissolution rate of drug is enhanced, onset of therapeutic action is increased, and the dose is reduced. The premature loss of drug through rapid clearance and metabolism can also be prevented [6]. They also increase retention due to bio-adhesion.

Nanoscale drug delivery systems such as nanoparticles, nanoliposomes, dendrimers, fullerence, nanopores, nanotubes, nanoshells, quantum dots, nanocapsule, nanosphere, nanovaccines, nanocrystals etc. are believed to have potentials to revolutionize drug delivery systems. Further nanomaterials on chips, nano robotics, and magnetic nanoparticles attached to specific antibody, nanosize empty virus capsids and magnetic immunoassay are new dimensions of their use in drug delivery. Thus nanomaterials can be used for strategic development of new drug delivery systems and reformulating existing drugs to enhance the effectiveness, patent protection, patient-compliance, safety of drugs and decreasing the cost of health care [9].

3. Various nanoscale drug delivery systems

3.1. Nanoparticles

Nanoparticles are submicron-sized polymeric colloidal particles with therapeutic agents of interest encapsulated or dispersed within their polymeric matrix or adsorbed or conjugated onto the surface. Commonly used synthetic polymers to prepare nanoparticles for drug delivery are generally biodegradable [10]. Nanoparticles may also be composed of or transport a variety of substances such as silica, gold or other heavy metals, medicaments, quantum dots, nanocrystals, quantum rods and various contrast agents [11]. Nanoparticle systems offer major improvements in therapeutics through site specificity, their ability to escape from multi-drug resistance and the efficient delivery of an agent. They can be used for active drug targeting attaching ligand such as antibody on their surface (Figure 1).

Solid lipid nanoparticles (SLNs) refer to as lipospheres or solid lipid nanospheres, or particles and are generally solid at human physiological temperature (37°C) and have a diameter less than 1000 nm [12]. They can be formed from a range of lipids, including mono-, di- and triglycerides, fatty acids, waxes and combinations there of. SLNs must be stabilized by surfactants to form administrable emulsions. SLNs form a strongly lipophilic matrix into which drugs can be loaded for subsequent release. SLNs have been investigated for the delivery of various cancer treatments like colon cancer, breast cancer [13].

Polymer-based nanoparticles have been extensively investigated as drug nanocarriers. The most widely researched synthetic polymers include polylactide (PLA), poly (D,L-lactide-co-

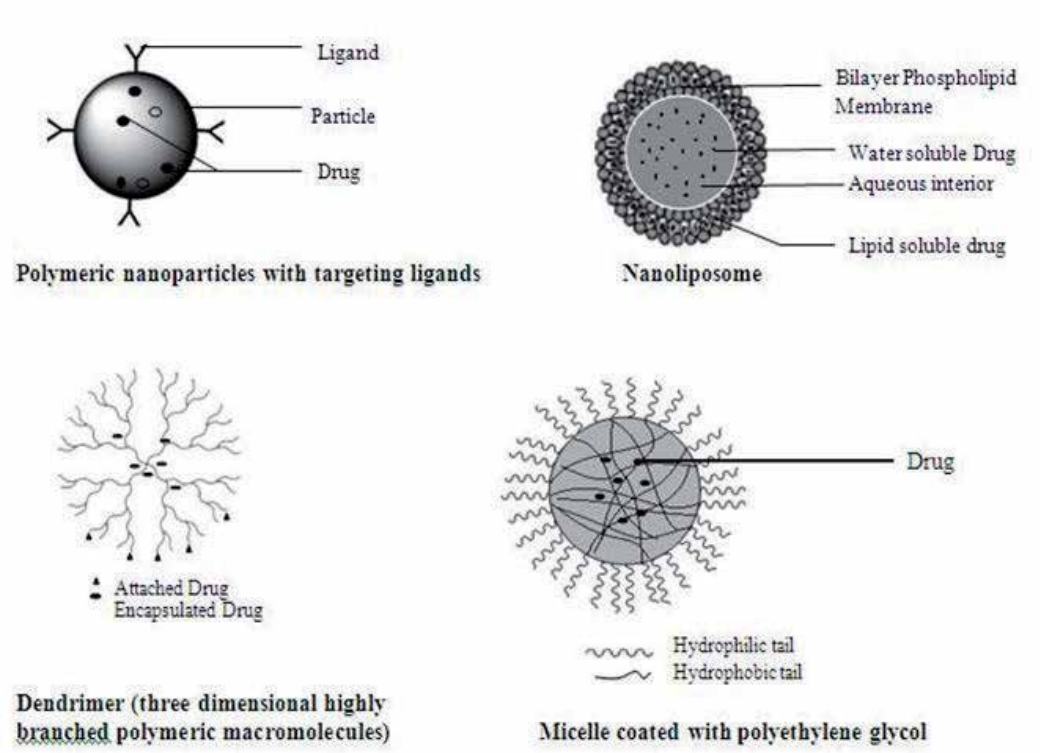

Figure 1. Different Types of Nanocarriers for drug delivery

glycolide) (PLGA) and poly ethylene glycol (PEG). All three polymers are hydrolized *in vivo* and are biodegradable. Other polymers based on biological polysaccharides have been extensively investigated, including chitosan, Clycodextrin and dextrans [14].

