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Meet the editor

Dr. Ali Demir Sezer has a Ph.D. from 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 is 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

Drug delivery is a method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. Drug delivery 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. Drug release comes from diffusion, degradation, swelling, and affinity-based mechanisms. Most common routes of administration include the preferred non-invasive peroral, topical, transmucosal and inhalation routes. Many medications such as peptide and protein, antibody, vaccine and gene based drugs, in general may not be delivered using these routes.

However, for all these exciting new drug and vaccine candidates, it is necessary to develop suitable dosage forms or drug delivery systems to allow the effective, safe and reliable application of these bioactive compounds to the patient. In the view of most experts pharmacology is on drugs, targets, and actions. In the context, the drug as a rule is seen as an active pharmaceutical ingredient and not as a complex mixture of chemical entities of a well defined structure. Today, we are becoming more and more aware of the fact that delivery of the active compound to the target site is a key. The present volume gives a topical overview on various modern approaches to drug delivery and targeting on covering today's options for specific carrier systems allowing successful drug treatment at various sites of the body that are difficult to address and allowing to increase the benefit-risk-ratio to the optimum.

On the other hand, the reader will be introduced to various aspects of the fundamentals of nanotechnology based drug delivery systems and the application of these systems 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.

The aim of this book was to gather all results coming from very fundamental studies. Again, this allows to gain a more general view of the various drug carrier systems and can prepare and apply, along with the methodologies necessary to design, develop and characterize them.

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 trough behavior of intermittent administrations with drug carrier systems. Novel drug delivery carriers 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.

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Section 1

Powder Technology in Drug Delivery

Chapter 1

Hot-Melt Extrusion (HME): From Process to Pharmaceutical Applications

Mohammed Maniruzzaman, Dennis Douroumis,
Joshua S. Boateng and Martin J. Snowden

Additional information is available at the end of the chapter

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1. Introduction

Over the last three decades hot-melt extrusion (HME) has emerged as an influential processing technology in developing molecular dispersions of active pharmaceutical ingredients (APIs) into polymers matrices and has already been demonstrated to provide time controlled, modified, extended and targeted drug delivery resulting in improved bioavailability [1, 2, 3, 4]. HME has now provided opportunity for use of materials in order to mask the bitter taste of active substances. Since industrial application of the extrusion process back in the 1930's HME has received considerable attention from both the pharmaceutical industry and academia in a range of applications for pharmaceutical dosage forms, such as tablets, capsules, films and implants for drug delivery through oral, transdermal and transmucosal routes [5]. This makes HME an excellent alternative to other conventionally available techniques such as roll spinning and spray drying. In addition to being a proven manufacturing process, HME meets the goal of the US Food and Drug Administration's (FDA) process analytical technology (PAT) scheme for designing, analyzing as well as controlling the manufacturing process through quality control measurements during active extrusion process [6]. In this chapter we review the hot-melt extrusion technique, based on a holistic perspective of its various components, processing technologies as well as the materials and novel formulation design and developments to its varied applications in oral drug delivery systems.

2. Hot-melt extrusion (HME): Process technology

Joseph Brama first invented the extrusion process for the manufacturing of lead pipes at the end of the eighteenth century [7]. Since then, it has been used in the plastic, rubber and food manufacturing industries to produce items ranging from pipes to sheets and bags. With the

advent of high throughput screening, currently more than half of all plastic products including bags, sheets, and pipes are manufactured my HME and therefore various polymers have been used to melt and form different shapes for a variety of industrial and domestic applications. The technology (HME) has proven to be a robust method of producing numerous drug delivery systems and therefore it has been found to be useful in the pharmaceutical industry as well [8]. Extrusion is the process of pumping raw materials at elevated controlled temperature and pressure through a heated barrel into a product of uniform shape and density [9]. Breitenbach first introduced the development of melt extrusion process in pharmaceutical manufacturing operations [10], however, Follonier and his co-workers first examined the hot melt technology to manufacture sustained release polymer based pellets of various freely soluble drugs [11]. HME involves the compaction and conversion of blends from a powder or a granular mix into a product of uniform shape [9]. During this process, polymers are melted and formed into products of different shapes and sizes such as plastic bags, sheets, and pipes by forcing polymeric components and active substances including any additives or plasticisers through an orifice or die under controlled temperature, pressure, feeding rate and screw speed [9, 12]. However, the theoretical approach to understanding the melt extrusion process could be summarized by classifying the whole procedure of HME compaction into the followings [13]:

1. Feeding of the extruder through a hopper
2. Mixing, grinding, reducing the particle size, venting and kneading
3. Flow through the die.
4. Extrusion from the die and further down-stream processing.

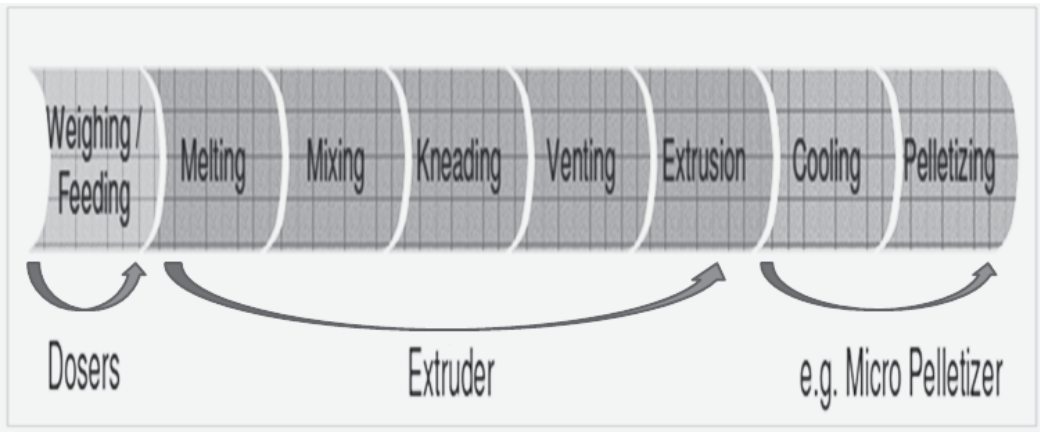

Figure 1. Schematic diagram of HME process [12].

The extruder generally consists of one or two rotating screws (either co-rotating or counter rotating) inside a stationary cylindrical barrel. The barrel is often manufactured in sections in order to shorten the residence time of molten materials. The sectioned parts of the barrel are then bolted or clamped together. An end-plate die is connected to the end of the barrel which is determined according to the shape of the extruded materials.

3. Equipment: Single screw and twin screw extruder

A single screw extruder consists of one rotating screw positioned inside a stationary barrel at the most fundamental level. In the more advanced twin-screw systems, extrusion of materials is performed by either a co-rotating or counter-rotating screw configuration [9]. Irrespective of type and complexity of the function and process, the extruder must be capable of rotating the screw at a selected predetermined speed while compensating for the torque and shear generated from both the material being extruded and the screws being used. However, regardless of the size and type of the screw inside the stationary barrel a typical extrusion set up consists of- a motor which acts as a drive unit, an extrusion barrel, a rotating screw and an extrusion die [13]. A central electronic control unit is connected to the extrusion unit in order to control the process parameters such as screw speed, temperature and therefore pressure [14]. This electronic control unit acts as a monitoring device as well. The typical length diameter ratios (L/D) of screws positioned inside the stationary barrel are another important characteristic to consider whether the extrusion equipment is a single screw or twin screw extruder. The L/D of the screw either in a single screw extruder or a twin screw extruder typically ranges from 20 to 40:1(mm). In case of the application of pilot plant extruders the diameters of the screws significantly ranges from 18-30 mm. In pharmaceutical scale up, the production machines are much larger with diameters typically exceeding 50-60mm [15]. In addition, the dimensions of a screw change over the length of the barrel. In the most advanced processing equipment for extrusion, the screws could be separated by clamps or be extended in proportion to the length of the barrel itself. A basic single screw extruder consists of three discrete zones: feed zone, compression and a metering zone (Fig. 2). Under the compression zone which is basically know as processing zone could be accompanied by few other steps such as mixing, kneading, venting etc [13, 15].

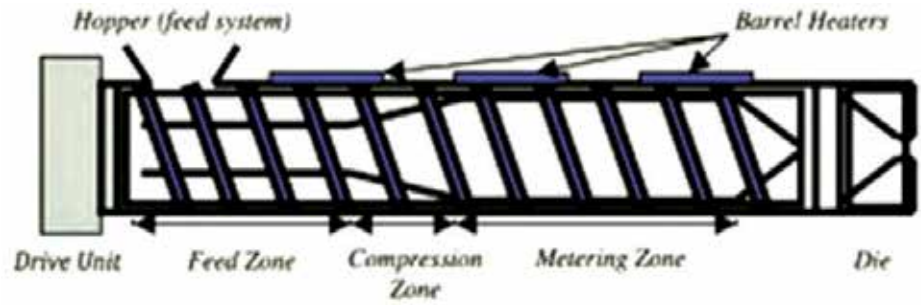

Figure 2. Schematic diagram of a single screw extruder [10].

The depth along with the pitch of the screw flights (both perpendicular and axial) differ within each zone, generating dissimilar pressures along the screw length (Fig. 3). Normally the pressure within the feed zone is very low in order to allow for consistent feeding from the hopper and gentle mixing of API, polymers and other excipients and therefore the screw flight depth and pitch are kept larger than that of other zones. At this stage of the process the pressure within the extruder is very low which subsequently gets increased in the

compression zone. This results in a gradual increase in pressure along the length of the compression zone which effectively imparts a high degree of mixing and compression to the material by decreasing the screw pitch and/or the flight depth [9, 15]. Moreover the major aim of the compression zone is not only to homogenize but also compress the extrudate to ensure the molten material reaches the final section of the barrel (metering zone) in a form appropriate for processing. Finally the final section which is known as the metering zone stabilizes the effervescent flow of the matrix and ensures the extruded product has a uniform thickness, shape and size. A constant and steady uniform screw flight depth and pitch helps maintain continuous high pressure ensuring a uniform delivery rate of extrudates through the extrusion die and hence a uniform extruded product.

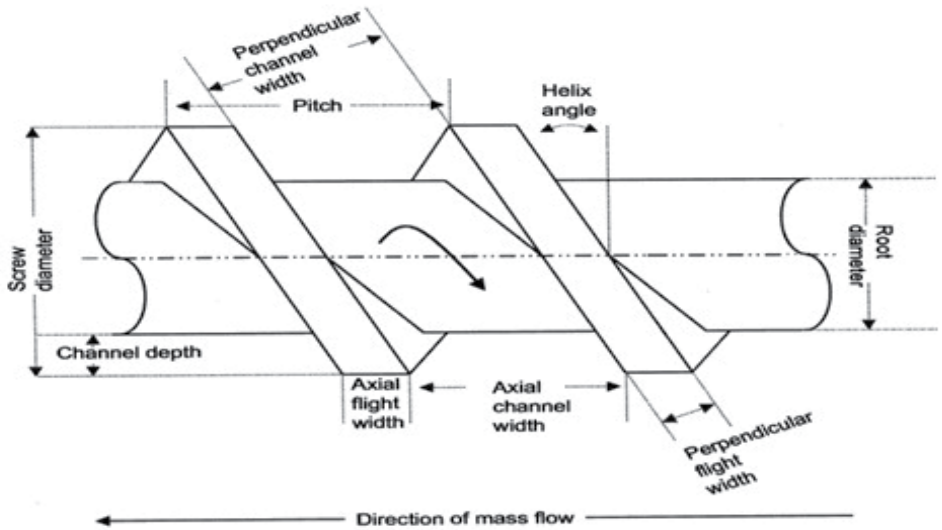

Figure 3. Screw geometry (extrusion) [9].

In addition to the above mentioned systems, downstream auxiliary equipment for cooling, cutting, collecting the finished product is also typically employed. Mass flow feeders to accurately meter materials into the feed hopper, pelletizers, spheronizer, roller/calendaring device in order to produce continuous films and process analytical technology such as near infra-red (NIR) and Raman, ultra sound, DSC systems are also options. Throughout the whole process, the temperature in all zones are normally controlled by electrical heating bands and monitored by thermocouples.

The single screw extrusion system is simple and offers lots of advantages but still does not acquire the mixing capability of a twin-screw machine and therefore is not the preferred approach for the production of most pharmaceutical formulations. Moreover, a twin-screw extruder offers much greater versatility (process manipulation and optimisation) in accommodating a wider range of pharmaceutical formulations making this set-up much more constructive. The rotation of the screws inside the extruder barrel may either be co-rotating (same direction) or counter-rotating (opposite direction), both directions being

equivalent from a processing perspective. A greater degree of conveying and much shorter residence times are achievable with an intermeshing set-up. Furthermore, the use of reverse-conveying and forward-conveying elements, kneading blocks and other intricate designs as a means of improving or controlling the level of mixing required can help the configuration of the screws themselves to be varied [16].

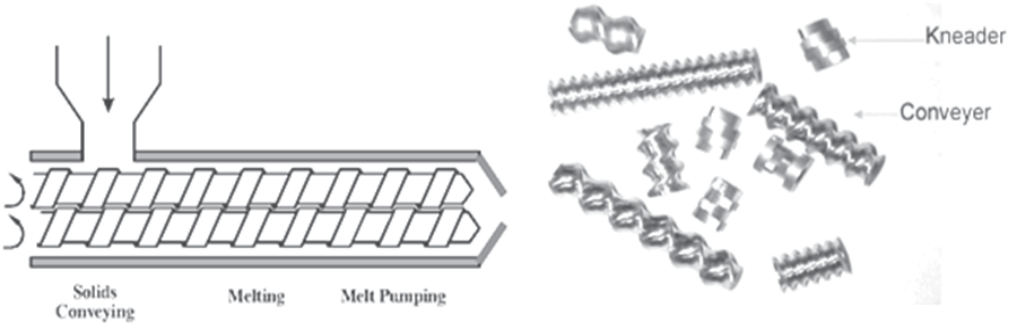

Figure 4. A twin screw extruder and screws [9].

4. Advantages of HME

HME offers several advantages over conventionally available pharmaceutical processing techniques including: (a) increased solubility and bioavailability of water insoluble compounds, (b) solvent free non ambient process, (c) economical process with reduced production time, fewer processing steps, and a continuous operation, (d) capabilities of sustained, modified and targeted release, (e) better content uniformity in extrudates, (f) no requirements on the compressibility of active ingredients, (g) uniform dispersion of fine particles, (h) good stability at changing pH and moisture levels and safe application in humans, (i) reduced number of unit operations and production of a wide range of performance dosage forms, and (j) a range of screw geometries [17, 18, 19, 20, 21].

However, HME has some disadvantages as well. The main drawbacks of HME include: thermal process (drug/polymer stability), limited polymers, high flow properties of polymers and excipients required and not suitable for relatively high heat sensitive molecules such as microbial species, proteins etc [20, 21].

5. Applications of HME

Extrusion technology is one of the most important fabrication processes in the plastic and rubber industries. Products made from melt extruded polymers range from pipes to hoses through to the insulated wires, cables, rubber sheeting and polystyrene tiles. Plastics that are commonly processed by HME technique include acrylics and cellulosics, polyethylene, poly propylene, polystyrene and vinyl plastics [9, 22]. In the food industry, extrusion has been utilized for pasta production with a widely used multitalented technique combines cooking and extrusion in a self-styled extrusion cooker [23]. In the animal feed industry and veterinary science, extrusion is commonly applied as a means of producing pelletized feeds,

implants or injection moulding [24]. HME has successfully been applied in the formulation of fast dispersing PVP melt extrudates of poorly soluble active agents as solid molecular dispersions in the crop protection field [25].

HME technology has already achieved a strong place in the pharmaceutical industry and academia due to several advantages over traditional processing methods such as roll spinning, grinding [18]. In addition to being an efficient manufacturing process, HME enhances the quality and efficacy of manufactured products and therefore over the past few years HME has emerged as a novel technique in the pharmaceutical applications [15, 28]. The main use of HME is to disperse active pharmaceutical ingredients (APIs) in a matrix at the molecular level, thus forming solid solutions [26]. In the pharmaceutical industry, HME has been used for various applications, such as i) enhancing the dissolution rate and bioavailability of poorly soluble drug by forming a solid dispersion or solid solution, ii) controlling or modifying the release of the drug, iii) taste masking of bitter APIs, and iv) formulation of various thin films [27].

The bioavailability of an active ingredient is controlled by its aqueous solubility. Therefore increasing the solubility of water insoluble drugs is still a real challenge in the formulation development process [26]. Due to the advent of high throughput screening (HTS) in the drug discovery process, the resultant compounds are often high molecular weight and highly lipophilic and therefore exhibit poor solubility [29]. Scientists have already tried to address solubility issues by various pharmaceutical interventions. Among the many methods available to improve solubility and dissolution rate, preparation of solid dispersions and solid solutions has gained vast attention. For that reason HME has been successfully applied to prepare solid molecular dispersion of APIs into different hydrophilic polymer matrices [26, 29].

6. Formulation research and developments to dates

Despite the fact that initial research developments have focused on the effects of formulation and processing variables on the properties of the final dosage forms, [9, 30, 31, 34, 35] more recent investigations have focused on the use of HME as a novel manufacturing technology of solid molecular dispersions through to the development of mini-matrices, taste masked formulations and also sustained release formulations as well as paediatric formulations [26, 48]. Early work by De Brabander *et al.* (2000) described the preparation of matrix mini-tablets which was followed by further investigations into the properties of sustained release mini-matrices manufactured from ethyl cellulose, HPMC and ibuprofen [32, 33]. Extruded mini tablets showed minimised risk of dose dumping, reduced inter- and intra-subject variability. Very recently, Roblegg *et al.* (2011) reported the development of retarded release pellets using vegetable calcium stearate (CaSt) as a thermoplastic excipient processed through HME, where pellets with a drug loading of 20% paracetamol released only 11.54% of the drug after 8 hours due to the significant densification of the pellets. As expected, the drug release was influenced by the pellet size and the drug loading [36]. A microbicide intravaginal ring (IVRs) IVR was prepared and developed from polyether

urethane (PU) elastomers for the sustained delivery of UC781 (a highly potent nonnucleoside reverse transcriptase inhibitor of HIV-1). PU IVRs containing UC781 were fabricated using a hot-melt extrusion process [37].

Moreover, a fourfold increase in the availability of propanolol in the systemic circulation was observed when the HME formulation was compared with a commercially available formulation (Inderal®). Over the last five years HME has been used largely to manufacture granules, pellets, immediate and modified release tablets, transmucosal/ transdermal films and implantable reservoir devices [3, 4, 9,35]. For instance, with respect to drug administration through the oral route, molecular solid dispersions of nifedipine [38], nimodipine [29] and itraconazole [39, 40,41] have been successfully produced using HME technology. Amorphous indomethacin dispersions have been manufactured using pharmaceutically acceptable hydrophilic polymers by using HME technology [26, 42,43].

Furthermore, HME research developments have driven targeted drug delivery systems including enteric matrix tablets and capsule systems over the last few years [44, 45]. Miller *et al.* (2007) have demonstrated the ability of HME to act as an efficient dispersive process for aggregated, fine engineering particles to improve dissolution rate properties by enhancing particles' wettability [46]. A very interesting investigation of Verreck and co-workers (2006) [47] determined the use of supercritical carbon dioxide (scCO2) as a temporary plasticiser during the manufacture of ethylcellulose through HME. A significant reduction in the processing temperature was achieved using scCO2 without any disadvantageous effects on the extrudate. Macroscopic morphology was significantly altered due to expansion of the scCO2 in the die. The use of scCO2 increased the surface area, porosity and hygroscopicity of the final dosage forms. More recently Douroumis and co-workers used HME technique to effectively enhance the solubility of ibuprofen, indomethacin and famotidine [26, 42].