Gold nanoparticles (NPs) consist of a core of gold atoms that can be functionalized by addition of a monolayer of moieties containing a thiol (SH) group. Gold NPs can be synthesized using $NaBH_4$ to reduce $AuCL_4^-$ salts in the presence of thiol containing moieties that subsequently form a monolayer around the core gold atom, depending on the stoichiometric gold/ thiol ratio [15]. Drug delivery using gold NPs has been made in DNA delivery for gene therapy and imaging [16]. PEG coated micelles containing drug are also used to deliver drug as new delivery system (Figure 1). Many other nanoparticulate synthetic, semisynthetic, natural and metals are under investigation to know their potentials as drug delivery materials.

Polymeric nanoparticles may adhere to the cell surface and release drug molecules by diffusion which may enter inside the cell to work. However the entire polymeric nanoparticles can also enter the cell by endocytosis. They bind with the cell surface receptor and formation of endosome takes place. Endosome may be lysed with the help of lysosomal enzymes and the nanoparticles release in the cytoplasm (Figure 2).

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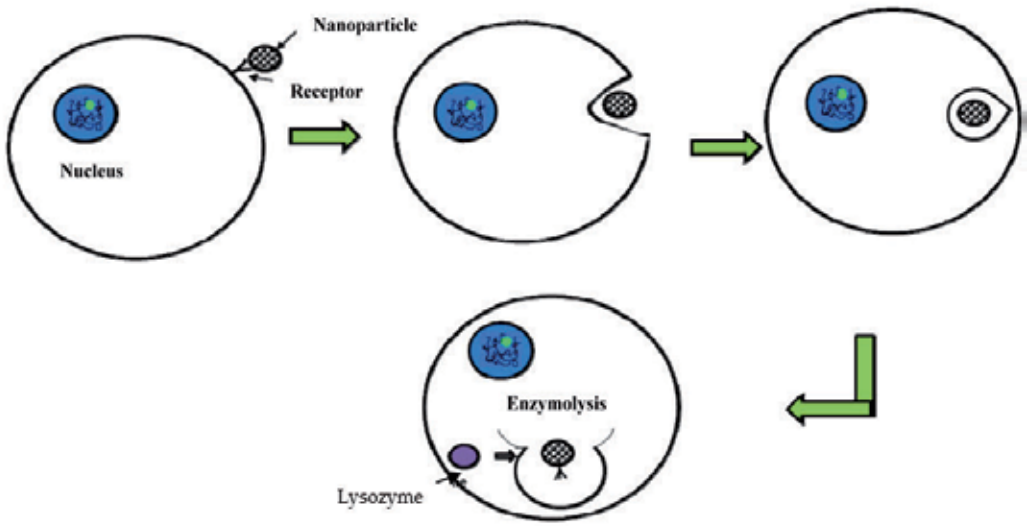

Figure 2. Endocytosis mediated cellular internalization of drug nanocarriers

3.1.1. Nanoliposomes

Nanoliposomes are the nanosize vesicles made of bilayered phospholipid membranes generally unilamellar with an aqueous interior (Figure 1) [17]. They can be used for the delivery of low molecular weight drugs, imaging agents, peptides, proteins, and nucleic acids. Different anticancer, antiviral drugs are incorporated within the liposomes [18]. Nanoliposomes can also provide slow release of an encapsulated drug, resulting in sustained exposure to the site of action and enhanced efficacy. Usually hydrophilic drugs can be loaded in aqueous compartment and lipophilic drugs are incorporated in the phospholipid layer [19]. However unlike liposome nanoliposome does not undergo rapid degradation and clearance by liver macrophages. As for the targeted drug delivery, nanoliposome plays an important role. It can be used for passive targeting or active targeting [20]. Due to the leaky vascular structure of the tumor tissue nanoliposomes get predominantly accumulated in the tumor and release the drug for a prolonged period of time in passive targeting. Active targeting is achieved by incorporating antibody, ligands etc. on the nanoliposomal surface. By active targeting liposomes directly go to the targeted organs or tissues, and release drug for a prolonged period of time, so that the normal cells are not affected and only the diseased cells are affected [21]. Targeted nanoliposomal drug delivery is more efficacious than the non-targeted drug delivery systems. C6-ceremide ligand induced nanoliposome used to treat the blood cancer directly targets the over expressed lukemic cells and decreases the high epxpression of survivin protein in leukemic cells [22]. The concept of long-circulating or sterically stabilized nanoliposomes is derived for novelibility of delivery systems which can circulate in the blood for a long period of time. Nanoliposomal formulations containing polyethylene glycol (PEG) alter the pharma-

cokinetic properties of various drug molecules leading to long elimination half-life [23]. Nanoliposomes are expected to bring lots of change in drug delivery in near future.

3.1.2. Dendrimers

Dendrimers are branched polymers, resembling the structure of a tree (Figure 1). Dendrimers represent three dimensional highly branched polymeric macromolecules with the diameter varying from 2.5 to 10 nm. It can be synthesized from both synthetic and natural monomers e.g. aminoacids, monosaccharides and nucleotides. Two classes of dendrimers commonly used for biomedical applications are polyamidoamines and polypropyleneimines [24]. A dendrimer is typically symmetric around the core, and when sufficiently extended it often adopts a spheroidal three-dimensional morphology in water. A central core can be recognized in their structure with at least two identical chemical functionalities. Starting from those groups, repeated units of other molecules can originate with at least one junction of branching. The repetitions of chains and branching result in a series of radially concentric layers with increased crowding [25].

The overall shapes of dendrimers range from spheres to flattened spheroids (disks) to amoeba-like structures, especially in cases where surface charges exist and give the macromolecule a ''starfish''-like shape. Branching of dendrimers depends on the synthesis processes. Low molecular weight drugs can be placed into the cavities within the dendrimer molecules and are temporarily immobilized there with hydrophobic forces, hydrogen and covalent bonds [26]. The two processes for the synthesis of dendrimers are divergent and convergent methods. In the divergent method dendrimer grows outwords from a multifunctional core molecule. The core molecule reacts with monomer molecules containing one reactive and two dormant groups giving the first generation dendrimer. The convergent method is developed as a response to the weakness of the divergent synthesis. In the convergent approach, the dendrimer is constructed stepwise, starting from the end groups and progressing inwards. When the growing branched polymeric arms, called dendrons, are large enough, they are attached to a multifunctional core molecule. The convergent method is relatively easy to purify the desired product and the occurrence of defects in the final structure is minimised [27]. Due to classical polymerization dendrimers have a negligible degree of polydispersity. They are random in nature and produce molecules of various sizes. The size of dendrimers can be carefully controlled during the process of synthesis of dendrimers. Scientists are focusing on newer approaches for speeding up the synthesis process by preassembly of oligomeric branches which can be linked together to reduce the number of synthesis steps involved and also increase the dendrimer yield [28].

Dendrimers are popularly used for transfer of genetic materials in cancer therapy or other viral diseases in different organs because of their monodisperisity, high density of functional groups, well-defined shape and multivalency. In gene delivery polyamidoamines (PAMAM) dendrimer is widely used. Some other types of dendrimers are peptide dendrimers, glyco-dendrimers, polypropilimine dendrimers, Polyethyleneimine (PEI) dendrimers etc.

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3.1.3. Nanoshells

Nanoshells (100-200 nm) may be used for drug carrier of both imaging and therapy. Nanoshells consist of nanoparticles with a core of silica and a coating of thin metallic shell [29]. They can be targeted to a tissue by using immunological methods. Nanoshells can also be embedded in a hydrogel polymer [30]. Nanoshells are currently being investigated for prevention of micrometastasis of tumors and also for the treatment of diabetes. Nanoshells are useful for diagnostic purposes in whole blood immunoassays [31].

3.2. Fullerenes and nanotubes

Fullerenes composed of carbon in the form of a hollow sphere or ellipsoid tube. These are also known as 'bucky balls' because of their resemblance to the geodesic dome design of Buck minster Fuller. Fullerenes are being investigated for drug transport of antiviral drugs, antibiotics and anticancer agents [32]. Fullerenes have the potential to stimulate host immune response and productions of fullerene specific antibodies. Soluble derivatives of fullerenes such as C60 have shown great utility as pharmaceutical agents.

Nanotubes are nanometer scale tube like structure and they are of different types like carbon nanotube, inorganic nanotube, DNA nanotube, membrane nanotube etc. [33]. Carbon nanotubes can be made more soluble by incorporation of carboxylic or ammonium groups to their structures and can be used for the transport of peptides, nucleic acids and other drug molecules. The ability of nanotubes to transport DNA across cell membrane is used in studies involving gene therapy. DNA can be attached to the tips of nanotubes or can be incorporated within the tubes [34].