The taste masking of bitter APIs is a major challenge especially for the development of orally disintegrating tablets (ODT). HME has been reported to be an effective technique to mask the bitter tastes of various APIs by the use of taste masking polymers that create solid dispersions to prevent bitter drugs from coming in contact with the patient's taste buds. Breitkruitz et al. (2008) successfully applied HME in taste masking of sodium benzoate for the formulation of paediatric drugs [48]. More recently Grycze *et al.* (2011) and Maniruzzaman *et al.* (2012) developed taste masked formulations of ibuprofen and paracetamol, respectively [26, 48]. Basically taste masking is achieved through intermolecular forces (e.g. hydrogen bonding) between the active substance and the polymer matrix by processing oppositely charged compounds through HME [49, 50]. The extrusion of solid lipids using twin–screw extruders was introduced for the preparation of immediate or sustained release taste masked matrices [51]. In this process, occasionally called "solvent – free cold extrusion" the lipids are extruded below their melting ranges. Consequently, the lipids are not melted during extrusion and build a coherent matrix with low porosity. In these few studies the effect of lipid composition and processing parameters such as the die diameter, the size of the extruded pellets, the screw speed and the powder feeding rates on the obtained drug release patterns were thoroughly investigated. Very

recently, Breitkreutz *et al.* (2012) applied solid lipid extrusion at room temperature for the taste masked formulation development of the BCS Class II drug NXP 1210. In this study, the authors investigated powdered hard fat (Witocan® 42/44 mikrofein), glycerol distearate (Precirol® ato 5) and glycerol trimyristate (Dynasan® 114) as lipid binders. The lipid based formulations design in this study was feasible for taste-masked granules or pellets containing poorly soluble drugs [52].

6.1. Films by hot-melt extrusion

Only a handful of researchers have reported the use of hot-melt extrusion for the manufacture of films. Films can be defined as thin sheets containing one or more polymers with or without a plasticiser and may be used as a drug delivery system (device) or directly applied to facilitate a therapeutic effect as in wound dressings. Films are currently being produced mainly by solvent casting in which polymers (and excipients such as plasticisers) are dissolved in a suitable solvent until they form clear viscous solution (gel). While film preparation using the solvent–casting approach allows film uniformity, clarity, flexibility and adjustable thickness to accommodate drug loadings they are limited by decreased elongation or elasticity and increased film tensile strength when physical aging is applied [53]. Another, limitation associated with solvent cast films is the use of organic solvents for water insoluble polymers. The hazardous nature of most organic solvents and the residual solvents after drying affect the selection of the appropriate solvent [54–57] as well as complicated processing conditions and disposal of the associated waste, all of which create significant environmental concerns. As a result, alternative technologies are needed in the pharmaceutical industry to overcome some of challenges described above. The two commonly used approaches include spray coating and hot melt extrusion with the latter becoming increasingly popular due to the many advantages it provides. Firstly, no solvents are used and fewer processing steps are required. In fact one of the key advantages of HME is the fact that extrudates can be obtained in a single processing step making it very economical. As far as films are concerned, there is no requirement for compressing of the active ingredients together with the excipients. The melting of the polymer into the molten state, coupled with the thorough initial mixing allows a more uniform dispersion of fine particles. Further, molecular dispersion of the drug helps improve its bioavailability [58]. Hot melt extruded films are produced through a simple process involving blending of appropriate amounts relevant polymer, drug and plasticiser into a uniform powdered mixture prior to feeding through the hopper of the preheated extruder and transferred into the heated barrel by a rotating extruder screw. Homogeneous films are obtained with thickness generally expected to be in the range less than 1mm. Generally three main ingredients are required for successful formulation of hot melt films i.e. film forming polymer, active ingredient and plasticiser [59]. The latter is required to impart flexibility to the final film which ensures ease of handling and application to the site of action. Occasionally, other additives are added to affect other functionally important properties such as bioadhesive agent which ensures that the film adheres to the mucosal surface for a long enough time to allow drug absorption or action. Different polymers and drugs have been employed and reported in the literature for obtaining drug loaded hot-melt extruded films for various indications and are summarised in table 1.

Film	Main polymer(s)	Plasticiser/additive	Main active ingredient(s)	Author
1	Acrylic Eudragit	- Triacetin - Triethylcitrate	Lidocaine	[60]
2	Hydroxypropylcellulose Polyethylene oxide	N/A	Ketoconazole	[61]
3	Hydroxypropylcellulose Hydroxypropylmethylcellulose Polyethylene oxide	Polyethylene glycol 3350	Lidocaine	[62]
4	Hydroxypropylcellulose	Polyethylene glycol 400	Hydrocortisone	[65]
5	Hydroxypropylcellulose Polycarbophil	Polyethylene glycol 3350	Clotrimazole	[58]
6	Polyethylene oxide	N/A	Ketoprofen	[59]

Table 1. Different hot-melt extruded films comprising different polymeric materials, plasticisers and active ingredients for various indications.

Repka and co-workers have conducted extensive research on the use of HME for the manufacture of mucoadhesive buccal films. They successfully evaluated different matrix formers and additives for the processing of the blend prior to extrusion [61, 62, 63, 64]. In an early investigation, it was found that even though films containing exclusively HPC could not be obtained, the addition of plasticizers, such as triethyl citrate, PEG 2000/8000, or acetyltributyl citrate, allowed for the manufacture of thin, flexible, and stable HPC films [65]. It has also been found that increasing the molecular weight of HPC decreased the release of drugs from hot-melt extruded films which resulted in dissolution profiles exhibiting zero-order drug release. According to the models applied in the research, the drug release was solely determined by erosion of the buccal film [66, 67, 68].

Development of films by HME may present future opportunities to develop gastro-retentive films for prolonged drug delivery and multi-layer films to modulate drug release for oral and transdermal applications. The growing market in medical devices, including incorporating drugs such as biodegradable stents and drug-loaded catheters will undoubtedly require HME manufacturing processes. These are required to be commercialised and perhaps may lead to new areas of collaboration across pharmaceutical, medical device and biotechnology research.

7. HME in commercial products

HME related patents which have been issued for pharmaceutical systems have steadily increased since the early 1980's. So far, the USA and Germany hold approximately more than half (56%) of all issued patents for HME in the market [69]. Despite this increased interest, only a handful of commercialized HME pharmaceutical products are currently

marketed. Several companies have been recognized to specialize in the use of HME as a drug delivery technology, such as PharmaForm and SOLIQS (Abbott). Recently, SOLIQS has developed a proprietary formulation which is known as Meltrex® and re-developed a protease-inhibitor combination product, Kaletra®. Kaletra is mainly used for the treatment of human immunodeficiency virus (HIV) infections. The formulated, melt extruded product was shown to have a significant enhancement in the bioavailability of active substances [70]. Furthermore, HME Kaletra® tablets were shown to have significant advantages for patient compliance (i.e. reduced dosing frequency and improved stability) compared to the previous soft-gel capsule formulation as recognized by the FDA decision to fast-track approval. Additionally, Nurofen (Meltlets® lemon) is available on the market as a fast dissolving tablet prepared by HME [42]. Ibuprofen has been used as active substance in the Meltlets® tablets where its bitter taste was successfully masked by similar technique to HME. Moreover, SOLIQS has also developed a fast-onset ibuprofen system and a sustained-release formulation of verapamil (Isoptin® SR-E) through a HME related technology called 'Calendaring' that was the first directly shaped HME product on the market.

8. Summary

HME has proven to be a robust method of producing numerous drug delivery systems and therefore it has been found to be useful in the pharmaceutical industry enlarging the scope to include a range of polymers and APIs that can be processed with or without plasticizers. It has also been documented that HME is a solvent-free, robust, quick and economy favoured manufacturing process for the production of a large variety of pharmaceutical dosage forms.

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Chapter 2

Development and Investigation of Dry Powder Inhalers for Cystic Fibrosis

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Additional information is available at the end of the chapter

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1. Introduction

Cystic Fibrosis (CF) is the most common lethal monogenic disorder in Caucasians, estimated to affect one *per* 2500-4000 newborns. CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) [1, 2]. CFTR acts mainly as a chloride channel and has other regulatory roles, including inhibition of sodium transport through the epithelial sodium channel, regulation of ATP channels and intracellular vesicle transport, acidification of intracellular organelles and inhibition of endogenous calcium- activated chloride channels [3-5]. CFTR is also involved in bicarbonate-chloride exchange [6]. In the airways, loss of functional CFTR promotes increase of oxidation status, tissue injury, modification of intracellular signaling pathways, cell apoptosis and inflammatory processes.

Clinically, the reduced volume of the epithelial lining fluid and the increased viscoelasticity of the mucus lead to a dysfunction of the mucociliary clearance, and as a consequence, patients suffer from recurrent and chronic infections caused mainly by bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, and especially *Pseudomonas aeruginosa*. Moreover, the chronic *P. aeruginosa* lung infection causes a sustained inflammatory response in the lung. Antibiotics are administered to CF patients in long-term treatment with the hope of maintaining quality of life, weight and lung function, as well as to decrease the number of exacerbations and hospital admission [7, 8].

Today there are few formulations, mostly solutions, approved for inhalation in CF patients and there is a continuous research in the development of new inhaled antibiotic therapeutic systems for management of chronic CF lung disease. New formulations and delivery devices are needed to improve efficiency, portability and possibly increase the dose locally available.

Besides chronic bacterial infections, chronic airway inflammation is uniformly observed in patients with CF [9, 10], as a consequence of over-expression of proinflammatory enzymes. Thus, the lung, dipped in an environment rich in oxygen as well as a defective antioxidant system, is susceptible to injury mediated by oxidative stress. Reactive oxygen species, ROS, such as super oxide anion ($O^{2\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), and reactive nitrogen species, RNS, such as nitrogen dioxide and peroxynitrite, are unstable molecules with unpaired electrons, capable of initiating oxidation. In order to prevent tissue damage, lungs are endowed with several antioxidant defences, including glutathione, heme oxygenase, superoxide dismutase, vitamins C and E, beta-carotene, uric acid [11]. However, when the presence of ROS and RNS overcomes the physiologic antioxidant defences, an oxidative stress status, occurs. Thus, as an adjunct to optimal antibiotic therapy, antioxidant/anti-inflammatory therapy is warranted to avoid a decline in lung function and tissue damage.

1.1. Respiratory drug delivery

Inhalation drug therapy consists of drug administration directly to the lung in form of micronized droplets or solid microparticles, highly recommended especially in pathologies affecting the lung (i.e. asthma, cystic fibrosis, chronic obstructive pulmonary disease). The administration of the active compound directly in the airways can be of great advantage: after inhalation, the site where the drug is deposited is less aggressive in terms of pH and enzymatic attach; additionally, the hepatic first-passage effect is bypassed. Both aspects influence the dose administered, which can be decreased compared to oral route. Moreover, the permeability of pulmonary epithelium is higher than the intestinal mucosa, due to a reduced resistance to substance transport. Finally, the drug dissolution, critical for many compounds, is less relevant in the case of solids, since the active compound is a very fine powder that impacts with a high surface area. In addition to biopharmaceutical aspects, inhalation bioavailability requires the deposition of the dose in the lung i.e., the active compound must be formulated in a respirable form. Development of formulations for inhalation is particularly challenging since the preparation of a respirable formulation and the selection of an adequate device for the administration are both required. Formulation and device constitute the dosage forms and affect the bioavailability of the inhaled drug. Concerning the formulation, dry powder inhalers (DPI) are preferred to solutions/suspensions due to drug stability, high concentration at the site of action and lack of propellant. The biggest issue encountered when formulating a dry powder for inhalation is its size which has to be small enough to guarantee the aerosolization and the deposition at the appropriate site of the respiratory tract. A failure in deposition may result in a failure of efficacy. Given that any discussion about the right size of particles for inhalation is meaningless without the consideration of their geometry and density, the concept of aerodynamic diameter has been introduced. The aerodynamic diameter (Dae) is a spherical equivalent diameter and derives from the equivalence between the inhaled particle and a sphere of unit density (ρ_0] undergoing sedimentation at the same rate (Eq. 1).

$$Dae = \mathrm{Dv}\sqrt{\frac{\rho}{\chi\rho_0}} \quad (1)$$

where Dv is the volume-equivalent diameter, ρ is the particle density and χ is the shape factor.

Hence, the aerodynamic behaviour depends on particle geometry, density and volume diameter: a small spherical particle with a high density will behave aerodynamically as a bigger particle, being poorly transported in the lower airways. The Dae can be improved reducing the volume diameter and the density or increasing the shape factor of the particles, by means of different processes, i.e. dry or wet milling, spray-drying, spray-freeze drying, and supercritical fluid technology. Among these, spray drying is a commonly used technique for the preparation of dry powders for inhalation.

1.2. Spray drying

Spray drying is a one-step process able to convert liquid feeds (i.e., solutions, suspensions and emulsions) in a dry powder. Firstly, the liquid is broken into droplets by means of a nozzle atomizer (atomization step); then, droplets come in contact with a heated gas in the drying chamber and the drying step starts; finally, the dried particles are separated from the heated gas by means of a cyclone (separation step) and collected into a glass container. The optimization of the aerodynamic properties of the powders produced *via* spray drying can be achieved modulating process parameters, solvent composition, solute concentration, liquid feed rate, inlet temperature, gas pressure and aspiration.

1.3. The challenge of excipients for dry powder inhalers

The primary function of the lung is respiration. To fulfil this purpose, the lung has a large surface area and a thin membranes. Many compounds have been tested to overcome drug delivery outcomes related to the small particle size requested for deposition. For example, in the spray drying process the powder properties can be modulated adding excipients able to affect the evaporation of spray droplets during the drying and consequently the particle shape. The safety of an inhalation drug product has to be taken into account: the structural and functional integrity of respiratory epithelium must be respected. This hardly limits the choice of excipients available for the formulation to few compounds, like sugars (lactose, mannitol and glucose) and hydrophobic additive (magnesium stearate, DSPC). As a matter of fact, natural amino acids (AAs) possess good safety profiles and, recently, showed to enhance flow aid properties when co-spray-dried with active compounds. As a support to AAs pulmonary safety, a formulation of Aztreonam and lysine (Cayston®, powder for instant solution and inhalation) has been recently approved by FDA for CF patients.

2. Aerosolized antioxidant and anti-inflammatory agents in Cystic Fibrosis

Oxidative stress has been identified as an early complication in the airways of infants and young children affected by CF [12, 13]. Recent clinical data suggest that oxidative damage of

pulmonary proteins during chronic infection may contribute to the decline of lung function in CF patients [14]. The massive infiltration of neutrophils in lungs of CF patients leads to the generation of oxygen-derived reactive oxygen species (ROS) and, in particular, H_2O_2 that contributes to irreversible lung damage and, ultimately, to patient death. Activated neutrophils migrate to the airways and release large amounts of ROS. On the other hand in CF epithelial cells, antioxidant defense systems appear to be defective in their ability to control the amount of ROS produced [15]. Therefore over-abundance of ROS and their products may cause tissue injury-events and modify intracellular signalling pathways leading to cell apoptosis and enhanced inflammatory processes. In addition to its Cl^- channel function, CFTR has been proposed to carry antioxidant-reduced glutathione. A recent study demonstrated that oxidative stress can suppress CFTR expression and function while increasing the cellular GSH content. Chronic lung inflammation with episodes of acute exacerbations initiates several physiological and metabolic changes with harmful effects including weight loss and metabolic breakdown. Antioxidants (glutathione, vitamins, beta-carotene, selenium and flavonoids) as dietary support or pharmacological treatment can be a promising approach. Great attention has been focused on flavonoids [16, 17], polyphenolic compounds with antioxidant, anti-inflammatory and antibacteric activity, hugely present in fruits and vegetables. Among natural flavonoids, naringin (N, Fig. 1) extracted from grapefruits has shown anti-inflammatory, antioxidant and anticarcinogenic effects [18].

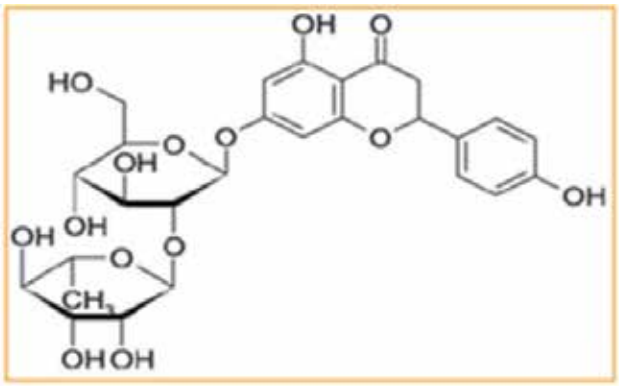


Figure 1. Naringin 4,5,7-trihydroxyflavanone 7-rhamnoglucosyde

In addition, recent studies have reported that flavonoids may act as CFTR direct activators, stimulating transepithelial chloride transport [19-21]. Although flavonoids are inhibitors of tyrosine kinases and phosphatases, their effects on CFTR are probably independent of these activities, resulting from direct binding to an NBD of phosphorylated CFTR [22].

With the aim to discover more effective activators of G551D-CFTR [19], some investigators have begun to examine the relationship between the chemical structure of flavonoids and their effects on CFTR Cl¦ channels. This study served to identify the pharmacophore portion of the skeletons molecular basis for interaction with the NBD. The well-documented antioxidant effect of flavonoids is unfortunately more evident *in vitro* than *in vivo*, due to the

high concentration needed, the susceptibility to oxidation and instability to the gastric pH in which they undergo hydrolysis and enzymatic degradation. Moreover, flavonoids show a very slight solubility in water, which leads to a very low dissolution rate, an irregular absorption of the drug from oral solid dosage forms in the gastrointestinal tract and a limited bioavailability. Despite a number of publications focused on the antioxidant effect of flavonoids, rather no attention has been addressed yet to their formulation in order to increase bioavailability. Recently, oral hydrophilic swellable matrices for a controlled release of some flavonoids [23, 24] and gastroresistant microparticles aiming at overcoming the acid environment have been formulated [25, 26]. An alternative strategy may be the direct aerosol delivery to the lung, which has the advantage to achieve higher locally available concentration of the antioxidant in the airways.

2.1. Naringin dry powders production and characterization

Naringin is a very slightly soluble molecule: its lipophilia can affect the dissolution of the drug when in contact with the liquids lining the lung. The micronization by means of spray drying process and addition of opportune additives able to improve powder wettability seem to be a valid strategy for the formulation of an efficacious dry powder inhaler. Micronized particles were produced by completely dissolving the active naringin (N) alone (#NET3) or with 5% w/w of leucine (#NET3-leu5) as dispersibility enhancer in 7/3 water/ethanol solutions [27, 28]. Spray drying conditions were: inlet temperature 110°C, drying air flow 500L/min, aspirator 100%, feed rate 5ml/min, nozzle 0.5mm. Aerodynamic properties were determined by means of both single stage glass impinger (SSGI) and Andersen cascade impactor (ACI). The device used for the DPI deposition tests was the Turbospin (kindly donated by PH&T SpA) in which the dose to be aerosolized was pre-metered in a size 2 gelatine capsule. Results demonstrated that the presence of leucine in the feed solution influenced particle size distribution, as well as powder density and morphology. Firstly, NET3-leu5 showed a d_{50} sensibly lower than NET3, evidencing a positive effect of leucine on particle diameter.

Code #	Leu content (% w/w)	Spray yield (%)	d_{50} (μm) and span	FPF (%)
NET3	0	59.4 ± 0.3	5.2 [1.6]	44.5 ± 1.5
NET3-leu5	5	60.7 ± 2.5	3.3 [1.7]	51.3 ± 1.6

FPF, fine particle fraction

Table 1. Composition, spray drying yield, particle size distribution and fine particle fraction after SSGI of Naringin powders.

Moreover, as showed by thermograms of NET3-leu5, DSC analyses indicated that spray-dried powders containing the AA were amorphous materials. Spray drying process caused the loss of crystalline habitus of both N and leu raw material as evidenced by the absence of

the endotherms corresponding to N crystal melting point (247°C, Fig. 2b) and leu crystal melting point (275°C, Fig. 2a).