3.3. Nanopores

Nanopores (20 nm in diameter) consist of wafers with high density of pores which allow entry of oxygen, glucose and other chemicals such as insulin to pass through. Nanopores can be used as devices to protect transplanted tissues from the host immune system, at the same time, utilizing the benefit of transplantation [35]. β-Cells of pancreas can be enclosed within the nanopore device and implanted in the recipient's body. Nanopores can also be employed in DNA sequencing. Nanopores are also being developed with an ability to differentiate purines from pyrimidines [36].

3.4. Quantum dots

Quantum dots (QD) are tiny semiconductor nanocrystals type of particles generally no larger than 10 nanometers that can be made to fluoresce in different colours when stimulated by light. The biomolecule conjugation of the QD can be modulated to target various biomarkers [37]. They can be tagged with biomolecules and used as highly sensitive probes. QD can also be used for imaging of sentinel node in cancer patients for tumour staging and planning of therapy. This technology also outlines some early success in the detection and treatment of breast cancer [38]. QD may provide new insights into understanding the pathophysiology of cancer and real time imaging and screening of tumors.

Bioconjugated QD are collections of variable sizes of nanoparticles embedded in tiny beads made of polymer material. In a process called "multiplexing," they can be finely tuned to a myriad of luminescent colors that can tag a multitude of different protein biomarkers or genetic sequences in cells or tissues [39]. The new class of quantum dot conjugate contains an amphiphilic triblock copolymer layer for *in vivo* protection and multiple PEG molecules for improved biocompatibility and circulation, making it highly stable and able to produce bright signals. Another advantage is that quantum dot probes emitting at different wavelengths can be used together for imaging and tracking multiple tumor markers simultaneously, potentially increasing the specificity and sensitivity of cancer detection [40]. Recent progress in the surface chemistry of QD has expanded their use in biological applications, reduced their cytotoxicity and rendered quantum dots a powerful tool for the investigation of dinstinct cellular processes, like uptake, receptor trafficking and intracellular delivery. Another application of QD is for viral diagnosis. Rapid and sensitive diagnosis of Respiratory Syncytial Virus (RSV) is important for infection control and development of antiviral drugs. Antibody-conjugated nanoparticles rapidly and sensitively detect RSV and estimate relative levels of surface protein expression. A major development is the use of dual-colour QD or fluorescence energy transfer nanobeads that can be simultaneously excited with a single light source [41]. QD linked to biological molecules, such as antibodies, have shown promise as a new tool for detecting and quantifying a wide variety of cancer-associated molecules. In the field of nanomedicine, QD can make a worthy contribution to the development of new diagnostic and delivery systems as they offer unique optical properties for highly sensitive detection and they are well defined in size and shape and can be modified with various targeting principles.

4. Applications of Nanoscale drug delivery systems

4.1. Nanotechnology for brain drug delivery

The blood brain barrier (BBB) is a structure formed by a complex system of endothelial cells, astroglia, pericytes, and perivascular mast cells, preventing the passage of most circulating cells and molecules [42]. The tightness of the BBB is attributed mainly to the vascular layer of brain capillary endothelial cells which are interconnected side-by-side by tight and adherens junctions. Among the different nanodevices, nanosize drug delivery systems between 1 and 100 nm work as a whole unit in terms of transport to cross BBB [43]. Nanosize brain drug delivery systems may promote the targeting ability of drug in brain and at the same time enhance the permeability of molecules through BBB. However crossing of BBB by the nano drug carriers will depend completely on the physicochemical and biomimetic features and does not depend on the chemical structure of drug, inside the nanoparticles [44]. Nanosize drug carriers which do not cross BBB generally can be made "stealth" coated with some polymeric materials or other chemicals to avoid the reticuloendothelial system, to display long circulation time and stability in blood, and may be functionalized to successfully cross the BBB and target brain [45].

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4.2. Nanosize drug carriers in ocular drug delivery

Drug loaded nanoparticles with favourable biological properties include prolonging the residence time, decreasing toxicity and high ability of drug penetration into the deeper layers of the ocular structure and minimizing precorneal drug loss by the rapid tear fluid turnover [46]. Nanoparticles could target at cornea, retina and choroid by surficial applications and intravitreal injection. Nanocarrier based drug delivery is suitable in the case of the retina, as it has no lymph system, hence retinal neovascularisation and choroidal neovascularization have similar environments to that of solid tumors, and the EPR effect as available for solid nanoparticles in case of solid tumor may be also available for drug delivery targeted to eyes by nanoparticles [47]. Nanoparticles can deliver ocular drugs to the target sites for the treatment of various diseases such as glaucoma, corneal diseases, diabetic retinopathy etc. The uses of nanotechnology based drug delivery systems like nanosuspensions, SLNs and nanoliposomes have greater effect for ocular therapeutic efficacy [48]. Nanotechnology-based drug delivery is also very efficient in crossing membrane barriers, such as the blood retinal barrier in the eye.