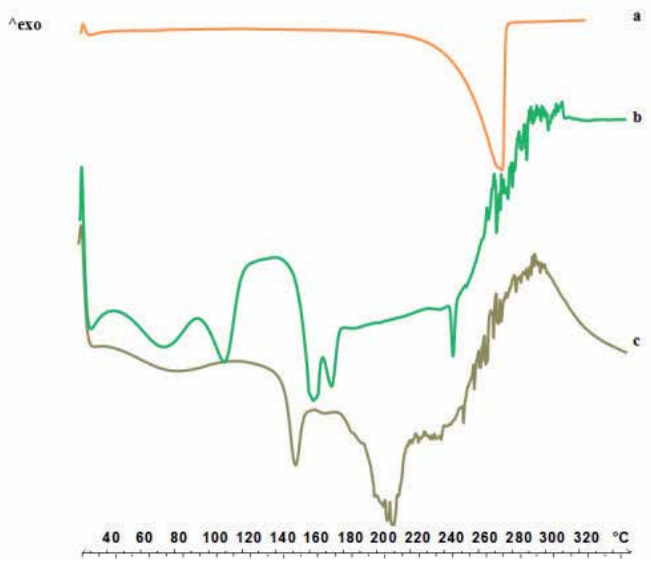


Figure 2. Differential scanning calorimetry thermograms of Naringin raw material (a), Leucine raw material (b) and NET3-leu5 (c).

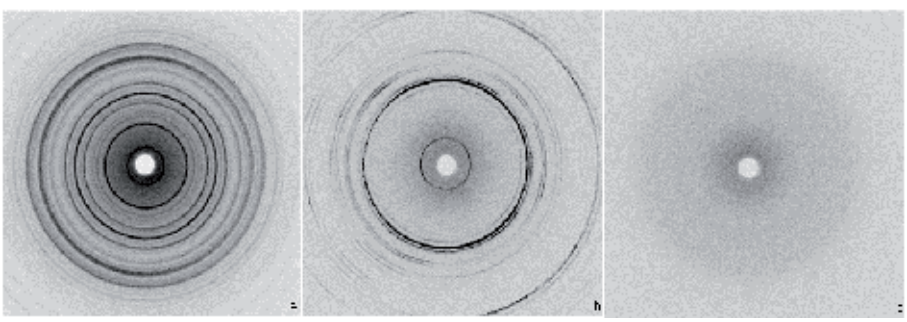


Figure 3. X-ray patterns of Naringin raw material (a), Leucine raw material (b) and NET3-leu5(c).

DSC results were confirmed by X-ray assessments, showing no crystalline state in NET3-leu5 powder. In figure 3 X-ray patterns of N (Fig. 3a) and leu (Fig. 3b) as raw materials were reported in comparison with X-ray patterns of NET3-leu5 (Fig. 3c). The loss in crystallinity is an important issue for drugs, such as N, very slightly soluble in water, bringing to an increase of solid solubility.

Microscopy observation revealed that particle morphology was affected by leucine content in the liquid feed: samples containing only N appeared as small particles, spherical in shape or very slightly corrugated and their SEM micrographs showed widespread aggregation (Fig. 4a).

On the contrary, micrographs of samples produced with 5% leu displayed well separated particles with corrugated, raisin-like surfaces (Fig. 4b), beneficial for particles intended for inhalation.

In fact, previous reports suggested that improvement of the respirable fraction may be obtained not only by lowering the size or the density of a powder, but also reducing interparticulate cohesion [29, 30]. Corrugated particles might also be more appropriate for dissolution in the lung fluid due to a larger area.

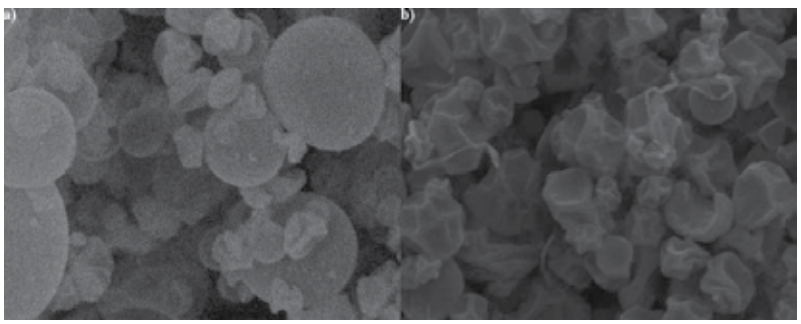


Figure 4. SEM picture of (a) NET3 and NET3-leu5

Regarding the *in vitro* deposition test by means of SSGI, the AA affected the aerodynamic properties of spray-dried powders as reported in table 1. NET3-leu5 showed an improvement of FPF due to both a reduction in the capsule and device retention and an increase in powder dispersibility. The latter is likely to be related to the absence of aggregates and high degree of particle corrugation, as observed by SEM analyses. These data were confirmed by ACI experiments (Fig. 5).

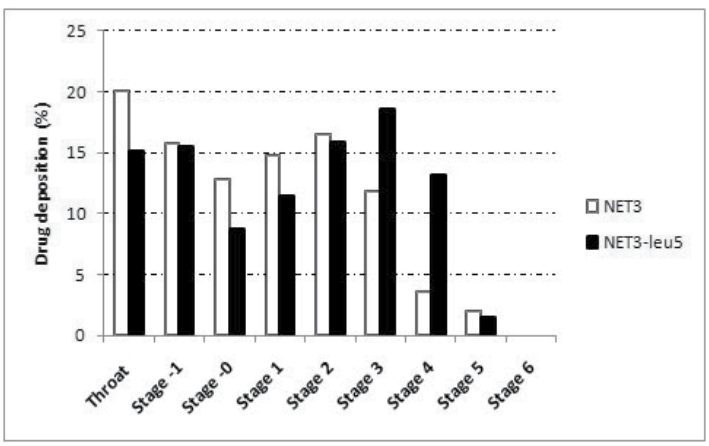


Figure 5. ACI deposition patterns of NET3 (white bars) and NET3-leu5 (black bars).

The powder containing the dispersibility agent (NET3-leu5) showed a lower deposition in the throat compared to NET3, with a resulting higher quantity of drug recovered from the deeper stages and an improving of the fine particle fraction.

These results are in agreement with previous report on the ability of surface corrugation to decrease interparticulate cohesion by reducing Van der Waals forces between particles and, consequently, increase powder respirability.

In conclusion, the use of leucine as excipient was useful to reduce adhesion between particles and improve powder dispersion, when delivered from dry powder inhalers. Therefore, a careful formulation plays a key role in the aerosol performance of N dry powders: NET3-leu5 showed optimezed bulk and aerodynamic behaviour.

Moreover, the spray drying process, reducing particle size while improving particle superficial area exposed to fluids, caused a greater (up to 30 fold higher, Fig. 6) immediate solubility of micronized powders (NET3 and NET3-leu5] when in contact with water at 37°C, compared to unprocessed commercial batch (rawN). Leucine addition to powder formulation (NET3-leu5) further increased N solubility which started declining very quikly, reaching a nearly constant value after 30 minutes, due to recrystallization of the amorphous material.

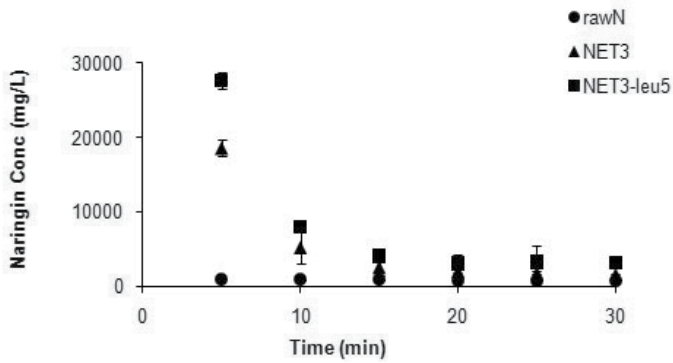


Figure 6. Aqueous solubility at 37°C of rawN (commercial batch, circles), NET3 (spray-dried batch without excipient, triangles) and NET3-leu5 (spray-dried batch with 5% leu, squares).

2.2. *In vitro* biological activities of N dry powders in bronchial epithelial cells

The developed dry powders have to be tested for verifying the ability to control airways inflammation. Two immortalized cell lines were selected as in vitro models: one, called CuFi1 (CF cells), was derived from human airway epithelial (HAE) cells of CFTR ΔF508/ΔF508 mutant genotype, the other, called NuLi1 (normal lung), was derived from a non-CF subject and used as control. These cell lines exhibited transepithelial resistance, maintained the ion channel physiology expected for the genotypes and retained NF-κB responses to inflammatory stimuli [31, 32] Cytotoxicity and effects on NF-κB pathway and on IL-6 and IL-8 release were examined.

2.2.1. Effect of N and its formulations on cell viability

Cytotoxicity (MTT assay) and cell viability (BrUd) evaluations (from 15 to 150μm) showed that neither rawN nor NET3 and NET3-leu5 are cytotoxic or cytostatic in both CF and non-

CF bronchial cells. After a 24 h treatment, rawN did not significantly affect cell viability, as determined by MTT assay in the concentration ranging from 15 to 150 μM (data not shown), but it caused a dose-dependent reduction of cell growth of different extent in NuLi1 and CuFi1 cells, from 60 to 150 μM (Fig. 7a). Interestingly, spray-dried powders containing leucine induced a dose-dependent and significant cell growth inhibition only in normal bronchial NuLi1 cells (Fig. 7c), while it determined a 14% increase of cell proliferation in CuFi1 cells at the highest dose (150 μM). To evaluate the contribution of the AA to the increased cell proliferation induced by NET3-leu5 in CuFi1, Leu spray-dried alone was also tested. The AA did not show any significant effect in NuLi1 cells while it was able to increase CuFi1 cell proliferation at all the concentrations tested (Fig. 7d).

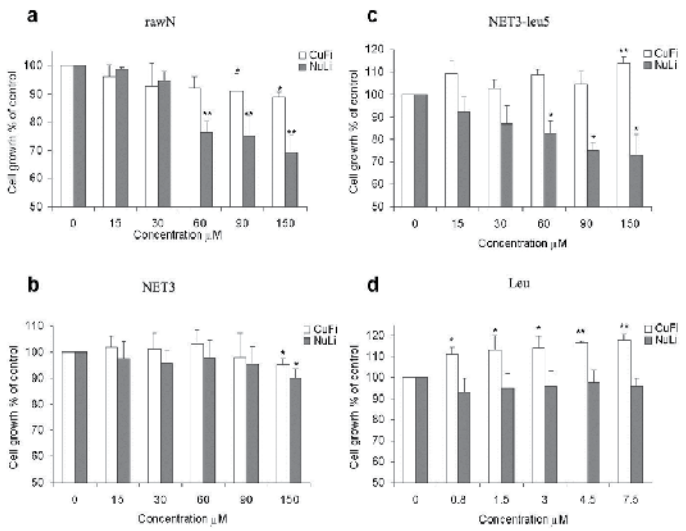


Figure 7. Naringin and its DPI formulations do not inhibit CuFi1 and NuLi1 cell proliferation at concentrations lower than 60 μM. Cells were treated for 24 h with: (a) raw Naringin (rawN), (b) spray-dried Naringin (NET3), (c) N co-sprayed with 5% leucine (NET3-leu5) (from 0.8 to 7.5 μM). Cell growth was determined using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit. The histograms report the percentage of growing cells in comparison with untreated cells (control, 100% proliferation). All data are shown as mean ± SD of three independent experiments each done in duplicate (*P < 0.05 and **P < 0.01 vs control).

This finding suggests that the technological improvement of immediate drug solubility and powder flowability, as well as the presence of the AA, may increase the drug uptake and improve the CF cell altered metabolism, reducing the toxicity observed for unprocessed rawN (Fig. 7a). In accordance, increased and altered basal protein catabolism has been reported in CF patients by many reports [33-35].

2.2.2. Effect of N and its formulations on NF-κB pathway

To study the anti-inflammatory effects of N in CuFi1 cells, we investigated the main molecular targets of NF-κB pathway in CuFi1 in comparison to normal bronchial NuLi1

cells. The NF-κB pathway is well known to play a crucial role in inflammatory process (36). In resting cells, the transcription factor NF-κB exists as homo- or heterodimer, maintained inactive in the cytosol by a family of inhibitor proteins named IκBs (IκBα, β, ε). In response to a wide range of stimuli such as cytokines and bacterial or viral products, IκB proteins are phosphorylated by IκB kinases (IKKα and β), ubiquitinated and degraded by the 26S proteasome. As a consequence, NF-κB dimers can localize into the nucleus and positively regulate the transcription of proinflammatory genes (37). This pathway is overactivated also in absence of any infection (38-40) in CF cells. In our experiments, CuFi1 cells exhibit higher expression levels of IKKβ and phosphoIKBα proteins compared to their normal counterpart NuLi1 cells (data from Western Blot analysis not shown). The effects of rawN, NET3 and NET3-leu5 at sub-toxic concentrations (30 μM) were evaluated at 2, 6 and 24 h on IKKβ and IκBα kinases, measuring both the expression levels and the phosphorylation status of the main molecular targets of the NF-κB pathway (i.e. IKKα, IKKβ and IκBα). Results are reported in figure 8.

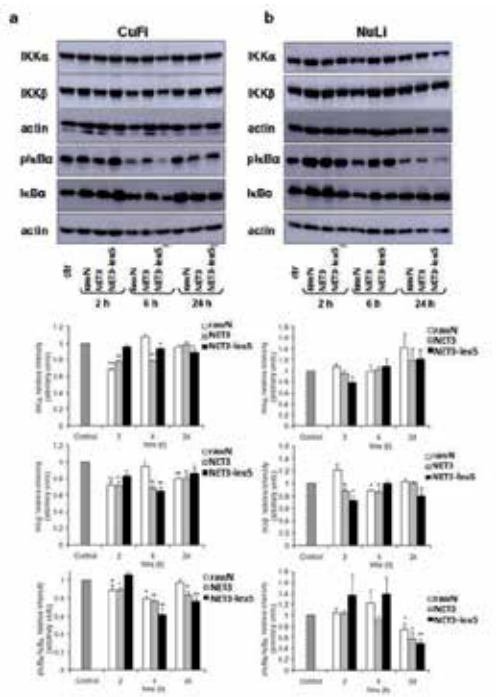


Figure 8. Naringin and its DPI formulations inhibit the key enzymes of the NF-κB pathway in CF bronchial epithelial cells. CuFi1 (a) and NuLi1 (b) cells were treated with raw Naringin (rawN), spray-dried Naringin (NET3) and N co-sprayed with 5% leucine (NET3-leu 5) at 30 μM concentration for the indicated time points. Cell lysates were analyzed by Western blot with antibodies against IKKα, IKKβ and pIκBα. Same filters were stripped and re-probed with total IκBα and anti-actin used as loading control. More representative results are shown (upper panels). Immunoreactive bands were quantified using Quantity One program. Densitometric analyses (mean ± SD) of three independent experiments are reported as relative intensity of IKKα, IKKβ or pIκBα/IκBα on actin and expressed as arbitrary units vs control (lower panels). (*P < 0.05 and **P < 0.01 vs control).

As regards to IKKα, NET3 and NET3-leu5 caused a reduction of IKKα but rawN did not in CuFi1 cells, while all powders did not cause any significant effect in normal airways epithelial cells (Fig. 8b). As IKKβ, its expression was generally reduced in CuFi1 cells: the highest decrease was observed at 6 h in NET3-leu5-treated cells (Fig. 8b). Interestingly, the observed reduction of expression levels of both the enzymatic subunits of the IKK complex in CuFi led to a significant and prolonged decrease of IκBα phoshorylation. In fact, this effect started early (2 h) and was retained all over the treatment time (24 h) in CF bronchial epithelial cells (Fig. 8a). On the contrary, in normal bronchial epithelial cells only a delayed (24 h) decrease of IκBα phosphorylation was observed as a consequence of the reduction of IKKβ subunit only expression level. Leucine spray-dried alone did not give any significant result in all Western Blot analyses (data not shown).

Previous evidence indicates that IKKβ plays a more crucial role for NF-κB activation in response to pro-inflammatory cytokines and microbial products [40], even though both the catalytic subunits of the IKK complex are able to regulate NF-κB activation and have a complementary role in the control of inflammation [41]. N formulations are effective in inhibiting both IKK subunits expression, and therefore caused a prolonged reduction of IκBα phosphorylation in CuFi1 cells.

2.2.3. Effect of N and its formulation on Interleukin-8 (IL-8) and interleukin-6 (IL-6) release

The direct effect of NET3-leu5 on the main cytokines involved in inflammatory response, interleukin 8 (IL-8) and interleukin 6 (IL-6) was also investigated. To this aim, CuFi1 cells were treated with NET3-Leu5 at 30 and 60 μM in the presence and absence of LPS-stimulation from *Pseudomonas aeruginosa*. Results (Fig. 9) showed that NET3-leu5 inhibited both cytokine production in unstimulated as well as in LPS-stimulated CuFi1 cells and the production of IL-8 more than IL-6.

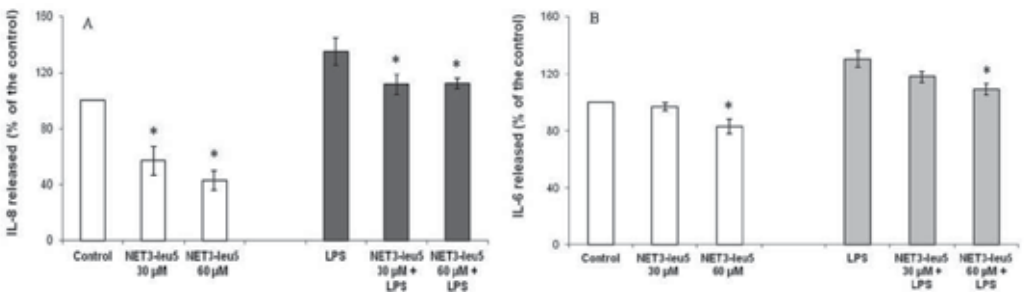


Figure 9. Effect of Naringin co-sprayed with 5% leucine (NET3-leu5) on basal and LPS-induced secretion of IL-8 (**A)** and IL-6 (**B)** in Cystic Fibrosis bronchial epithelial (CuFi1) cells. Data are presented as mean percentage of released cytokines in the control supernatants (untreated and unstimulated) ± SD of two independent experiments each done in duplicate. (*P < 0.05 vs control supernatants).

These data indicate that the inhibition of NFkB pathway by NET3-leu5 results in a reduction of the release of pro-inflammatory cytokines. NET3-leu5 seems involved in controlling the

pro-inflammatory status of CF cells in the presence as well as in the absence of bacterial stimulation. However, LPS-stimulated cytokine secretion is dependent on Toll-like receptor-4 (TLR-4] signaling which expression is reduced in the CF airway epithelial cells, promoting the bacterial colonization and chronic infection in CF lung (42).