4.3. Nanoparticle loaded contact lenses

Contact lenses loaded with nanoparticles can be effective for topical administration of ophthalmic drugs. Drug loaded contact lenses can also provide continuous drug release because of slow diffusion of the drug molecules through the lens matrix. The soaked contact lenses also delivered drugs only for a period of few hours for some typical drugs [49]. The duration of drug delivery from contact lenses can be significantly increased if the drug is first entrapped in nanoformulations, such as nanoliposomes, nanoparticles, or microemulsions. Such drug nanocarriers can then be dispersed throughout the contact lens material. The entrapment of drug in nanocarriers also prevents the interaction of drug with the polymerization mixture. This provides additional resistance to drug release, as the drug must first diffuse through the nanocarriers and penetrate the drug carrier surface to reach the contact lens matrix [50].

4.4. Biodistribution of nanoparticles in the retina

The ocular biodistribution of nanoparticles can provide insights into the bioavailability, cellular uptake, duration of drug action and toxicity. Factors such as particle size, composition, surface charge and mode of administration influence the biodistribution in the retinal structures and also their drainage from the ocular tissues [51]. Larger particles (2 μm) were found to remain in vitreous cavity near the trabecular meshwork from which they are discharged out from the ocular tissue within 6 days, whereas the particles 200 nm were found evenly distributed in the vitreous cavity, and the inner limiting membrane. The smaller particles ~50 nm crossed the retinal barriers, and was detected in the retina even after 2 months post injection [52]. The surface chemistry can also affect nanoparticle distribution. Positively charged nanoparticles can adhere to the anionic vitreous network components and aggregate within the vitreous network. The surface chemistry can also affect nanoparticle distribution. Posively charged nanoparticles can adhere to the anionic vitreous network components and aggregate

within the vitreous humor [53]. Anionic nanoparticles were found to diffuse through the vitreous humor and could even penetrate the retinal layers to be taken up by Muller Cells [54]. Vitreous humor is regarded as the barrier for non-viral ocular gene therapy because of the strong interaction of conventional cationic nature of non-viral gene vectors with the anionic vitreous humor [53]. The cationic PEI nanoparticles aggregated within vitreous humor and were prevented from distributing to the retina by the vitreal barrier. In contrast, cationic glycol chitosan (GC) nanoparticles and GC/PEI blended nanoparticles could penetrate the vitreal barrier and even reach at the inner limiting membrane because of the existence of glycol groups on nanoparticles [55].

4.5. Nanoparticles in cancer

Cancer cells are more vulnerable than normal cells to the effect of chemotherapeutic agents and the most of the anticancer drugs can cause injury to the normal cells. Optimum dose and frequency are both important factors in the persistence of cancer cells during cancer chemotherapy [56]. Now attempts are focused on efforts to kill cancer cells by more specific targeting while sparing the normal cells.

Nanoparticulate delivery systems in cancer therapies provide better penetration of therapeutic and diagnostic substances within the cancerous tissue in comparison to conventional cancer therapies [57]. Nanoparticles are constructed to take advantages of fundamental cancer morphology and modes of development such as rapid proliferation of cells, antigen expression, and leaky tumor vasculature. Nanoparticulate drug delivery systems are being developed to deliver smaller doses of chemotherapeutic agents in an effective form and control drug distribution within the body [58]. Nanocarriers can offer many advantages over free drugs in cancer chemotherapy such as they protect the drug from premature degradation, prevent drugs from prematurely interacting with the biological environment, enhance absorption of the drugs into a selected tissue (solid tumour), control the pharmacokinetic and drug tissue distribution profile and improve intracellular penetration [59].

Nanoparticulate delivery systems utilize specific targeting agents for cancer cells minimizing the uptake of the anticancer agent by normal cells and enhance the entry and retention of the agent in tumor cells (Figure 3) [60]. Nanocarriers may actively bind to the specific cancer cells by attaching targeting agents with the help of ligand molecules to the surface of the nanocarriers that bind to specific receptor antigens on the cell surface. Nanocarriers will recognize and bind to target cells through ligand receptor interactions. It is even possible to increase the drug targeting efficacy with the help of antibodies by conjugating a therapeutic agent directly to it for targeted delivery [61].

Like receptor targeting, targeting of angiogenic factors also takes advantage of properties unique to cancer cells. Anti-angiogenic treatment is the use of drugs or other substances to stop tumors from developing new blood vessels. In a study nanoparticles were formulated comprising a water-based core of Vickers microhardness sodium alginate, cellulose sulphate, and anti-angiogenic factors such as thrombospondin (TSP)-1 or TSP-517, crosslinked with dextran polyaldehyde with calcium chloride or conjugated to heparin sulphate with sodium chloride. In addition bioluminescent agent, luciferase, or contrast agent, polymeric gadolinium

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was located within the polyanionic core [62] for drug targeting and detection. Similarly, many efforts are on for cancer cell targeting specifically with drug nanocarriers.. Thus the drug nanocarriers are of great hope for future cancer therapy.

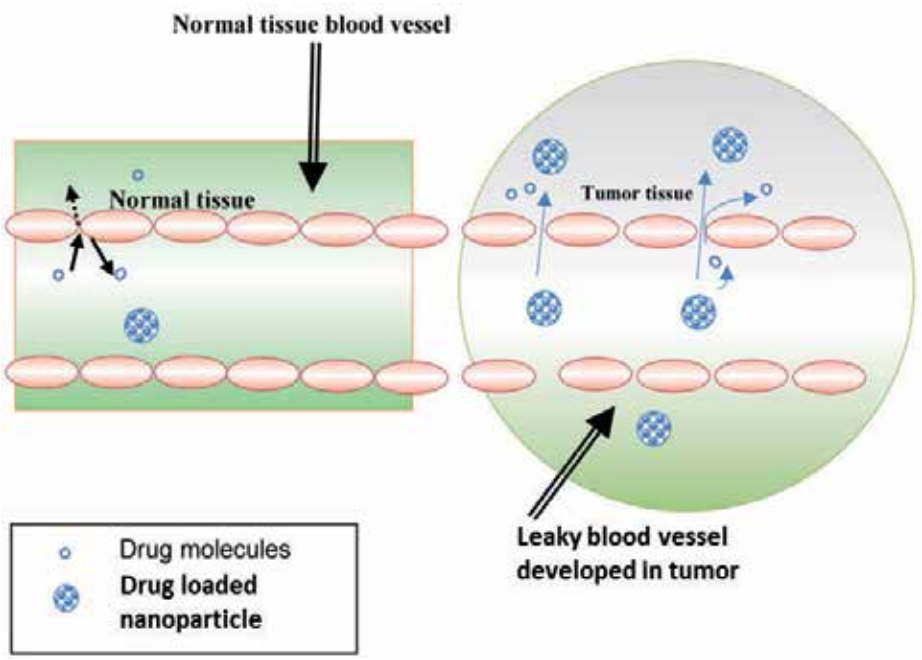

Figure 3. Schematic diagram of nanoparticle permeation and retention effect in normal and tumour tissues. Normal tissue vasculatures are lined by tight endothelial cells, hereby preventing nanoparticulate drug delivery system from escaping, whereas tumor tissue vasculatures are leaky and hyperpermeable allowing preferential accumulation of nanoparticles or nanoliposomes in the tumor interstial space by passive targeting

4.6. Gene delivery

Transfer of genetic material in nanocarriers may be an approach for the treatment of various genetic disorders such as diabetes mellitus, cystic fibrosis, alpha 1 antitrypsin deficiency and may more. A number of systemic diseases are caused by lack of enzymes factors that are due to missing or defective genes [63]. Previously gene therapy which was used to treat genetic disorders nowadays being contemplated as carrier systems which could be implanted for combating diseases other than genetic disorder like malignant form of cancer, heart diseases and nervous diseases [64]. Nanoliposomes can be used to deliver genetic materials into cells. Nanoliposomes incorporated with PEG and galactose target liver cells effectively due to their rapid uptake by liver Kupffer cells. Gene therapy may be tried with liposomal nanocarriers for liver disorders such as Wilson's and hereditary hemochromatosis. Cationic nanoliposomes have been considered as potential non-viral human gene delivery system [65]. Another effective method for administering nanoliposomes is by using ligand receptor complex using EGF-EGFR system for targeting purpose by nanoliposomes where EGF is a small protein which

binds with receptor EGFR. Also mixing cationic lipids with plasmid DNA leads to the formation of lipoplexes where the process is driven by electrostatic interactions [66]. The negatively charged genetic material (e.g. plasmid) is not encapsulated in nanoliposomes but complexed with cationic lipids by electrostatic interactions. Plasmid liposome complexes can enter the disease cells by infusion with the plasma or endosome membrane. Allovectin-7 (gene transfer product) is composed of a plasmid containing the gene for the major histocompatibility complex antigene HLA-B7 with B2 microglobulin formulated with the cytofectin [67]. The nature of a composed lipid decides the unloading of the gene from nanoliposomes which enables control over the mode of release, doping of nanoliposomes with neutral lipids such as 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) which helps in endosomal membrane fusion by recognizing and destabilizing the phospholipids in a flip flop manner which paves way for the liposomes to integrate in the membrane with the dissociation of nucleic acid into the cytoplasm [64].