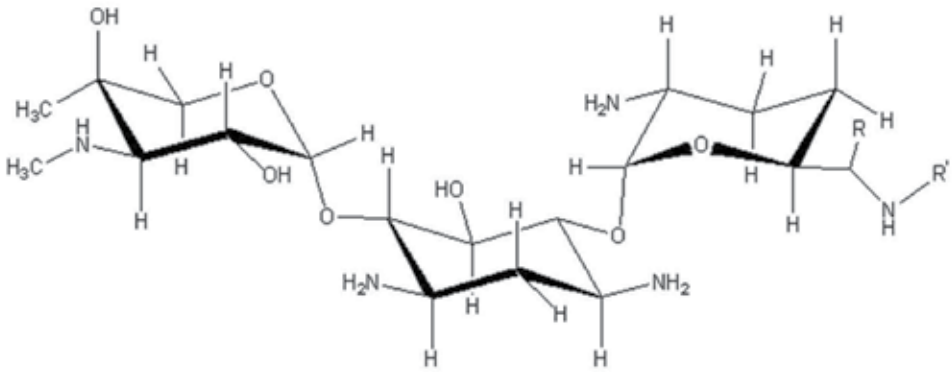
3. Aerosolized antibiotics in cystic fibrosis

Pulmonary infections are the major cause of morbidity and mortality in cystic fibrosis (CF), with Pseudomonas aeruginosa (Pa) acting as the princISOl pathogen. The viscous mucus lining the lung of CF patients impairs the mucociliary function, facilitating recurrent and chronic respiratory infections caused mainly by Pa but also by Haemophilus influenzae, Bulkolderia cepacia [7, 8]. Treatment of lung disease by antibiotics is an accepted standard in CF cure aiming at reducing decline in lung function and number of hospitalizations [43]. Aminoglycosides, such as gentamicin sulfate (G) (Fig. 10), are indicated in the management of acute exacerbations of CF as well as in the control of chronic infection and eradication of Pa infections. Various clinical studies on gentamicin inhalation treatment in cystic fibrosis patients chronically infected with Pseudomonas aeruginosa have shown that antibiotic solutions for aerosol treatment produce both subjective and objective improvement. Interestingly, among aminoglycosides, G has shown the ability to partially restore the expression of the functional protein CFTR (cystic fibrosis transmembrane conductance regulator) in CF mouse models bearing class I nonsense mutations [44-47]. In particular, Du and coll. [45] demonstrated that G was able to induce the expression of a higher CFTR level compared to tobramycin. Aminoglycoside antibiotics can suppress premature termination codons by allowing an amino acid to be incorporated in place of the stop codon, thus permitting translation to continue to the normal end of the transcript. Regarding the use of aminoglycosides in the treatment of airways infections and class I CFTR mutations, the main problem is their reduced penetration in the endobronchial space after intravenous (IV) administration, combined with their high systemic toxicity. Since aminoglycosides peak sputum concentrations are only 12 to 20% of the peak serum concentrations [48] to achieve adequate drug concentrations at the site of action, it is necessary to use large IV doses, which may produce serum levels associated with renal and oto-toxicity.

These problems can be overcome by the use of aerosolized aminoglycosides, which can deliver high dose of drug directly to the lungs, while minimizing systemic exposure. Therefore, the first aim of the research was to develop micronized gentamicin powders, easy to handle and stable for long time; the second goal was to obtain a dry powder suitable for pulmonary administration.

3.1. Design and development of a new dry powder inhaler of gentamicin

Differently from Naringin, Gentamicin is a very soluble drug: as its high hydrophilia guarantees a rapid drug solubility and diffusion in the fluids lining the lung, as it may cause high hygroscopicity and instability, preventing the formulation of a stable and respirable dry powder. As it is well known, hygroscopicity modulates the moisture content of the



	R	R'
Gent.C1	CH_3	CH_3
Gent.C2	CH_3	H
Gent.C1a	H	H

Figure 10. Gentamicin sulfate structure.

particles in the final dosage form prior to aerosol generation and it is correlated to chemical or physical instability of the product. For aerosols formulation, the agglomeration leads to an inability to generate particles of respirable size. Moreover, as aerosol particles enter the lungs, they experience a high-humidity environment (99.5% relative humidity at 37°C): inhaled particles may be subject to hygroscopic growth, increasing their dimensions and affecting lung deposition. In this case, excipients able to modify the hygroscopic properties of a drug need to be considered. A dry powder formulation was obtained by co-spray-drying Gentamicin and leucine from 7/3 hydro-alcoholic solutions, using an organic solvent less polar than ethanol, the isopropanol. Microparticles were designed while studying the effect of leu, feed composition and process parameters on particle formation, physicochemical properties and aerosol performance. In addition, the effect of the engineered particles on cell viability and cell proliferation of CuFi1 cells was investigated.

3.1.1. Manufacturing and characterization of G/leu co-spray-dried powders

Due to its high polarity, G raw material was deliquescent, becoming liquid after 1 hour of exposure to room conditions. In order to reduce hygroscopicity and to increase powder dispersibility, G was subject to spray drying process alone or with leu as flowability enhancer using water or water-isopropanol (ISO) mixtures.

Preliminarly, the solubilities of the drug and excipient in the feed systems were determined; G freely soluble in water exhibited the lowest solubility in water/ISO 7/3 (v/v) system, the poor solubility of leu is even lower in water-co-solvent systems (Table 2).

Liquid feed composition	G (mg/ml)	Leu (mg/ml)
Water	Freely soluble	24.2±1.0
Water/ISO 8/2 (v/v)	351.8±25.1	11.2±0.5
Water/ISO 7/3 (v/v)	135.9±24.6	9.5±0.2

Table 2. Gentamicin and L-leucine solubility in liquid feeds used for spray drying at pH 7.0±0.1.

As reported in Table 3, addition of the organic co-solvent into the water feed was extremely helpful in terms of process yield suggesting a reduction in powder cohesiveness and, therefore, a potential enhancement of the aerosolisation properties (49). Differently, leu addition did not have a linear effect on spray drying yield (Table 3).

	Code #	Leu content (%w/w)	Process yield (%)	d_{50} (μm) and span
20% v/v ISO	**GISO2**	0	78.0 ±3.8	4.74 [2.10]
	GISO2-leu5	5	73.9 ±0.5	6.19 [1.88]
	GISO2-leu10	10	65.0 ±5.5	4.07 [1.81]
	GISO2-leu15	15	84.6 ±3.3	3.72 [1.58]
	GISO2-leu5	20	77.5 ±0.6	4.82 [1.73]
30% v/vISO	**GISO3**	0	85.5 ±0.7	4.24 [1.97]
	GISO3-leu5	5	86.6 ±1.2	3.77 [1.36]
	GISO3-leu10	10	85.9 ±0.9	3.69 [1.51]
	GISO3-leu15	15	82.0 ±2.1	3.90 [1.62]
	GISO3-leu20	20	80.8 ±1.3	4.11 [1.90]

Table 3. Physical characteristics of spray dried particles: liquid fees composition, process yield, particle size and bulk density.

Optimized process parameters led to micronized powders with d_{50} (ranging from 3.7 μm to 4.8 μm) similar for all batches produced (Table 3), with no evident effect of solvent and leu content on the particles diameter.

Organic co-solvent had a massive effect on hygroscopicity too (Fig. 11). In particular, by adding 30% v/v of ISO into the aqueous feed, humidity uptake by G powders was reduced from 10.5% (water) to 4.8% (water/ISO) after exposure at room conditions. In the presence of 10% w/w leu, G lost its water avidity [0.9% weight gained after 80 min). These effects may be explained by the addition of the lower-soluble component (leu) into the liquid feeds, able to reach the critical concentration for shell formation as the droplet evaporation progresses during spray-drying process [50]. Such enrichment in leu at the particle surface seems to slow down water uptake of hygroscopic drug such as G, in agreement with previous observations [51] and, potentially, increase powder flowability.

Leu effect on spray-dried powders appears clearly, after microscopy studies, as an evident increase in particle corrugation. Morphology studies showed an increase in particle

corrugation as an effect of leu presence in spray-dried powders. As an example, SEM pictures of particles dried from 8:2 water/ISO ratio solutions were reported in figure 12.

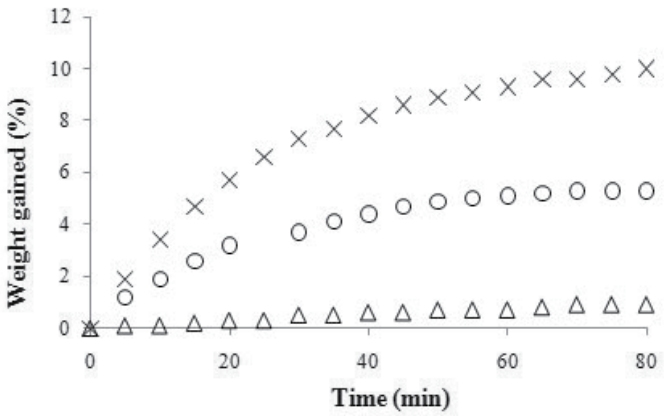


Figure 11. Weight gained after 80 min of exposure at room conditions by G raw material (cross), G spray-dried from 7:3 w water-ISO (circles) v/v systems, and G/10%leu spray-dried from water-ISO 7:3 v/v mixture (triangles).

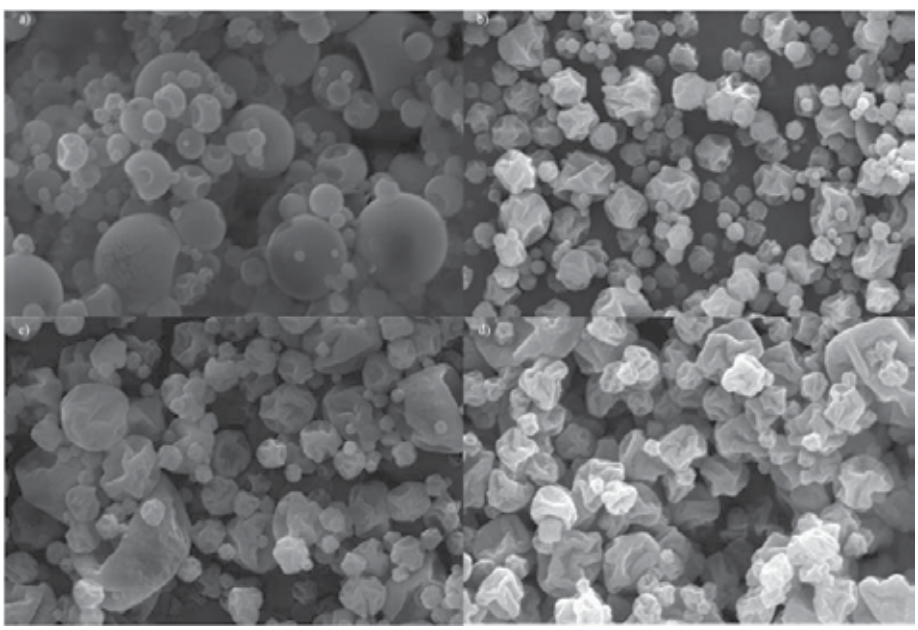


Figure 12. SEM pictures of powders dried from water/ISO 8:2 v/v systems containing: a) G; b) GISO2-leu5; c) GISO2-leu10; d) GISO2-leu5.

As well known, the morphology of spray-dried particles is strongly influenced by the solubility of the components and their initial saturation in the liquid feeds. G, freely soluble in water, led to the formation of spherical particles when spray dried alone (Fig. 12a, G). According to previous observations [52], during the co-spray drying process, the saturation of the lower-soluble component (leu) may increase faster than that of hydrophilic one (G), due to the preferential evaporation of alcohol and the associated change in the solvent/co-solvent ratio. This led to the formation of a primary solid shell which collapsed, hence corrugated microparticles were formed. As the relative amount of the less soluble component increased, particle corrugation was more and more evident; particles from almost spherical became raisins like (Fig. 12b, GISO2-leu5) or irregularly wrinkled (Fig.12d, GISO2-leu5). Such surface modification has been shown to be beneficial for particles intended for inhalation [29]: a corrugated surface improves powder dispersibility by minimizing contact areas and reducing interparticulate cohesion and, therefore, corrugated particles disperse better than spherical ones.

3.1.2. Aerodynamic behavior of G/leu powders

As aerodynamic properties, batches dried from water were hygroscopic, cohesive powders, difficult to insert into and come out from the capsule and with unsatisfying aerodynamic properties (data not shown). In particular, neat G dried from water was a cohesive and sticky material, unable to be aerosolized.

	Code #	Leu content (%w/w)	Charged Dose (mg)	ED (%)	FPF (%)	FPD (mg)
20% v/v ISO	GISO2	0	60	95.8±1.9	14.5±7.8	8.7±4.7
	GISO2-leu5	5	80	98.0±0.2	21.9±5.1	16.6±3.9
	GISO2-leu10	10	120	99.4±0.1	32.6±5.6	35.2±6.0
	GISO2-leu15	15	120	99.6±0.2	46.8±0.5	47.7±0.5
	GISO2-leu5	20	120	99.3±0.3	50.9±1.0	48.8± 0.9
30% v/vISO	GISO3	0	60	90.9±7.9	13.4±8.5	7.5±4.9
	GISO3-leu5	5	70	97.2±0.5	22.3±3.0	14.8±2.0
	GISO3-leu10	10	110	99.4±1.1	28.8±5.0	28.4± 5.0
	GISO3-leu15	15	120	99.1±0.3	49.4±0.8	50.4±0.8
	GISO3-leu20	20	120	99.2±0.0	50.2±1.0	48.2±0.9

ED, emitted dose; FPF, fine particle fraction; FPD, fine particle dose

Table 4. Aerodynamic properties of spray-dried powders after single stage glass impinger deposition experiments; device TURBOSPIN, charged with capsules type 2 (mean ± SD of three experiments).

G spray drying from hydroalcoholic solvent (GISO2 and GISO3) reduced powder cohesivity and enabled the aerosolization process; however, the resulting aerodynamic properties were still not satisfying (FPF less than 15%; Table 4). The inclusion of leu substantially increased emitted doses (ED up to 99.6% for #GISO2-leu15) and fine particle fractions (FPF up to 49.4% for #GISO3-leu15). Taking into account the relative reduction in drug content, further increase in the excipient/drug ratio up to 20/80 w/w did not improve DPI performance. The organic co-solvent led to the best

FPF and FPD values. As example, GISO3-leu15 formulations, containing 15% w/w of leu and obtained from 30% v/v of ISO/water feed, emitted 50.4 mg of fine G after one actuation of the Turbospin device. These results are in agreement with previous studies [29, 30, 53] evidencing the enhancement of powder aerosol performance as particle surface corrugation goes up to a certain degree; further corrugation enhancement did not improve aerodynamic properties. The plot in figure 13 allows to appreciate a dramatic increase in both particle corrugation (SEM micrographs) and FPF as the leu content increased.

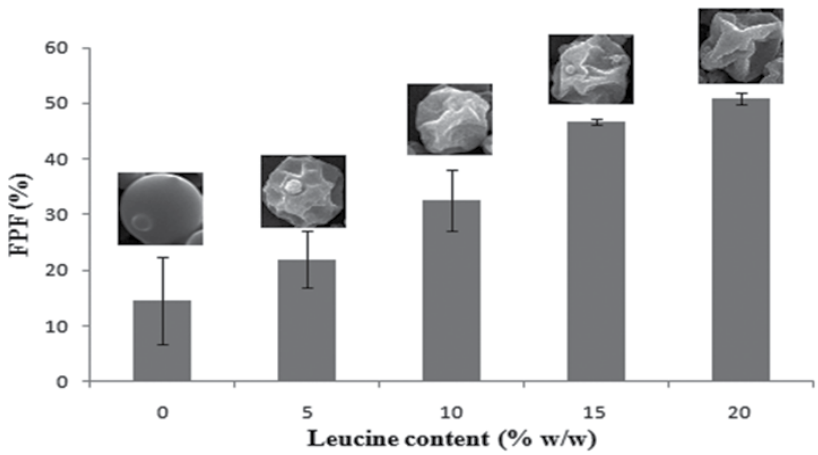


Figure 13. FPF and SEM images of G powders spray-dried from liquid feeds containing 20% ISO and increasing amount of leu.

MMAD, FPF and FPD values obtained by Andersen cascade impactor deposition studies (Table 5) confirmed the observed trend. Capsules charged with 120 mg of powder emitted almost the whole dose from the device after the pump actuation, as indicated by ED ≥ 99%. Among all formulations, GISO3-leu15 (G/15%leu from 30% v/v of ISO/water feed) showed very satisfying aerodynamic properties as proved by MMAD of 3.45 μm, FPF 58.1% and FPD of 56.4 mg (Table 5).

	Code #	ED (%)	MMAD (μm)	FPD (mg)	FPF (%)
20% v/v ISO	**GISO2-leu15**	99.7±0.3	4.0±0.1	49.3±1.7	46.0±2.7
	GISO2-leu5	99.6±0.4	4.2±0.1	39.3±0.3	42.5±0.2
30% v/v ISO	**GISO3-leu15**	99.2±0.3	3.4±0.2	56.4±1.1	58.1±3.6
	GISO3-leu20	99.2±0.2	3.3±0.1	54.7±2.2	58.0 ± 0.5

Table 5. Aerodynamic properties of G spray-dried powders containing 15 or 20% w/w leu after Andersen cascade impactor deposition experiments (mean ± SD)

3.1.3. Effect of G/leu powders on viability of cf airways epithelium

In order to establish whether the particle engineering has any cytotoxic or cytostatic effect on bronchial epithelial cells [31, 32], CuFi1 cells were treated for 24 h with increasing

concentrations (from 0.0002 to 2 μM expressed as G content) of GISO3 or GISO-Leu15 powders in comparison to raw G. Results indicated that neither raw G nor its formulations generally inhibited cells viability as determined by MTT assay (Fig. 14 B). Only Raw G at concentrations higher than 0.02 μM showed a slight but significant decrease in cell survival. An interesting observation is that an increase in leu content up to 15%, as in GISO3-leu15 , faintly but not significantly decreased CuFi1 viability at concentration ranging from 0.02 to 0.2 μM ($P<0.05$) (Fig. 13 B) whereas at 2.0 μM did not. As previously oserved in formulations for inhalation containing leucine [27], this effect seems to be related to leu ability to improve cell proliferation and metabolism of bronchial epithelial CF cells.

Furthermore ELISA BrdU immunoassay confirmed that raw G slightly reduced CF cell growth only at the highest concentration [2 μM, $P<0.01$] (Fig. 14 A).

Therefore, G/leu systems had no cytotoxic or cytostatic effect on CF epithelial lung cells (CuFi1 model), at concentrations up to 2 μM.

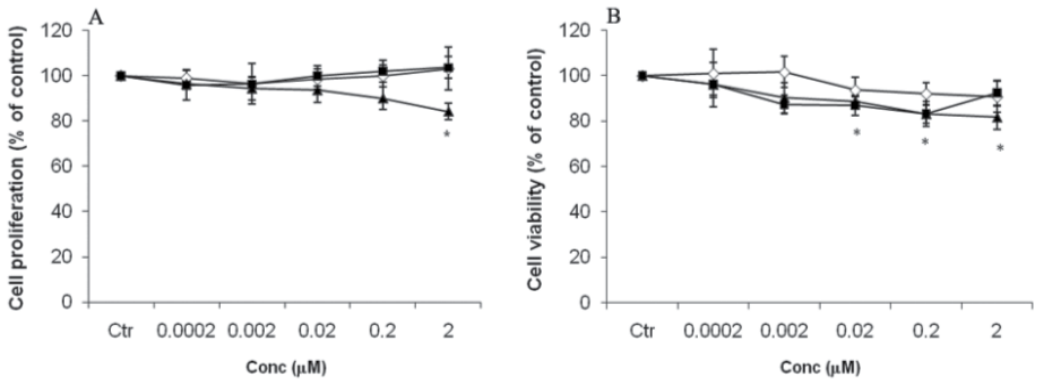


Figure 14. Effect of Gentamicin and its DPI formulations on CuFi1 cell proliferation and viability. Cells were treated for 24 h with: raw Gentamicin (rawG, ▲), spray-dried Gentamicin (GISO3 ◊) and G co-sprayed with 15%w/w leucine (GISO3-leu15 ■) at concentrations from 0.0002 μM to 2 μM. Cell growth (A) was determined using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit. Cell viability (B) was determined by MTT assay. All data are shown as mean ± SD of three independent experiments, each done in duplicate (*$P<0.05$ and **$P<0.01$ *vs* control).

An proper engineering process, use of hydro-alcoholic feeds and the AA addition, allow the preparation of micronized powders able to be aerosolized. The addition of small amount of the AA led to the production of dry formulations with excellent emitted dose and good aerodynamic properties after actuation of the Turbospin device. Finally, the engineered particles showed no cytotoxic or cytostatic effect on bronchial epithelial cells bearing a CFTR F508/F508 mutant genotype.

4. Conclusions

The engineering process by spray drying and the use of water-co-solvent systems as liquid feed allowed micronized powders to be produced with high yield, starting from Naringin or Gentamicin sulfate, drugs with different physicochemical properties. The addition of a small

amount of a safe excipient, as leucine, led to powders with excellent emission doses, counteracting both G high hygroscopicity and N cohesiveness and low solubility. In particular, N DPI containing 5% leu (NET3-leu5) and G DPI containing 15% leu (GISO3-leu15) were able to deliver almost the total dose of drug loaded in the capsules, with about 60% of FPF. Finally, N and G engineered powders showed no cytotoxic or cytostatic effect on bronchial epithelial cells bearing a CFTR F508/F508 mutant genotype. As to efficacy, NET3-leu5 powder, containing natural polyphenol and AA, were able to negatively modulate NF-κB pathways in absence of stimulation in bronchial epithelia and to reduce the overexpressed IL-8 and IL-6 production both in basically and in LPS-stimulated conditions. These findings, together with the well-known G antibiotic activity support the use of G-leu and N-leu DPIs in the treatment of infections and intrinsic inflammation of CF lungs.

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Chapter 3

The Role of Carrier in Dry Powder Inhaler

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Additional information is available at the end of the chapter

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1. Introduction

Increasing prevalence of pulmonary diseases with high mortality and morbidity such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis, infectious diseases, tuberculosis and lung cancer, makes pulmonary drug delivery as a non-invasive and attractive approach for the local drug administration and treatment of these pathologies (Marianecci et al., 2011). In this case, lower dosages than by the oral route can be used with comparable effectiveness which will reduce unwanted side effects (Timsina et al., 1994).The lung also provides a non-invasive route of delivery for the systemic circulation, due to its unique characteristics such as large surface area, thin epithelial barrier and high blood flow. Lack of first pass metabolism and less enzymatic activity make pulmonary delivery as an ideal administration route for extensively degraded drugs following oral delivery and for macromolecules, such as proteins and peptides, respectively(Hamishehkar et al., 2010; Rytting et al., 2008; Sakagami, 2006). Following approving the first inhaled therapeutic macromolecule for systemic delivery, human insulin (Exubera™) by the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products in 2006, the scientific community look for other candidates that would benefit from pulmonary delivery for systemic action (Patton et al., 2004; Furness, 2005). In contrast to injection therapy, inhalation therapy is not associated with pain and this should increase patient comfort and compliance, causing improved treatment outcome (Laube, 2005). Drug deposition in the lung is mainly controlled by its aerodynamic diameter (Wolff et al., 1993). Particles larger than 5 μm are mostly trapped by oropharyngeal deposition and incapable of reaching the lungs while smaller than 1 μm are mostly exhaled without deposition (Sakagami, 2006). Particles with aerodynamic diameters between 1 and 5 μm are expected to efficiently deposit in the lung periphery (Heyder et al., 1986). The effective inhalation performance of dry-powder products is dependent on the drug formulation and the inhaler device. Dry powder formulations are usually prepared by mixing the micronized drug particles with larger carrier particles. The aerosolization efficiency of a powder is highly dependent on the carrier characteristics, such as particle size distribution, shape and surface

properties. The main objective in the inhalation field is to achieve reproducible, high pulmonary deposition. This could be achieved by successful carrier selection and careful process optimization (Pilcer et al., 2012). Therefore, the purpose of this chapter is to review the used carriers in inhalable formulations, their production and the impact of the physicochemical properties of carriers on carrier-drug dispersion is discussed in detail. This chapter offers a perspective on current reported studies to modify carrier for its better performance. Several methodologies have been discussed.

2. Dry powder inhalers

Pressurized metered-dose inhaler (MDI), nebulizer and dry powder inhaler (DPI) are main delivery systems in pulmonary delivery (Timsina et al., 1994). Among these, DPI appears to be the most promising for future use (Todo et al., 2001).They are propellant-free, portable, easy to operate and low-cost devices with improved stability of the formulation as a result of the dry state(Carpenter et al., 1997; Prime et al., 1997).Spinhaler®, the first dry powder inhaler, came into the market in 1970 and since then a new are started in the subject of pulmonary drug delivery. Dry powder inhalers and dry powder inhalation technology became the second most frequently used inhalation devices for pulmonary drug administration after Montreal Protocol in 1987 in limitation of using CFC in products. Dry powder inhalers have even become the first choice of inhalation devices in European countries (Marriott et al., 2012).They are a widely accepted inhaled delivery dosage form where they are currently used by an estimated 40% of patients to treat asthma and chronic obstructive pulmonary disease (Atkins, 2005). Using the DPI system, respiratory delivery of potent drugs such as insulin (Edwards et al., 1997), antibiotics (Geller et al., 2007; Hickey et al., 2006), drugs for neurological disorders like Parkinson's disease(Stoessl, 2008), antituberculosis (Anon, 2008), antihypertensive nifedipine(Plumley et al., 2009), anticoagulant heparin (Rawat et al., 2008),drugs for sexual dysfunction (Cheatham et al., 2006), opioids and fentanyl for cancer pain (Farr et al., 2006; Kleinstreuer et al., 2008; Fleischer et al., 2005) and delivery of atropine sulphate nanoparticle as an antidote for organophosphorus poisoning with better bioavailability (Ali et al., 2009) have been studied. DPI formulations of measles vaccine (LiCalsi et al., 2001), mucosal vaccination for influenza virus (Edwards et al., 2005), have all been studied with considerable achievement. DPIs have to overcome various physical difficulties for effective drug delivery either local or systemic purposes (Prime et al., 1997). First, small size of inhalable particles subjected them to forces of agglomeration and cohesion, resulting in poor flow and non-uniform dispersion (Crowder et al., 2002).

3. The role of carrier on DPI performance

DPI is generally formulated as a powder mixture of coarse carrier particles and micronized drug particles with aerodynamic particle diameters of 1–5 μm (Iida et al., 2003). Carrier particles are used to improve drug particle flowability, thus improving dosing accuracy and minimizing the dose variability observed with drug formulations alone while making them easier to handle during manufacturing operations (Timsina et al., 1994; Schiavone et al., 2004). With the use of carrier particles, drug particles are emitted from capsules and devices

more readily, hence, the inhalation efficiency increases (Iida et al., 2001). Moreover, usually no more than a few milligrammes of a drug needs to be delivered (e.g., between 20 μg and 500 μg of corticosteroids for asthma therapy), and thus carrier provides bulk, which improves the handling, dispensing, and metering of the drug (Pilcer et al., 2012). The presence of the carrier material is the taste/sensation on inhaling, which can assure the patient that a dose has been taken (Prime et al., 1997).Consequently, the carrier forms an important component of the formulation and any change in the physico-chemical properties of the carrier particles has the potential to alter the drug deposition profile (Zeng et al., 2000). Therefore, the design of the carrier particle is important for the development of dry powder inhalations (Hamishehkar et al., 2010). Carrier particles should have several characteristics such as physico-chemical stability, biocompatibility and biodegradability, compatible with the drug substance and must be inert, available and economical. During insufflation, the drug particles are detached from the surface of the carrier particles by the energy of the inspired air flow that overcomes the adhesion forces between drug and carrier. The larger carrier particles impact in the upper airways, while the small drug particles go through the lower parts of lungs (Pilcer et al., 2012). Unsatisfactory detachment of drug from the carrier due to strong inter-particulate forces may be one of the main reasons of inefficient drug delivery encountered with most DPIs (Zeng et al., 2000; Zhou et al., 2011). Therefore, in the best case, the adjusted balance between adhesive and cohesive forces provides enough adhesion between drug and carrier to produce a stable formulation (homogeneous mixture with no powder segregation and proper content uniformity) yet allows for easy separation during inhalation. Consequently, it has been stated that the efficiency of a DPI formulation is extremely dependent on the carrier characteristics and the selection of carrier is a crucial determinant of the overall DPI performance (Pilcer et al., 2012). Obviously, the effect of the carrier material on DPI formulation should be carefully evaluated. The range of materials which can be proposed to be as carriers in inhaled products are restricted for toxicological reasons. Lactose and other sugars have been studied and used, therefore modifications to these materials may allow further formulation optimization (Prime et al., 1997).

4. Inhaler testing equipments

Cascade impactors operate on the base of inertial impaction. Each stage of the impactor contains a single or series of nozzles or jets through which the sample laden air is drawn directing any airborne particles towards the surface of the collection plate for that particular stage. Whether a particular particle impacts on that stage is dependent on its aerodynamic diameter. Particles having sufficient inertia will impact on that particular stage collection plate whilst smaller particles with insufficient inertia will remain entrained in the air stream and pass to the next stage where the process is repeated. The stages are normally assembled in a stack in order of decreasing particle size. As the jets get smaller, the air velocity increases and finer particles are collected. Any remaining particles are collected on an after filter (or by a –Micro-Orifice Collector). The term 'Impactor' is generally used for an instrument where the particles 'impact' on a dry impaction plate or cup. If the collection surface is liquid, as in the case of the Multi-Stage Liquid Impinger (MSLI), then the term

'impinger' is used. The general principles of inertial impaction apply to both 'impactors' and 'impingers'. The US and European Pharmacopoeia list no less than five different cascade impactors/impingers suitable for the aerodynamic assessment of fine particles. However, only the Andersen Cascade Impactor (ACI), the Next Generation Impactor (NGI) and the Multi-Stage Liquid Impinger (MSLI) appear in both pharmacopoeia. In research applications, in vitro/in vivo correlation and bioequivalence may be important and so detailed particle size data may be required. In routine quality control, where the concern is batch-to-batch variation a coarser test may be acceptable. The Glass Twin Impinger, for example has been retained as Apparatus A in the European Pharmacopoeia, because of its value as a simple and inexpensive quality control tool. In general however, it is accepted that an Impactor/impinger should have a minimum of five stages and preferably more, if it is to provide detailed particle size distribution data. The aerodynamic particle size distribution of the drug leaving an inhaler device can define the manner in which an aerosol deposits in the respiratory tract during inhalation. This characteristic of the aerosol is often used in judging inhaler performance and is particularly relevant in the development of inhalation formulations during research, production, quality assurance and equivalency testing. The results of characterizations using cascade impaction techniques are additionally used for the determination of fine particle fraction or fine particle dose which may be correlated to the dose or fraction of the drug that penetrates to the lung during inhalation by a patient. Dry Powder Inhaler (DPI) testing could require added options for preventing stage overloading and necessary to achieve the specified pressure drop through the device. Upper stage mass overloading can be prevented with the addition of a high capacity preseparator or pre-collector. The feature traps non-inhalable aerosols. To achieve the proper pressure drop of 4 kPa (40.8 cm water) in the inhaler, a higher vacuum flow rate at 60 or 90 L/minute may be needed.Impactors/impingers are specifically designed to meet the highest criteria laid down in the various Pharmacopoeia (e.g. United States Pharmacopeia Chapter <601>; European Pharmacopoeia Chapter 29.9.18 for characterizing aerosol clouds emitted by inhalers). By analyzing the drug deposited on the individual stages and the final filter, the Fine Particle Fraction (FPF), the Fine Particle Dose (FPD), the Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) can all be calculated (The Copley Scientific Limited, 2010).

5. Expressions used to define drug lung deposition

The American and European pharmacopoeias have explained methods based on inertial impaction to assess the in vitro inhalation performance of formulations by determination of the fine particles (Pilcer et al., 2012).

5.1. Fine Particle Dose (FPD) and fine particle fraction (FPF)

The aerodynamic evaluation methods of fine particles permit the determination of the fine-particle dose (FPD), which corresponds to the mass of drug particles that have an aerodynamic diameter less than 5 μm. Such particles can theoretically be deposited in the deep lung after inhalation. The fine-particle fraction (FPF), which is the percentage of the

FPD usually related to either the nominal dose (total drug mass contained in the device) or the recovered drug (sum of the drug collected in the device and in the different parts of the impingers or impactors after inhalation) (Pilcer et al., 2012).

5.2. Emitted dose

The emitted dose expresses the drug mass exiting the device after inhalation. In some cases, FPF can be calculated from emitted dose instead of total or recovered dose of drug from impingers or impactors. The ability of the powder to be fluidised by the airflow through an inhaler is usually indicated by the emission dose, whilst the FPD and FPF measure the capability of the formulation to be fluidised and deagglomerated in time to release the drug from the carrier to be deposited in the appropriate level of the impactors and impingers (Pilcer et al., 2012).

5.3. Dispersibility

The dispersibility is calculated as the ratio of FPD to emitted dose (Zeng et al., 2000).

5.4. Mass median aerodynamic diameter (MMAD)

Mass median diameter of an aerosol means the particle diameter that has 50% of the aerosol mass residing above and 50% of its mass below it. The concept of aerodynamic diameter is central to any aerosol measurements and respiratory drug delivery. The aerodynamic diameter relates the particle to the diameter of a sphere of unit density that has the same settling velocity as the particle of interest regardless of its shape or density. The mass–mean aerodynamic diameter (MMAD) is read from the cumulative distribution curve at the 50% point (Labiris et al., 2003).The theoretical mass–mean aerodynamic diameter (d_{aero}) was determined from the geometric particle size and tap density using the following relationship:

$$d_{aero} = d_{geo}\left[\frac{(\rho / \rho_{ref})^{0.5}}{\gamma}\right]$$

Where d_{geo}=geometric diameter, γ=shape factor (for a spherical particle, γ=1), ϱ=particle bulk density and ϱ_{ref}=water mass density (1 g/cm^3). Tapped density measurements underestimate particle bulk densities since the volume of particles measured includes the interstitial space between the particles. The true particle density, and the aerodynamic diameter of a given powder, is expected to be slightly larger than reported (El-Gendy et al., 2009).

5.5. Geometric standard deviation (GSD)

The degree of dispersity is an important consideration for both quality and efficacy of pharmaceutical aerosols (Chew et al., 2002). The nature of the aerosol distribution must be established accurately if its implications for deposition and efficacy are to be understood

(Telko et al., 2005). The degree of dispersion in a lognormally distributed aerosol is characterized by the geometric standard deviation (GSD). A larger GSD implies a longer large particle size tail in the distribution(Musante et al., 2002). GSD for a well-functioning stage should ideally be less than 1.2 (the GSD for an ideal size fractionators would be 1.0 and indicates a monodisperse aerosol) (Marple et al., 2003). GSD is a measure of the variability of the particle diameters within the aerosol and is calculated from the ratio of the particle diameter at the 84.1% point on the cumulative distribution curve to the MMAD. For a log-normal distribution, the GSD is the same for the number, surface area or mass distributions (Labiris et al., 2003). The GSD was determined as

$$GSD = \sqrt{\frac{sizes\ X}{sizes\ Y}}$$

where sizes X and Y are particle sizes for which the line crosses the 84% and 16% mark, respectively (Emami et al., 2009).Mostly, particle size distributions are log-normal, for which type of distributions the geometric mean diameter (GMD) and GSD are frequently used as the characteristic parameters. Aerosols from dry powder inhalers are not log-normal however, because they are nearly always a mixture of primary and secondary particles. Agglomerates in the aerosol are the reason for a tail-off at the side of the larger diameters. Therefore, the mass median diameter is a better parameter, although the size fraction for which mass median diameter is calculated should be defined also. For inhalation drugs, the mass median aerodynamic diameter (MMAD) is the most frequently used parameter(Bosquillon et al., 2001).

6. Carrier qualifications for application in DPIs

6.1. Carrier size

Different and controversy reports have been published about the suitable carrier size for inhalation purposes. Some previous studies have reported improvements in the amount of respirable drug delivered from a DPI by way of reducing the particle size of the carrier (Steckel et al., 1997; Gilani et al., 2004; Louey et al., 2003). For example, increased respirable fraction of salbutamol sulphate (Kassem et al., 1989; Zeng et al., 2000), terbutaline (Kassem et al., 1989), disodium chromoglycate(Braun et al., 1996) and budesonide (Steckel et al., 1997) were concluded with decreased carrier size. It was proposed that smaller agglomerates meet more forceful shear in the turbulent airstreams causing more effective deagglomeration (Islama et al., 2012). However, the use of too small a carrier will result in poor flow properties of the powder, which is one of the primary reasons for incorporating a coarse carrier within the formulation (Zeng et al., 2001).On the other hand, it was reported that larger carrier particles, normally exhibit larger surface discontinuities than fine crystals (De Boer et al., 2003). This may have the advantage of providing shelter to drug particles from the press-on forces during mixing, as the drug particles tend to assemble in these discontinuities during mixing (Iida et al., 2003; De Boer et al., 2005). Therefore, a high carrier particle size does not necessarily have a negative effect on the drug deposition profiles after

inhalation (Hamishehkar et al., 2010). In a study, formulations of respirable recombinant human granulocyte-colony stimulating factor with larger carriers (90–125 μm) showed a higher drug dispersion than the same formulation with 38–75 μm carriers, and it is interpreted that this is due to the lower inter-particle forces among the larger sized particles (French et al., 1996). Similar results was shown for enhanced inhalation performance of terbutaline sulphate from a formulation containing coarse lactose (53–105 μm) than the same drug containing fine lactose carriers with size less than 53 μm (Byron et al., 1990). Recently, an increase in carrier size resulted better aerosolisation behavior of insulin loaded PLGA microparticles mixed with mannitol carrier. The authors conclude that the use of larger particles of mannitol carrier with a lower carrier/microcapsule ratio leads to higher dispersion of the drug due to increase flowability (Hamishehkar et al., 2010).

6.2. Carrier shape

Although the influence of carrier particle shape on the drug dispersibility from the DPI formulation is not well recognized but it is known that the attractive forces between drug and carrier particles can be shape dependent (Mullins et al., 1992; Crowder et al., 2001). In fact most commonly used particles for DPI formulations have irregular shapes. In vitro inhalation studies have indicated that elongated (Larhrib et al., 2003; Zeng et al., 2000), needle-like (Ikegami et al., 2002), porous and wrinkled particles (Chew et al., 2005)have improved lung deposition properties of various formulations. Increasing the elongation ratio of the lactose carrier particles also appeared to increase the FPF of salbutamol sulphate (Zeng et al., 2000).Recently, it was reported pollen-shaped hydroxyapatite carrier increased dispersibility of budesonide particles due to reduction in particle interactions (Hassan et al., 2010; 2010). Definitely surface shape effects agglomeration strength but it was also discussed that the aerodynamic diameter of the agglomerates can be changed by shape factor. Because of their larger shape factor, elongated particles have a smaller aerodynamic diameter than spherical particles and thus agglomerations of active drug particles and elongated carriers remain aerosolized for a longer time, and greater distance along the inhalation path, then deagglomeration is enhanced (Islama et al., 2012). The figure 1 shows the presence of loose agglomerates in freeze dried mannitol samples adapted from reference (Hamishehkar et al., 2010), applied mannitol particles in different shapes for formulation of DPI form of insulin-loaded biodegradable polymeric microparticles. Spray dried and freeze dried mannitol, both showed smooth surfaces while formulation composed of freeze dried mannitol showed higher FPF and emitted dose than spray dried mannitol. This was interesting when it was found that spray dried mannitol had lower true density than freeze dried mannitol, possible resulting in better emitted dose and consequently improved FPF. This aerosolization behavior of freeze dried mannitol can be attributed to its needle shape particle morphology. Spatial hindering effect of rod shape particles can lead their easier aerosolization, and hence higher emission and higher FPF. The results of FPF and emitted dose for different formulations can partially be explained by elongation ratio and shape factors reported in this article. Freeze dried mannitol had higher elongation ratio and lower shape factor than spray dried mannitol which indicates

more irregular shape. In vitro inhalation studies have indicated that elongated, fibrous particles improve lung deposition properties (Chan et al., 1989; Fults et al., 1997).