Viral system based gene carrier had the ability to overcome the biological barriers in the body and then access to the host nucleus replicative machinery which resulted in the exploitations of the system for drug delivery using nanotechnology [64]. The development of a non-viral method for *in vivo* gene transfer was designed where the vector was packed into compact nanoparticles by successive additions of oppositely charged polyelectrolytes including an incorporation of ligands into the DNA-polyelectrolyte shells which were mixed with Pluronic F127 gel serving as a biodegradable adhesive to keep shells in contact with the targeted vessel [68].

A novel method of gene delivery is with viruses such as adeno associated virus (AAV) which have their virulent genes removed with lentiviruses, clearly showing their efficiency [64].

8. Drug delivery with the help of empty virus capsid

The viral nanoparticles (VNPs) consist of protein core which ranges in complexity from small capsid-protein homomers to larger protein-based heteromers capable of internalizing oligonucleotides and being enveloped by lipids. Chemical modification process and genetic mutation provide the viral coat proteins with receptor binding domain that helps in cell specific targeting of VNPs [69]. Even fusion of terminal / internal proteins on the surface or inside the VNPs can be utilized for introduction of heterologous peptides, and in some cases entire proteins. VNPs can be genetically engineered by inserting amino acids for bioconjugation, peptide based affinity tags and peptides as targeting ligands for stimulation of immune response. [70].

High sequence variability due to the influence of the immune system in viral life-cycles is often seen on the surface loops of viral capsid proteins. This variability makes the loops highly susceptible to insertion of foreign sequences. VP1, the major coat protein of viruses of Polyomaviridae family, when expressed in insect cells, yeast and *Escherichia coli* self-assembles as protein cages and shows natural affinity for a cell surface glycoprotein with a terminal a 2,3-linked N-acetylneuraminic acid and attaches to a4h1-integrin receptors [71]. Virus like particle

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(VLPs) constructed from the virus are used to deliver therapeutic genes to human fetal glial cells. Another technique Cell-docking involves attachment of antibodies to the surface of brain natriuretic peptide (BNPs). Coupling reaction between murine polyoma-virus and antitumor antibody B3 yielded polyoma VLPs with 30 to 40 antibody fragments bound to the surface, allowing the modified VLPs to bind to the breast carcinoma cells with high efficiencies [72].

9. A glimpse to future of nanosize drug delivery systems

Advancement of nanosize drug delivery systems establishes a new paradigm in pharmaceutical field. Convergence of science and engineering leads a new era of hope where medicines will act with increase efficacy, high bioavailability and less toxicity. Several nanoscale drug delivery systems are currently in clinical trials and few of them are already commercially available. Examples of such products are Abeicet (for fungal infection), Doxil (antineoplastic), Abraxane (metastatic breast cancer), Emend (antiemetic) etc. Despite the impressive progress in the field, very few nanoformulations have been approved by US-FDA (United States Food and Drug Administration) and even reached market in recent years. Although nanocarriers have lots of advantages because of the unique properties they have, there are many clinical, toxicological and regulatory aspects which are the matters of concern too. The biocompatibility of nanomaterials is of atmost importance because of the effect of the nanomaterials in the body ranging from cytotoxicity to hypersensitivity [8]. With the advancement of nanotechnology, the biological phenomenon such as host response to a specific nanomaterial should also be clinically transparent [9]. Therefore it is quite essential to introduce cost effective, better and safer nanobiomaterials which will provide efficient drug loading and controlled drug release of some challenging drug moieties for which there is no other suitable delivery available yet.

Nanoliposomes are well developed and presently possess the highest amount of clinical trials among other nanomaterials with some formulations currently in the market. This may be due to the fact that other materials have not been investigated for the same duration and are relatively newer in comparison. However polymer based nanomaterial, carbon nanotubes, gold nanoparticles etc. should not be overlooked because of less number of clinical trials [7].

Genexol-PM is an example which was undergone recent clinical trial. This is an amphiphilic diblock co-polymer (PEG-D, L-Lactic acid) that delivers paclitaxel. Clinical trial currently is in phase IV using Genexol-PM for recurrent breast cancer and phase III for breast cancer. Fungal infections associated with acute leukemia and for central line fungal infections, amphotericin B containing nanoliposomes are in phase IV clinical trial. ThermoDox (Doxorubicin loaded nanoliposome) is currently in phase III trials for hepatocellular carcinoma. Similarly Caelyx, a doxorubicin HCl loaded nanoliposome that is pegylated, is currently in phase IV trials for ovarian neoplasms [7]. Some recent clinical trials are shown in Table 1.