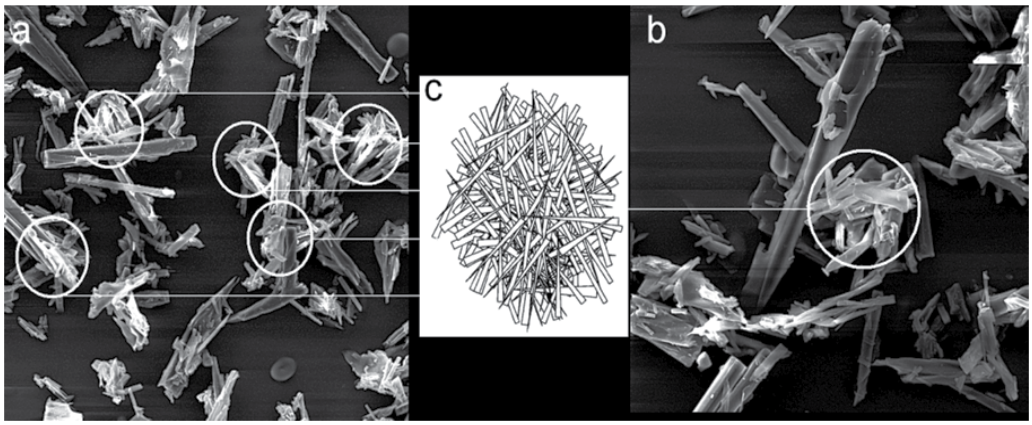

Figure 1. Scanning electron micrographs of freeze-dried mannitol (FDM) (a, b) and schematic image of formed loose agglomerates of needle shape FDM particles (c) adapted from reference (Hamishehkar et al., 2010)

6.3. Carrier surface

The in vitro inhalation properties of DPI are reported to be related to the surface properties of the carrier particles (Zeng et al., 2000; Heng et al., 2000; Iida et al., 2001; 2003). Surface morphology has been demonstrated to directly influence the contact area between drug particle and carrier, leading to variations in interparticulate adhesion. Several studies have reported that variations in contact area, as a result of differing surface structure, could potentially compromise the aerosolization performance of the drug particles (Zeng et al., 2000; Flament et al., 2004; Young et al., 2002). Some surface modifications of carrier particles have been reported to improve inhalation performance of DPI (Kawashima et al., 1998; Chan et al., 2003). Previous investigations reported that the carrier surface morphology directly affected the aerosolization efficiency from a DPI (Podczeck, 1998; Larhrib et al., 1999). In general terms, a decrease in roughness is believed to improve aerosolization efficiency of a drug-carrier blend (Ganderton et al., 1992; Kawashima et al., 1998; Zeng et al., 2001). However, it was shown that coarse carrier particles which normally exhibit large surface discontinuities may provide shelter to drug particles from the press-on forces during mixing, as the drug particles tend to assemble in these discontinuities during mixing (Iida et al., 2003). Therefore, high carrier rugosity drug-to-carrier interaction (Kawashima et al., 1998; Podczeck, 1998; Zeng et al., 2000). These interaction forces have to be strong enough to guarantee good mixture stability during handling and proper drug deaggregation does not necessarily have a negative effect on the drug detachment from carrier crystals during inhalation, providing that inertial detachment forces are applied. Chan et al. also reported that a positive linear trend was established between the roughness of the lactose surface and the FPF and dispersibility of the drug (Chan et al., 2003). Therefore, an important balance between the surface morphologies of both the drug and carrier can exist (Young et al., 2002). It was shown by Heng et al. that an optimum lactose surface roughness (Ra) was required

for an increased fine particle fraction of salbutamol sulphate (Heng et al., 2000). All these studies demonstrated that the different surface roughness of the carrier led to different adhesion forces between the drug and carrier, which was reflected in the in vitro deposition results. The carrier surface plays an important role in, but weak enough to enable the separation forces during inhalation to detach a substantial fraction of the drug dose from the carrier crystals. This requires that the size distributions of the interaction forces (during mixing) and separation forces (during inhalation) are balanced properly (De Boer et al., 2003).The assumed role of carrier surface on the separation of microcapsules aggregation during preparation of DPI formulations and detachment of microcapsules after aerosolization from the surface of carriers is shown schematically in Figure 2 which is adapted from reference (Hamishehkar et al., 2010).This figure shows the dry powder inhalation formulations containing the blend of microcapsules and carriers. It can be seen that there are not enough active sites on the surface of spray dried mannitol and freeze dried mannitol for microcapsules to be deposited on, so carriers cannot disaggregate microcapsules. In the case of sieved sorbitol, microcapsules immersed on the surface of carrier and did not detach easily from its surface after aerosolization. Therefore in spite of better emission of microcapsules from the formulation containing sieved sorbitol, the FPF of the drug decreased. In this article, sieved mannitol showed higher FPF for insulin-loaded PLGA microparticles due to its appropriate surface roughness characteristics.

6.4. Fine carrier particles

The addition of fine particles to DPI formulations was shown to improve the inhalation efficiency of drugs (Zeng et al., 1998; Lucas et al., 1998). Islam et al. confirmed that the presence of fine lactose associated with large carriers or added as an excipient, played a key role in the drug dispersion process in this study (Islam et al., 2004). Similar observations were made by Louey et al. using salbutamol sulphate with various lactose carriers (Louey et al., 2003). The addition of ternary components like magnesium stearate and leucine (French et al., 1996; Islam et al., 2004; Staniforth, 1996) has also enhanced drug dispersion by decreasing the cohesive forces between drug particles. The addition of 10% fine carriers (lactose, glucose, mannitol and sorbitol) in the interactive mixtures of salmeterol xinafoate and coarse carriers demonstrated the same conclusion that fines enhance the detachment of the drug from the large carriers (Adi et al., 2007). On the other hand, the opposite results have been concluded about the role of fines from few studies. It was reported that the concentration of added fine lactose has to be carefully controlled such that a desired dispersibility of the drug can be achieved without substantially affecting powder flow properties (Zeng et al., 1998). The presence of ''fines'' tend to inhibit flow because fines can fit into the voids between larger particles and encourage packing and consequent powder densification, and fines are inherently poor flowing (Augsburger, 1974) due to various surface forces (Hickey et al., 2007) and high cohesive energy. It was recently reported that the presence of fines caused a decrease in FPF (Steckel et al., 2004; Hamishehkar et al., 2010). Also the use of micronized fine carrier may introduce extra amorphous content to the powder because a large portion of micronized lactose is in the amorphous form, which is thermodynamically unstable and will convert to the more stable crystalline form on

exposure to moisture (Saleki-Gerhardt et al., 1994). Such a transformation is likely to change the performance characteristics of the bulk powder, such as flowability and drug dispersion (Ward et al., 1994).

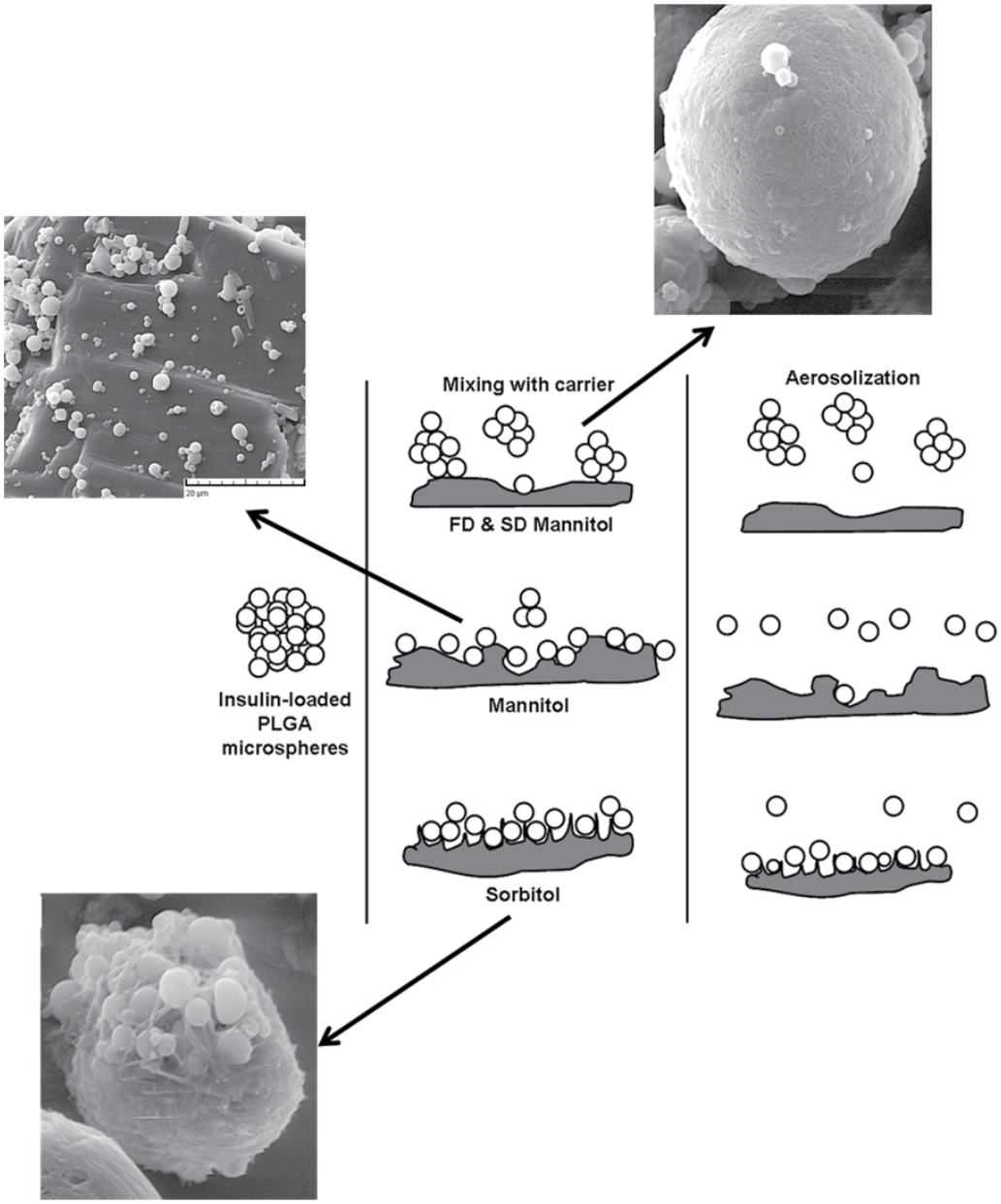

Figure 2. Schematic representation of the role of carrier surface asperities on the drug entrapment and its fluidization capabilities adapted from reference (Hamishehkar et al., 2010).

6.5. Carrier crystallinity

The amorphous part shows a higher surface adhesion energy compared to crystalline surfaces (Young et al., 2004). As a result of increase in adhesion energy, poor deaggregation of drug particles is observed (Podczeck et al., 1997). Therefore it can be hypothesized that, the presence of amorphous material may cause problems, for example, due to the fusion of particles, resulting in poor dispersion (Young et al., 2004; Ward et al., 1995; Podczeck et al., 1997). In addition, the amorphous regions can present difficulties such as decreased chemical stability (Pikal et al., 1978). Partially amorphous or unstable polymorphic forms and changes therein (Harjunen et al., 2002) make the interparticulate contact quite unpredictable and the powder formulation rather unstable (De Boer et al., 2003). It was reported that the maximum fine particle dose of terbutalinesulphate is obtained with the crystallized form of mannitol comparing to different polymorphs of mannitol (Saint-Lorant et al., 2007). Furthermore, previous reports have suggested increased amorphous content in DPI systems resulted in decreased aerosolization performance (Young et al., 2004; Ward et al., 1995).

7. Lactose as the most frequently used carrier in DPIs

Lactose, 4-(b-D-galactosido-)-D-glucose, can be obtained in either two basic isomeric forms, α and β-lactose, or as an amorphous form (Zeng et al., 2000). Historically, lactose monohydrate was an obvious choice for use as a carrier excipient. Lactose accompanying with glucose and mannitol is allowed as carriers in DPIs by the US Food and Drug Administration department (Labiris et al., 2003).Lactose is the most common and frequently used carrier in DPIs formulations and nowadays various inhalation grades of lactose with different physico-chemical properties are available on the market. The advantages of lactose are its well-investigated toxicity profile, physical and chemical stability, compatibility with the drug substance, its broad availability and relatively low price (Steckel et al., 2004; Pilcer et al., 2010).α-lactose monohydrate is the most common lactose grade used in the inhalation field.Almost all DPI formulations on the market are based on a-lactose monohydrate as a carrier. Therefore, wealth of literatures refers to the optimization of lactose carrier particles for better inhalation performance. More than 250 articles have been published in the past 40 years regarding the role of lactose in adhesive mixtures used in DPIs. However, in spite of these extensive investigations, the relationship between physico-chemical properties of the lactose in adhesive mixtures and the performance of the DPIs remains largely indistinct (Marriott et al., 2012).

8. Lactose engineering for application in DPIs

8.1. Surface modification

Iida et al. prepared lactose carrier particles for dry powder inhalations by its surface modification with aqueous ethanol solution and evaluated the inhalation efficiency of salbutamol sulfate from its mixture with modified lactose. The degree of adhesion between drug particles and carrier particles and the separation characteristics of drug particles from carrier particles in air flow were assessed by the ultracentrifuge separation and the air jet

sieve methods, respectively. It was shown that the average adhesion force between the surface-treated lactose carrier and drug particles was considerably lower than that of powder mixed with the un-treated lactose carrier, indicating better drug separation from carrier and consequently an improvement of in vitro inhalation properties. The authors claimed that surface-smoothing of lactose by aqueous ethanol solution resulted a well balanced drug-carrier adhesion force so that the drug particles could be emitted together with the carrier particles and efficiently separated in airflow after emission(Iida et al., 2003). Fine lactose particles were immobilized on the lactose surface by spray coating with liquid suspensions consisting of micronized lactose dispersed in isopropyl alcohol and/or water mixtures to modify surface of lactose. The produced lactose was used as a carrier in the formulation of inhalable salbutamol sulfate powder. It was found that the roughness of the lactose surface established by immobilization of fine lactose increased the FPF and dispersibility of the drug. The authors claimed that unlike crevices and valleys, these microscopic undulations did not accommodate the drug particles and instead enhanced the detachment of drug from the lactose surface and improved the drug inhalation efficiency (Chan et al., 2003). Spray dried amorphous, spray dried crystallized and fluidized bed granulated lactose were prepared and used as carrier for inhalation of pranlukast hydrate. Fluidized bed granulated lactose emitted drug particles effectively from the inhalation device, whereas most part of drug captured in the upper stage of twin impinger, resulting in lower inhalation efficiency, due to strong adhesion of drug to the carrier lactose. The spray dried amorphous lactose, smoothed sphere particle, did not so improve the inhalation efficiency as expected, because of fairly strong adhesion between drug and lactose particles. But the spray dried crystallized lactose having lots of microscopical projection on its surface increased the respirable particle percent of the emitted particles. The conclusion was that the surface roughness should be optimized to gain improved inhalation efficiency of particles (Kawashima et al., 1998). In the other study, a Wurster fluidized bed was used for surface-coating of lactose particles with lactose aqueous solution containing hydroxypropyl methyl cellulose. The authors could be able to increase the inhalation performance of salbutamol sulfate 2.5 times more than commercial lactose (Pharmatose® 200M) and reported that surface coating of carrier particles may be an effective technique for improving the inhalation performance of DPI (Iida et al., 2005).Lactose carrier particles were layered with vegetable magnesium stearate by physical mixing and their effect of on the dry powder inhalation properties of salbutamol sulfate was investigated. The in vitro inhalation performance of drug was enhanced compared with the powder mixed with unlayered lactose carrier. It was stated that using this surface layering system would thus be valuable for increasing the inhalation properties of dry powder inhalation (Iida et al., 2004). For example, increasing the surface smoothness of lactose carrier particles was shown to improve the potentially respirable fraction of albuterol sulfate from the Rotahaler® (Littringer et al., 2012).

8.2. Shape modification

Lactose crystallization from Carbopol gel in different conditions (named Carbo lactose) produced a more regular shape lactose with smoother surface as compared with the control lactose. The Carbo lactose caused a higher and reproducible salbutamol sulphate emission

and FPF after aerosolisation via a Rotahaler® tested in multi-stage impinge. It was concluded that engineered crystal growth under controlled conditions can enhance the potential inhalable fraction of drug from dry powder inhalers (Zeng et al., 2001).More recently, the use of more elongated crystals of lactose was also found to produce a higher inhalation efficiency of albuterol sulfate(Zeng et al., 2000).However, the improvement in drug dispersion that can be achieved by increasing the elongation ratio of the carrier particles is limited (Zeng et al., 2001).

8.3. Composite lactose

Lactose carrier particles were prepared by fusing sub units of lactose (either2, 6 or 10 μm prepared by spray drying) in saturated lactose slurry, sieve fractioned to obtain a 63–90 μm carriers and used for inhalation of salbutamol sulfate. The surface morphology and physico-chemical properties of the composite carriers were considerably different from regular α-lactose monohydrate.In all cases the composite carriers resulted in improved drug aerosol performance. It was suggested that composite based carriers are a potential route to control drug–carrier adhesion forces and variability thus allowing more precise control of formulation performance (Young et al., 2009).

8.4. Engineered lactose-mannitol mixture

Mannitol and lactose were co-crystallised to prepare crystals with more desirable characteristics than either lactose or mannitol alone appropriate for application as carriers in the formulations of salbutamol sulfate DPIs. In vitro deposition evaluation showed that crystallized carriers resulted more efficient delivery of salbutamol sulphate compared to formulations containing commercial grade carriers. It was concluded that simultaneous crystallization of lactose-mannitol can be a new approach to enhance inhalation performance of DPI formulations (Kaialy et al., 2012).

9. Mannitol

Because of clinical considerations lactose or other sugars cannot be used for drug delivery to diabetic patients. Moreover, for some drugs, e.g., formoterol, or for specific applications, e.g., peptide or protein drugs, lactose monohydrate may not be the carrier of choice due to its reducing sugar function that may interact with functional groups of the drug or the protein (Patton et al., 1992)In addition, lactose monohydrate is produced from bovine or with bovine-driven additives so that the transmissible spongiform encephalopathy (TSE) is still an issue for this compound (European Commission-Health & Consumer Protection Directorate, 2002)Lactose intolerance is a problem that necessitates the patient to use lactose-free formulations (Glasnapp, 1998).It is therefore logical to look for substitute carriers that still possess the positive aspects but overcome the above mentioned drawbacks of lactose monohydrate (Hamishehkar et al., 2010). Mannitol, a hexahydric alcohol, has been mainly used as a pharmaceutical excipient. Its potential use as a carrier for aerosol delivery has been reported (Tee et al., 2000; Steckel et al., 2004). Mannitol does not have a reducing sugar

function, is less hygroscopic than lactose (Saint-Lorant et al., 2007)and gives a suitable sweet after-taste which has a benefit to patients confirming them that a dose has been properly administered (Kaialy et al., 2010).Mannitol proved to be the most promising candidate for this application than the more hygroscopic sugar alcohols such as sorbitol, xylitol and maltitol. D-mannitol is currently marketed in some countries as a pulmonary diagnostic dry powder inhalation aerosol (Aridol™) and as a therapeutic dry powder inhalation aerosol for the treatment of cystic fibrosis and chronic bronchitis (Bronchitol™) which are recently approved by US food and drug administration office and a European regulatory committee, respectively(Mansour et al., 2010).Spherical mannitol particles used in Aridol™ were produced by spray drying (Tang et al., 2009).A DPI formulation for inhalation of ciprofloxacin hydrochloride (an antibacterial fluoroquinolone) was prepared by its co-spray drying with different percentages of mannitol. The combination formulation containing 50% (w/w) mannitol showed the best inhaltion performance, good stability and lowest particle cohesion (as measured by colloid probe microscopy). It was proposed that the combination of co-spray-dried mannitol and ciprofloxacin from a DPI is an attractive approach to promote mucous clearance in the respiratory tract while simultaneously treating local chronic infection, such as chronic obstructive pulmonary disease and cystic fibrosis (Adi et al., 2010). Spray drying method was used to prepare several types of mannitol differ in shape and surface roughness. In this study besides the introducing the spray drying method as a proper technique for preparation of mannitol as a carrier for inhlation purposes, it was concluded that the highest fine particle fraction was achieved with carrier particles of spherical shape and a rough surface (Littringer et al., 2012).Despite the above mentioned study, in another study it was shown that the use of elongated mannitol as the carrier particle produced improved DPI performance. It was also indicated that mannitol particles crystallized from either acetone or ethanol (α-mannitol) showed the best aerosolisation performance while poorest aerosolisation performance was obtained from grounded mannitol (β-mannitol) (Kaialya et al., 2011).The same research group in another study again reported the better inhalation performance of salbutamol sulfate from elongated mannitol. In this study mannitol was crystallized from different binary mixtures of acetone/water and its carrier role in DPI formulations of salbutamol sulfate was investigated (Kaialy et al., 2010).Mannitol particles, produced by spray drying, have been used commercially (Aridol™) in bronchial provocation test (mannitol particle itself as an active ingredient not as a carrier). In a study, a confined liquid impinging jets (CLIJs) followed by jet milling was applied and introduced as an alternative method for spray drying. Although the inhalation performance of mannitol particles prepared by CLIJ method was not higher than those prepared from spray drying method (FPF 30% for particles prepared by CLIJ method compared to FPF 47% of those prepared by spray dying method) but CLIJ method offers several advantages. A main advantage of using the CLIJ method is that it can be scaled up with an acceptable yield as the precipitate can be largely collected and recovered on a filter, compared with spray drying which has a low collection efficiency for fine particles below 2 μm (Tang et al., 2009).Lactose, mannitol and glucose were used as the carriers in the formulations of inhalation powders of budesonide and salbutamol sulphate. The highest respirable fraction of drugs were achieved when mannitol was used as the carrier (Harjunen et al., 2003).

10. Sorbitol

Reducing sugars such as lactose may have an impact on the stability of proteins and peptides (Li et al., 1996; Dubost et al., 1996). Actuality, the use of lactose with protein powders may cause a reaction with lysine residues present in the protein, generating lactosylated protein molecules (Cryan, 2005).In this case, polyols such as sorbitol can play a crucial role in the formulation of respirable protein powder. Sorbitol can also serve as stability enhancers during processing. It was reported that the stability of interferon β to jet milling, required to produce a respirable powder, was found to be dependent on the presence of sorbitol in the formulation (Platz et al., 1991; Platz et al., 1994).Sorbitol showed a comparable inhalation outcome for salbutamol sulfate with lactose and mannitol but failed to produce efficient dispersion of drug (FPF less than10%) (Tee et al., 2000).

11. Erythritol

Erythritol, a meso-compound of 1,2,3,4-butanetetrol, is a natural occurring sugar alcohol existing in various fruits and fermented foods, as well as in body fluids of humans and animals. Industrially, it is prepared by glucose fermentation (Lopes Jesus et al., 2010). Erythritol has been administered as a suitable excipient in pharmaceutical formulations due to its thermal stability, very low hygroscopicity (Cohen et al., 1993) sweetness taste, low toxicity (Munro et al., 1998) and high compatibility with drugs (Endo et al., 2005; Gonnissen et al., 2007).Due to above desirable characteristics, erythritol was recently entered in the European Pharmacopoiea (Traini et al., 2006). Endo et al. used erythritol as carrier in the DPI formulation of glucagon, a key regulatory element of glycogen metabolism which is known to be effective in the clinical treatment of hypoglycemia and the maintenance of normal circulating glucose levels in patients with total pancreatectomy. This hormone has been restricted to parenteral administration. The in vitro and in vivo studies were indicated the suitability of erythritol for application in DPI formulation. Moreover, it was claimed that this dry powder inhalation of glucagon can be administered to the clinical treatment of hypoglycemia, and the maintenance of normal circulating glucose levels in patients with total pancreatectomy (Endo et al., 2005). In a comparative study, erythritol andlactose monohydrate were used as carriers in the DPI formulation of salbutamol sulfate. Drug-carrier adhesion was measured using atomic force microscope colloid probe technique and showed a higher adhesion force for erythritol than lactose. Consequently lower inhalation performance of salbutamol sulfate was resulted from DPI formulations containing erythritol than lactose. However, it was concluded that even though erythritol may show a reduced DPI functionality, with this drug, it may offer some potential advantages in terms of its reproducible chemical structure and stability (Traini et al., 2006).

12. Trehalose

Trehalose dihydrate is a disaccharide sugar and crystalline hydrate like lactose. However, trehalose dihydrate is a non-reducing sugar and lactose monohydrate is a reducing sugar. As a reducing sugar, it participates in solid-state chemical degradation by the Maillard

reaction with certain types of small molecular weight drugs (such as formoterol and budesonide) and polypeptide/protein-type drugs. Therefore, some attempts have been carried out on the application of trehalose in the formulation of DPIs. In a study, trehalose was used as a carrier for DPI formulation of albuterol sulfate, ipratropium bromide monohydrate, disodium cromoglycate, and fluticasone propionate (Mansour et al., 2010).The highest inhalation performance was reported for the blend of albuterol and ipratropium with trehalose dihydrate in comparison with lactose monohydrate and mannitol (Cline et al., 2002).

13. Other carriers

Recently, the feasibility of using pollen-shape hydroxyapatite particles as carrier in DPI formulation of budesonide is investigated. The hydroxyapatite carriers showed better flowability and capability of higher drug attachment than commonly used lactose carrier with similar size range. Consequently, DPI formulations with hydroxyapatite carriers gave higher drug emission and respirable fraction than traditional lactose carriers (Hassan et al., 2010). Many different non-lactose carriers have been investigated, such as cyclodextrins, dextrose, glucose monohydrate, maltitol, maltose, raffinosepentahydrate and xylitol.Anhydrous glucose is already used in the marketed product Bronchodual® (Boehringer) (Pilcer et al., 2010).Sucrose, trehalose and raffinose are non-reducing sugars and as such have the advantage that they will not undergo the Maillard browning reaction with proteins (Ógáin et al., 2011). But in contrast to lactose monohydrate which was found to only take up moisture on its surface, and showing only a small reduction in its FPF after storage at 75% RH (Young et al., 2007; Zeng etal., 2007), a main difficulty with these sugars, especially with the more hygroscopic substances – sorbitol, maltitol and xylitol – has attributed to their sensitivity to humidity. In fact, the capillary forces, arisen from the dynamic condensation of water molecules onto particle surfaces, seem to be less prominent with mannitol and lactose as carriers, probably because of their less hygroscopic characteristics than the other carbohydrates (Zeng et al., 2007; Young et al., 2007). However, it was proposed that the difficulties arising from their hygroscopicity can be overcome by adding an ultrafine hydrophobic excipient to the powder blend (Steckel et al., 2004).Comparing the different forms of mannitol, lactose and maltitol mixed with terbutaline sulfate resulted in higher FPF with crystallized mannitol for terbutaline sulfate (Saint-Lorant et al., 2007).Hooton et al, used beta cyclodextrin, lactose, raffinose, trehalose and xylitol for the formulation of salbutamol sulfate DPI and applied the cohesive-adhesive balance technique for analyzing quantitative AFM measurements to interpret inhalation behavior of drug. The rank order of the FPF of the salbutamol sulfate based carrier DPI formulations was beta cyclodextrin> lactose >raffinose>trehalose> xylitol which had a linear correlation with cohesive-adhesive ratios of the AFM force measurements (Hooton et al., 2006).

14. Large porous particles

A new type of aerosol formulation is the large porous hollow particles. These particles have the mean diameters >5 μm and mass densities <0.1g/cm^3 (Edwards et al., 1997). Although

these particles have large geometric diameters because of their low density, they exhibit aerodynamic diameters comparable to smaller particles having higher densities (Koushik et al., 2004). They may be ideal for pulmonary drug delivery because of their low density and large surface area which causes excellent dispersibility (Labiris et al., 2003). Furthermore, their large geometric size may reduce clearance by macrophage action, thereby improving the bioavailability of inhaled pharmaceuticals (Musante et al., 2002).To show the ability of large porous aerosols to increase systemic bioavailability as well as to provide sustained-release capability in the lungs, Edwards et al. encapsulated insulin into a biodegradable polymers and indicated the better inhalation performance and controlled-release capability of these particles in the lung (Edwards et al., 1997).Ungaro et al. confirmed the same observations with delivery of insulin into rat lung by preparation of PLGA large porous particle with the aid of cyclodextrins (Ungaro et al., 2009).These particles can be prepared using polymeric or nonpolymeric excipients, by solvent evaporation and spray-drying techniques (Edwards et al., 1998). Pulmospheres™ is an example which is made of phosphatidylcholine, the primary component of human lung surfactant (Labiris et al., 2003). In two interesting studies, highly porous large biodegradable polymeric particles were fabricated using ammonium bicarbonate as an effervescent porogen (Ungaro et al., 2010; Yang et al., 2009).

15. Conclusion

The number of diseases that are being considered candidates for the aerosol therapy has increased considerably. Until recently, asthma and chronic obstructive pulmonary diseases were only the apparent examples of diseases that could be treated via drug delivery to lungs. But now other pulmonary diseases such as cystic fibrosis, lung cancer and pulmonary infectious diseases and also systemic disorders such as diabetes, cancer, neurobiological disorders are considered to be managed by pulmonary drug delivery. Interest in DPIs has increased in the last decade due to its numerous advantages over other pulmonary drug delivery dosage forms. Currently, the inhalation performance of DPIs are being improved by changing formulation strategy, drug and carrier particle engineering. Regarding formulation development, micronised drug particles are cohesive with poor flow properties. Addition of large carrier particles into powders to enhance their flow characteristics has been an appropriate approach. The main goal in the inhalation field is to obtain reproducible, high pulmonary deposition which can be highly effected by physico-chemical characteristics of carrier. This could be achieved by successful carrier selection and careful process optimization. Technologies for engineering carrier particle shape, density, and size will continue to develop to enhance the effectiveness of pulmonary drug formulations. This approach may enable more drugs to be delivered through this route for local treatment of lung diseases or systemic therapy.

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Section 2

Nanocarriers in Drug Delivery

Chapter 4

Nanotechnology in Drug Delivery

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Additional information is available at the end of the chapter

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1. Introduction

Nanoscience has been variously defined at different fora, books, journals and the web, yet one thing is common; it involves the study of the control of matter on an atomic and molecular scale. This molecular level investigation is at a range usually below 100 nm. In simple terms, a nanometer is one billionth of a meter and the properties of materials at this atomic or subatomic level differ significantly from properties of the same materials at larger sizes. Although, the initial properties of nanomaterials studied were for its physical, mechanical, electrical, magnetic, chemical and biological applications, recently, attention has been geared towards its pharmaceutical application, especially in the area of drug delivery.

This is because of the challenges with use of large size materials in drug delivery, some of which include poor bioavailability, in vivo stability, solubility, intestinal absorption, sustained and targeted delivery to site of action, therapeutic effectiveness, generalized side effects, and plasma fluctuations of drugs. Of recent, several researches in nanodrug delivery have been designed to overcome these challenges through the development and fabrication of nanostructures. It has been reported that, nanostructures have the ability to protect drugs from the degradation in the gastrointestinal tract, the technology can allow target delivery of drugs to various areas of the body. The technology enables the delivery of drugs that are poorly water soluble and can provide means of bypassing the liver, thereby preventing the first pass metabolism Nanotechnology increases oral bioavailability of drugs due to their specialized uptake mechanisms such as absorptive endocytosis and are able to remain in the blood circulation for a long time, releasing the incorporated drug in a controlled fashion, leading to less plasma fluctuations and minimized side-effects. Nanoscale size nanostructures are able to penetrate tissues and are easily taken up by cells, allowing for efficient delivery of drugs to target sites of action. Uptake of nanostructures has been reported to be 15–250 times greater than that of microparticles in the 1–10 um range. Nanotechnology improves performance and acceptability of dosage forms by increasing

their effectiveness, safety, patient adherence, as well as ultimately reducing health care costs. It may also enhance the performance of drugs that are unable to pass clinical trial phases. Nanotechnology definitely promises to serve as drug delivery carrier of choice for the more challenging conventional drugs used for the treatment and management of chronic diseases such as cancer, asthma, hypertension, HIV and diabetes.

2. Nanotechnology-based drug delivery systems

2.1. Smart drug delivery systems

Ideally, nanoparticulate drug delivery system should selectively accumulate in the required organ or tissue and at the same time, penetrate target cells to deliver the bioactive agent. It has been suggested (1, 2) that, organ or tissue accumulation could be achieved by the passive or antibody-mediated active targeting (3, 4), while the intracellular delivery could be mediated by certain ligands (5, 6) or by cell-penetrating peptides (7, 8). Thus, a drug delivery system (DDS) should be multifunctional and possess the ability to switch on and switch off certain functions when necessary. Another important requirement is that different properties of the multifunctional DDS are coordinated in an optimal fashion. Thus, for example, if the system is to be constructed that can provide the combination of the longevity allowing for the target accumulation and specific cell surface binding allowing, two requirements must be met; the half-life of the carrier in the circulation should be long enough and second, the internalization of the DDS by the target cells should proceed fast enough not to allow for the carrier degradation and drug loss in the interstitial space. Intracellular transport of bioactive molecules is one of the key problems in drug delivery. Nanoparticulate DDS, such as liposomes and micelles, are frequently used to increase the efficacy of drug and DNA delivery and targeting (9, 10). So far, very few successful attempts have been made to deliver various drug carriers directly into the cell cytoplasm, bypassing the endocytic pathway, to protect drugs and DNA from the lysosomal degradation, thus enhancing drug efficiency and DNA incorporation into the cell genome (11-14). Within the multifunctional DDS, it has been postulated that, the development of a DDS built in such a way that during the first phase of delivery, a nonspecific cell-penetrating function is shielded by the organ/tissue-specific delivery will be possible. Upon accumulating in the target, protecting polymer or antibody attached to the surface of the DDS via the stimuli-sensitive bond should detach under the action of local pathological conditions such as abnormal pH or temperature and expose the previously hidden second function allowing for the subsequent delivery of the carrier and its cargo inside cells. While such DDS should be stable in the blood for a long time to allow for an efficient target accumulation, it has to lose the protective coat inside the target almost instantly to allow for fast internalization thereby minimizing the washing away of the released drug or DNA. Intracellular trafficking, distribution, and fate of the carrier and its cargo can be additionally controlled by its charge and composition, which can drive it to the nuclear compartment or toward other cell organelles.

It has been reported within the past few years, that certain proteins and peptides (such as TAT peptide) can enter cell cytoplasm directly and even target cell nuclei (15, 16). Certain

proteins and peptides have also been used for the intracellular delivery of small drug molecules, large molecules (enzymes, DNA), and nanoparticulates (quantum dots, iron oxide nanoparticles, liposomes) (13, 17-22). The mechanism of this phenomenon is currently a subject of investigation, although important progress has been made, as some reports show that electrostatic interactions and hydrogen bonding lay behind certain proteins and peptides-mediated direct transduction of small molecules (23, 24), while the energy-dependent macropinocytosis is responsible for certain proteins and peptides-mediated intracellular delivery of large molecules and nanoparticulates with their subsequent enhanced release from endosomes into the cell cytoplasm (25 – 28).

One of the most outstanding achievements in the drug delivery field was the development of smart drug delivery systems (SDDSs), also called stimuli-sensitive delivery systems. The concept is based on rapid transitions of a physicochemical property of polymer systems upon a stimulus. This stimulus includes physical (temperature, mechanical stress, ultrasound, electricity, light), chemical (pH, ionic strength), or biological (enzymes, biomolecules) signals and such stimuli can either be internal, resulting from changes in the physiological condition of a living subject, or ''external'' signals, artificially induced to provoke desired events. SDDS provides a programmable and predictable drug release profile in response to various stimulation sources. Fig 1 below shows a typical smart drug delivery system;

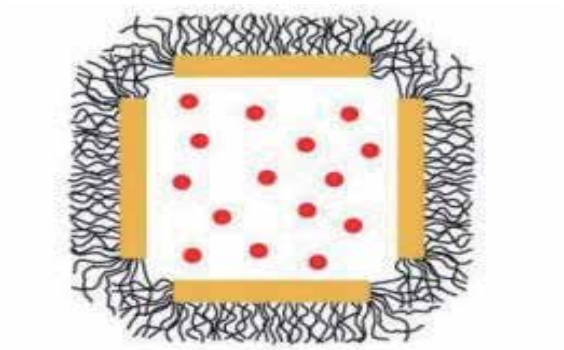


Figure 1. Smart drug delivery system -- Gold nanocage covered with polymer

Depending on the desired applications, one may design different drug delivery systems for enhanced therapeutic efficiency with low systemic toxicity and side effects. SDDS has several advantages compared to conventional drug delivery systems. The conventional controlled release systems are based on the predetermined drug release rate irrespective of the environmental condition at the time of application. On the other hand, SDDS is based on the release-on-demand strategy, allowing a drug carrier to liberate a therapeutic drug only when it is required in response to a specific stimulation. The best example of SDDS has been self-regulated insulin delivery systems that can respond to changes in the environmental glucose level (29, 30). One of the most widely used SDDSs has been polymeric micelles. Many polymeric micelles consisting of hydrophobic and hydrophilic polymer blocks have been developed. They have been found to dissolve water-insoluble drugs, such as

doxorubicin or paclitaxel, at high concentrations. When administered to the body, drug release from polymeric micelles usually depends on simple diffusion, degradation of the micelle blocks, or disruption of the micelles by body components.

The release kinetics of the loaded drug can be modulated by varying the degradation rate of hydrophobic polymer blocks, but because the degradation rate is usually very slow, the loaded drug is released by diffusion from polymeric micelles. This slow release by passive diffusion may not be desirable, as the polymeric micelles reaching the target site need to release their contents fast. To solve this problem, smart polymeric micelles have been designed to liberate the loaded therapeutic agent at the targeted site fast. For example, Lee et al, (31) reported that Poly (ethylene glycol)-b-polyhistidine (PEG-b-PHis) forms micelles only over the pKb of the polyhistidine block (pH 6.5–7.0). It is interesting to know that, the pKb can be adjusted by varying the molecular weight of polyhistidine. Since solid tumors have a slightly acidic environment, a small reduction in pH to less than 7 at the tumor site triggers dissociation of the polymeric micelle to release its contents. In a separate study, Lee et al (32) reported that, PEG-b- polyhistidine micelles containing doxorubicin effectively killed multi-drug resistant MCF-7 cells at pH 6.8. Similarly, Hruby et al, (33) reported that, SDDS can achieve a highly localized drug accumulation at target sites even though it is administered parenterally. It is therefore postulated that, SDDS with enhanced targeting property is highly promising in increasing the efficiency and efficacy of therapy while at the same time minimizing side effects.