Ligand or antibody conjugated nanoformulation, bifunctional and multifunctional nanoparticles are the newer research approaches through which detection and treatment of cancerous cells can be achieved. Nanomachines are also largely in the research-and-development phase, but some primitive molecular machines have been tested. An example is nanorobot which is

capable of penetrating the various biological barriers of human body to identify the cancer cells. Thus, nanodrug delivery systems have a leading role to play in nanomedicine in near future.

Product name	Delivery material	Phase	Condition	Therapeutic Delivered	Sponsor	Clinicaltrials. gov Identifier
Genexol-PM	Amphilic diblock Copolymer forming micelle	I	Non small Cell lung cancer	Paclitaxel	Samyang Biopharmaceutical Corp	NCT01023347
Docetaxel-PNP	Polymeric nanoparticles	I	Advanced solid malignancies	Docetaxel	Samyang Biopharmaceutical Corp	NCT01103791
CYT-6091	AuNP	I	Unspecified adult solid tumor	TNF	NCI	NCT00356980
Paclitaxel poliglumex	Drug Polymer Conjugate	II	Prostate cancer	Paclitaxel	OHSU Knight Cancer Institute	NCT00459810
Kogenate FS	PEG-liposome	I	Hemophilia A	Recombinant factor VIII	Bayer	NCT00629837
Long-circulating liposomal prednisolone disodium phosphate	Liposome	II	Rheumatoid arthritis	Prednisolone	Radboud University	NCT00241982
LE-DT	Liposome	II	Pancreatic cancer	Doxetaxel	Insys Therapeutics Inc	NCT01186731
Cisplatin and Liposomal Doxorubicin	Liposome	I	Advanced cancer	Cisplatin and doxorubicin	M.D. Anderson Cancer Center	NCT00507962
Liposomal doxorubicin and bevacizumab	Liposome	II	Kaposi's sarcoma	Doxorubicin and bevacizumab	NCI	NCT00923936
AP5346	Drug polymer conjugate	Not stated	Head and neck cancer	AP5346 and oxaliplatin	University of California, San Diego	NCT00415298

Abbreviations: PEG-Polyethylene glycol, TNF-Tumor necrosis factor, NCI-National Cancer Institute, AuNP-Gold nanoparticles

Table 1. Recent Nanodrug Carriers in Clinical Trials (Source: Clinicaltrials.gov)

Nanocarriers may lead to a solution to major unsolved medical problems which will aggressively enhance quality of life.

Regulatory aspect: One of the main areas related to the safety aspects of drug-nanocarrier systems is to encourage academic organizations, industry and regulatory governmental agencies to establish convincing testing procedures on the safety aspects of the nanomaterials.

The global importance of trade for nanomaterials has established new international organizations, such as the International Council on Nanotechnology (ICON), the International Organization for Standardization (Geneva, Switzerland) etc. for sharing responsibilities in this field. In the year 1996 the NNI was established in the United States of America to coordinate governmental multi-agencies such as the Food and Drug Administration (FDA), the Department of Labor through the Occupational Safety and Health Administration (OSHA), the National Institute for Occupational Safety and Health (NIOSH), and the Environmental Protection Agency (EPA), for the development of nanoscience and technology.

10. Conclusion

Last few years several new technologies have been developed for the treatment of various diseases. The use of nanotechnology in developing nanocarriers for drug delivery is bringing lots of hope and enthusiasm in the field of drug delivery research. Nanoscale drug delivery devices present some advantages which show higher intracellular uptake than the other conventional form of drug delivery systems. Nanocarriers can be conjugated with a ligand such as antibody to favor a targeted therapeutic approach. The empty virus capsids are also being tried to use for delivering drugs as a new therapeutic strategy. Thus, nanoscale size drug delivery systems may revolutionize the entire drug therapy strategy and bring it to a new height in near future. However, toxicity concerns of the nanosize formulations should not be ignored. Full proof methods should be established to evaluate both the short-term and long-term toxicity analysis of the nanosize drug delivery systems.

Author details

Biswajit Mukherjee*, Niladri Shekhar Dey, Ruma Maji, Priyanka Bhowmik,
Pranab Jyoti Das and Paramita Paul

*Address all correspondence to: biswajit55@yahoo.com

Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

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Parliament, the council and the European and Economic Social Committee on the Second Regulatory Review on Nanomaterials. SWD 2012; 288 Final.

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This book collects reviews and original articles from eminent experts working in the interdisciplinary arena of nanotechnology use in drug delivery. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentialities of nanotechnology application of drug delivery. Since the advent of analytical techniques and capabilities to measure particle sizes in nanometer ranges, there has been tremendous interest in the use of nanoparticles for more efficient methods of drug delivery. On the other hand, this reference discusses advances in design, optimization, and adaptation of gene delivery systems for the treatment of cancer, cardiovascular, pulmonary, genetic, and infectious diseases, and considers assessment and review procedures involved in the development of gene-based pharmaceuticals.

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