2.2. Polymer–drug conjugates

Polymer–drug conjugates are a class of polymer therapeutics that consists of a water-soluble polymer that is chemically conjugated to a drug through a biodegradable linker. The idea started in 1975 when Ringsdorf proposed the use of polymer–drug conjugates to deliver hydrophobic small molecules (34). The reasoning was that, small molecule drugs, especially hydrophobic compounds, have a low aqueous solubility and a broad tissue distribution profile such that, administration of the free drug may result in serious side effects. Therefore, conjugation of these compounds to hydrophilic, biocompatible polymers would significantly increase their aqueous solubility, modify their tissue distribution profile and enhance their plasma circulation half-life. An important attribute of colloidal systems is their hydrodynamic diameter, which are typically about 3–20 nm for polymer–drug conjugates (35) and between 10 and 200 nm for colloidal particles such as micelles or liposomes. The colloidal nature or size of these vehicles can facilitate their retention within the circulation for prolonged periods, in comparison to low molecular weight small molecules. One major difference between polymer–drug conjugates and delivery systems that contain physically entrapped drug (e.g., micelles and liposomes) is that the drug is chemically conjugated to the polymer and therefore these systems qualify as new chemical entities (NCE). Classification as an NCE is often accompanied by additional development and regulatory hurdles that must be met in order to receive approval. Over the last decade, polymer-conjugate technology has proven to be a viable formulation strategy. There have been reports (36 – 38) of bioconjugation of protein and peptide to PEG been able to significantly

improve the efficacy of these macro- molecular drugs by increasing their stability in the presence of proteases and decreasing their immunogenicity. Studies have also shown that by using PEG in a specific molecular weight range, the fast renal clearance and mononuclear phagocytic system uptake of the drugs can be prevented or delayed leading to a prolonged plasma half-life for the conjugated molecules. Successful applications have led to several FDA- approved products e.g. Neulasta®. The first practical use of polymer therapeutics that resulted in an FDA-approved anti-cancer treatment was the introduction of PEG-L-asparaginase (Oncaspar1) in 1994. This conjugate is composed of PEG polymer (MW ~ 5 kD) attached to the enzyme, L-asparaginase, and is used for the treatment of acute lymphoblastic leukemia (39). In fact, polymer–drug conjugate itself can be considered as a nanovehicle. Various conjugates have been developed and clinically tested. Recent advances in polymer–drug conjugates are well described in reviews (Duncan, 2006; Duncan, Vicent, Greco, & Nicholson, 2005). One of the major advantages of polymer–drug conjugates is prolonged circulation in the blood stream by retarding degradation/metabolism/excretion rates of the conjugated drugs. Many peptide and protein drugs cannot be delivered by oral administration because of their large molecular weights. Even when administered directly into the blood stream, they do not remain in the blood for a long time due to fast degradation and metabolism, limiting the clinical applications. The circulation times of these drugs have increased substantially by conjugation with polymers, such as PEG. A good example is the glucagon-like peptide-1, which regulates food uptake and insulin release. The peptide is a very useful therapeutic agent for diabetic patients, but it is liable to degradation by a plasma enzyme, dipeptidyl dipeptidase IV, but by introducing one PEG chain, Lee et al (40) showed that its half-life could be increased up to 40 folds over the natural form. Very often, low molecular weight drugs with high hydrophobicity are used for conjugation with attendant reduction in the degradation/clearance rate as well as the toxicity of the conjugated drug. The therapeutic effect is achieved upon hydrolysis inside the target cells to release the original drug. The polymers used in conjugation usually have stimuli-responsiveness, imparting unique properties into the conjugated drug such that, its activities can be turned on or off by external signals. For example, Shimoboji et al, (41, 42) reported that, the catalytic activity endoglucanase 12A upon conjugation could be turned on by application of UV light or high temperature because, it was conjugated with either photo-sensitive or thermo-sensitive polymers. The active site of the enzyme was exposed by collapsing the conjugated long polymer chain by external stimuli. Once visible light was turned on or the temperature was lowered, the enzyme activity vanished due to the blocking of the active site by the extended polymer chain. (43, 44)

2.3. Multifunctional drug carriers

A multifunctional drug delivery system (MDDS) refers to drug carrier that has multiple properties of prolonged blood circulation, passive or active localization at specific disease site, stimuli-sensitivity, ability to deliver drug into intracellular target organelles, and/or imaging ability (45). Technically therefore, it has two or more functions, infact, SDDS and polymer–drug conjugates discussed above can be considered MDDS. In addition to

delivering drugs, MDDS can carry out the second function, such as stimuli-responsiveness or hydrolysis inside cells. Some reported MDDS include the biotin-tagged pH-sensitive polymeric micelles based on a mixture of PLA-b-PEG-b-PHis-biotin (PLA=poly (L-lactic acid)) and PEG-b-PHis block copolymers by Lee et al (46) in which the targeting moiety, biotin, was masked until the carrier was exposed to an expected environment of pH 7.0. Once the nanocarrier was internalized to cancer cells by ligand– receptor interactions, lowered pH (< 6.5) destabilized the carrier resulting in a burst release of the loaded drug and that of Lukyanov et al (47), where a pH-degradable PEG-b-phosphatidylethanolamine (PE) liposome had anti-myosin monoclonal antibody as well as TAT or biotin attached on its surface.

2.4. Organic/inorganic composites

An inorganic-organic composite usually comprises an inorganic phase and a film forming organic phase. A typical green approach to developing an inorganic-organic composite involves the selection of film forming organic phase from starches having a degree of polymerization; degree of substitution and viscosity such that the substituted starches are insoluble in water during mixing but dissolve at a higher processing temperature during forming, setting or drying of the composite. Thus, excessive migration of the starch is prevented and the composite is substantially strengthened. There has also been reports on the lab-on-a-chip approach (48 – 54), which embodies micron- or nano-sized machines composed of sophisticated circuits. Small devices have many advantages including portability/disposability, low cost, high reproducibility, high-throughput screening, and multiple functionalities in a single device. Recently, combined with other technologies such as optics, single molecular imaging, or cell/protein-based assay systems, biomedical lab-on-a-chip devices have become an important part of drug discovery and diagnosis, but its application in drug delivery systems based on are just beginning to appear (55 – 57).

As rightly noted by several authors, to release a drug from a nanodevice is more complicated than to perform assay or screening drug candidates, this is because, successful drug delivery requires at least four components namely; drug reservoir, pump, valve, and sensor (58). Drugs can be placed either in a fabricated reservoir or in conventional micro-/nanoparticles. Other important organic/inorganic composites are metal nanoparticles, such as silver, iron oxide, or gold nanoparticles, coated with hydrophilic polymers. Their major application has been as theranostics. Only recently, Hirsch et al, (59) developed gold nanoshell, which provided tunable emission light for bioimaging. Importantly, is the fact that, gold nanoparticles can be detected by X-ray and emit thermal energy by excitation making it very useful for medical imaging and thermal therapy (theranostics). In a related report, Corot et al, (60) developed super paramagnetic iron oxide nanoparticles for magnetic resonance imaging (MRI) of the whole body. Mechanistically, these nanoparticles are primarily engulfed by monocyte or macrophage after intravenous administration. However, uptake of super paramagnetic iron oxide by macrophage does not induce activation of nearby cells making it suitable for diagnosis of inflammatory or degenerative diseases.

3. Nanoparticulate drug delivery systems

3.1. Liposomes

Use of micro and nano particles in biomedicine and especially in drug delivery has a great deal of advantages over conventional systems such as: the enhanced delivery, high performance characteristics of the product, use of lesser amounts of expensive drugs in the delivery systems, extension of the bioactivity of the drug by protecting it from environmental effects in biological media, more effective treatment with minimal side effects. In addition, research for the design of more effective delivery systems is more economical for the discovery of a new bioactive molecule. Micro and nano colloidal drug delivery systems such as emulsions, suspensions and liposomes have been used for decades for this purpose and recently, nanosized systems with dimension of less than 100 nm gained significant attention. Nanotechnology promises to generate a library of sophisticated drug delivery systems that integrate molecular recognition, diagnostic and feedback. Nanotechnology is expected to create lots of innovations and play a critical role in various biomedical applications including the design of drug and gene delivery systems, molecular imaging, biomarkers and biosensors. By understanding the signaling and interaction between the molecules at nano levels, it would be possible to mimic biological systems.

Liposomes are small spherical vesicles (Fig. 2) in which one or more aqueous compartments are completely enclosed by molecules that have hydrophilic and hydrophobic functionality. Liposomes vary with composition, size, surface charge and method of preparation. They can be single or in multiple bilayers. Those containing one bilayer membrane are termed small unilamellar vesicles or large unilamellar vesicles based on their sizes (61). If more than one bilayer is present then they are called multilamellar vesicles. Liposomes are commonly used as model cells or carriers for various bioactive agents including drugs, vaccines, cosmetics and nutraceuticals. Drugs associated with liposomes have markedly altered pharmacokinetic properties compared to free drugs in solution. Liposomes are also effective in reducing systemic toxicity and preventing early degradation of the encapsulated drug after administration. They can be covered with polymers such as polyethylene glycol (PEG) – in which case they are called pegylated or stealth liposomes, in this form, they usually will exhibit prolonged half-life in blood circulation. Liposomes can also be conjugated to antibodies or ligands to in order enhance target-specificity. For example, Visser et al (62) studied pegylated horse –radish-peroxidase loaded liposomes, tagged with transferrin to the blood-brain barrier. The authors showed an effective targeting of liposomes loaded with protein or peptides to the brain capillary endothelial cells and suggested that the system could be an attractive approach for targeting drug delivery to brain. In another report, Lopez-Pinto and coworkers (63) examined the dermal delivery of a lipophilic drug, minoxidil, from ethosomes versus classic liposomes by applying the vesicles non-occlusively on rat skin, yet in a separate study, Ozden and Hasirci (64) prepared small unilamellar vesicles composed of phosphatidyl- choline, dicetyl phosphate and cholesterol and entrapped glucose oxidase in them. Liposomes are also studied as carriers for cells, genes or DNA fragments. Ito et al (65) studied the effect of magnetite cationic liposomes which have

positive surface charge to enrich and proliferate Mesenchymal stem cells (MSCs) in vitro. Kunisawa et al (66) established a protocol for the encapsulation of nanoparticles in liposomes, which were further fused with ultra violet-inactivated Sendai virus to compose fusogenic liposomes and observed that fusogenic liposome demonstrated a high ability to deliver nanoparticles containing DNA into cytoplasm.

Foco et al (67) studied the delivery of sodium ascorbyl phosphate (SAP), an effective oxygen species scavenger to prevent the degenerative effects of UV radiation on skin. SAP was encapsulated into liposomes to improve its penetration through the stratum corneum into the deeper layers of the skin. Sinico et al (68) studied transdermal delivery of tretinoin and examined the influence of liposome composition, size, lamellarity and charge on transdermal delivery. They studied positively or negatively charged liposomes of different types. It was reported that negatively charged liposomes strongly improved newborn pig skin hydration and tretinoin retention. Arcon et al (69) encapsulated an anticancer agent, cisplatin, in sterically stabilized liposomes and studied the systems with extended X-ray absorption fine structure method, and concluded that the liposome-encapsulated drug is chemically stable and does not hydrolyze.

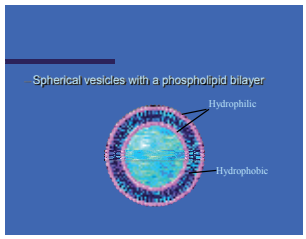


Figure 2. Spherical vesicles with a phospholipid bilayer

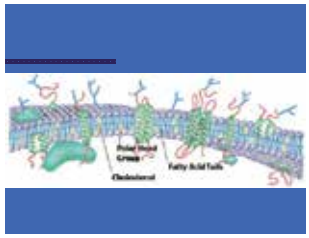


Figure 3. Cell Membrane

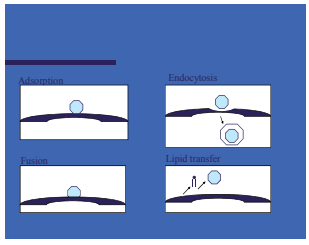


Figure 4. Modes of Liposome/Cell Interaction

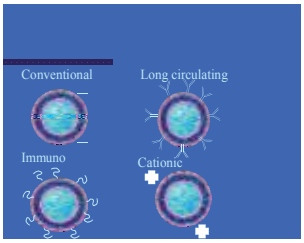


Figure 5. Classes of Liposomes

Type of Agents	Examples
Anticancer Drugs	Duanorubicin, Doxorubicin*, Epirubicin Methotrexate, Cisplatin*, Cytarabin
Anti bacterial	Triclosan, Clindamycin hydrochloride, Ampicillin, peperacillin, rifamicin
Antiviral	AZT
DNA material	cDNA - CFTR*
Enzymes	Hexosaminidase A Glucocerebrosidase, Peroxidase
Radionuclide	In-111*, Tc-99m
Fungicides	Amphotericin B*
Vaccines	Malaria merozoite, Malaria sporozoite Hepatitis B antigen, Rabies virus glycoprotein

*Currently in Clinical Trials or Approved for Clinical Use

Source: Jessica Scott, www.nanobiotec.iqm.unicamp.br

Table 1. Current liposomal drug preparations

3.2. Microemulsions

Microemulsions are isotropic, thermodynamically stable systems composed of oil, water, and surfactant. Thermodynamic stability rather than size, is the defining hallmark of a microemulsion, although the droplet sizes are still below 100 nm (and many times much smaller) (70). Be that as it may, what is critical about microemulsions is that, they contain two phases consisting of two immiscible liquids that are mixed together and stabilized with the aid of a surfactant with or without a co-surfactant. They may have droplets in the range

Liposome Utility	Current Applications	Disease States Treated
Solubilization	Amphotericin B, minoxidil	Fungal infections
Site-Avoidance	Amphotericin B – reduced nephrotoxicity doxorubicin – decreased cardiotoxicity	Fungal infections, cancer
Sustained-Release	Systemic antineoplastic drugs, hormones, corticosteroids, drug depot in the lungs	Cancer, biotherapeutics
Drug protection	Cytosine arabinoside, interleukins	Cancer, etc.
RES Targeting	Immunomodulators,vaccines, antimalarials,macophage-located diseases	Cancer, MAI, tropical parasites
Specific Targeting	Cells bearing specific antigens	Wide therapeutic applicability
Extravasation	Leaky vasculature of tumors, inflammations, infections	Cancer, bacterial infections
Accumulation	Prostaglandins	Cardiovascular diseases

Source: Handbook of Biological Physics Volume 1, (ed. R. Lipowsky and E. Sackmann). Elsevier Science B. V.

Table 2. Some applications of liposomes in the pharmaceutical industry

of 5–100 nm. The difference between microemulsions and emulsions is that, the later are opaque mixtures of two immiscible liquids, thermo- dynamically unstable and usually require the application of high torque mechanical mixing or homogenization to produce dispersed droplets in the range of 0.2–25 mm. Both types can be made as water-in-oil (w/o) or oil-in-water (o/w) (70). Choice of the dispersed and continuous phases for microemulsions formulations is based on the hydrophilicity of the model drug. Also, surfactants that have hydrophilic–lipophilic balances (HLB) of 3–6 tend to promote the formation of w/o microemulsions while those with HLB values of 8–10 tend to promote the formation of o/w microemulsions. It has been reported (70) that, the formation and stability of microemulsions are dependent on the interfacial tension between the dispersed and continuous phases. Microemulsion instability can lead to Oswald ripening leading to dissolution of the small droplets with a resultant increase in the size of the large droplets, therefore, stabilization against Ostwald ripening is very critical, this is because, the resultant change in the size of the droplets could lead to loss of physical stability of the dosage form. Choice of the components of microemulsions affects its stability (70). Safety is also another important factor that must be considered during component selection. Attwood, 1994 had opined that, the irritant and toxic properties of some alcohols (1-butanol and 2-butanol)

could limit their potential use. Microemulsions have been proposed as drug delivery systems to enhance the absorption of drug across biological membranes (70). Some of the advantages of microemulsions include (i) Increased solubility and stability of drugs (ii) ease and economy of scale-up. Some of the disadvantages are; (a) premature leakage/release of incorporated drug (b) phase inversion (c) Many of the effective surfactants and/or co-surfactants do not have a pharmaceutically acceptable toxicity profile; and (d) microemulsion systems often require development of complex systems that may be time consuming.

Product	Developed	Applications
Rapamune®	Elan's	An immunosuppressant
Emend®	Elan's	Anti-nausea
Estrasorb®	Elan's	Topical estrogen therapy
Megace® ES	Elan's	Stimulate appetite
TriCor®	Elan's	Cholesterol-lowering
Abraxane®	APP	Breast cancer
Doxil®	Alza	Anti-cancer
Acticoat®	Smith & Nephew	Antimicrobial
SilvaGard	AcryMed, Inc.,	Antimicrobial

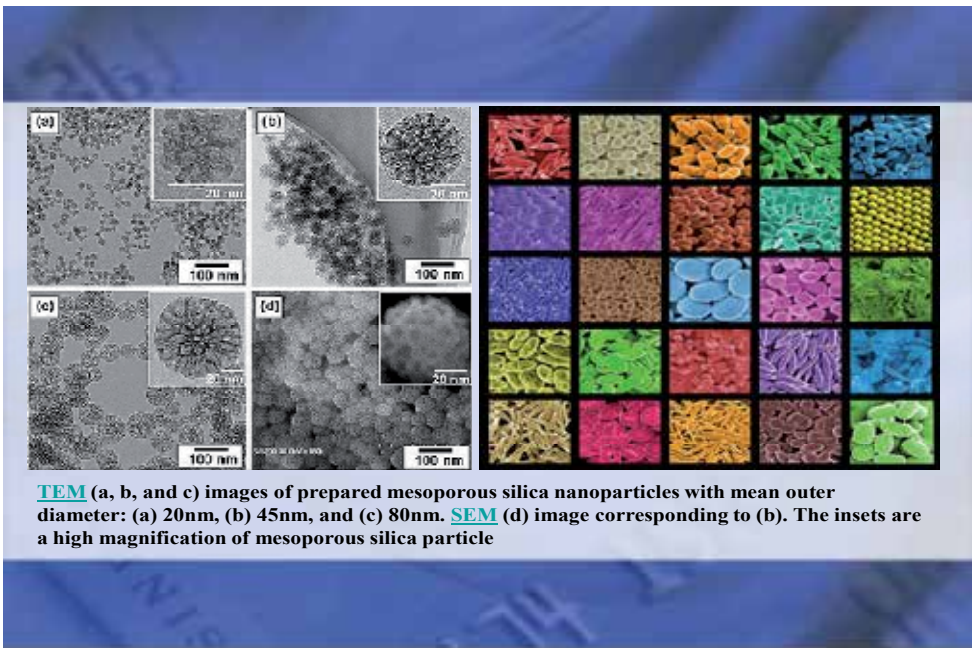
Source: B. K. Nanjwade, Department of Pharmaceutics, KLE University College of Pharmacy, BELGAUM-590010

Table 3. Some medical applications of liposomes

3.3. Nanoparticles

Nanoparticle drug delivery systems are nanometeric carriers used to deliver drugs or biomolecules. Generally, nanometeric carriers also comprise sub-micron particles with size below 1000 nm and with various morphologies, including nanospheres, nanocapsules, nano- micelles, nanoliposomes, and nanodrugs, etc. (71, 72). Nanoparticle drug delivery systems have outstanding advantages, some of which include; (1) they can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged; (2) they can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal

cord and lymph; (3) they could show controlled- release properties due to the biodegradability, pH, ion and/or temperature sensibility of materials; (4) they can improve the utility of drugs and reduce toxic side effects. As drug delivery system, nanoparticles 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. Over the years, nanoparticle drug delivery systems have shown huge potential in biological, medical and pharmaceutical applications (73). Currently, the researches on nanoparticle drug delivery system focus on: (1) the selectness and combination of carrier materials to obtain suitable drug release speed; (2) the surface modification of nanoparticles to improve their targeting ability; (3) the optimization of the preparation of nanoparticles to increase their drug delivery capability, their application in clinics and the possibility of industrial production; (4) the investigation of in vivo dynamic process to disclose the interaction of nanoparticles with blood and targeting tissues and organs, etc. One type of nanoparticle, which is differentiated from any of the above terms, is a solid lipid nanoparticle (SLN) with a lipid core that is solid at room temperature. During formation of SLNs the solid lipid is first melted, then emulsified as a liquid to form an o/w emulsion, and cooled to allow the lipid to solidify. Due to the similarity in formation and content, these particles have been referred to as ''emulsions with solid fat globules''.



Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

Figure 6. Transmission and electron micrographs of silica nanopartcles

Nanoparticles are solid colloidal particles, ranging in size from 1 to 1000 nm, consisting of various macromolecules in which the therapeutic drugs can be adsorbed, entrapped or

covalently attached. The distinct advantages offered by solid nanoparticles in drug development can be ascribed to their physical stability and the possibility of modifying the formulating materials in order to achieve controlled release characteristics. The ability to formulate nanoparticles to achieve sustained release offers an opportunity for product life cycle management by developing formulations with decreased dosing frequency for drugs that are going off patent. There has been a variety of materials used to engineer solid nanoparticles both with and without surface functionality. Perhaps the most widely used are the aliphatic polyesters such as poly (lactic acid) (PLA), the more hydrophilic poly (glycolic acid) (PGA) and their copolymers poly (lactide-coglycolide) (PLGA). The degradation rate of these polymers and often the corresponding drug release rate can vary from days (PGA) to months (PLA). The effectiveness of nanoparticles in drug delivery can be attributed to many factors such as physical and biological stability, good tolerability of the components, simplicity of the manufacturing process, possibility of facile scale-up of the manufacturing process, amenability to freeze drying and sterilization.

4. Some natural polymers in nanodrug delivery

4.1. Starch

Starch is a common polysaccharide. It occurs majorly in plants where they act as storage materials. Chemically, it is composed of recurring units of glycopyranose in an alpha D-(1, 4) linkage and on hydrolysis yields the monosaccharide, glucose (Heller et al., 1990). The use of starch in pharmaceutics is extensive. It is used as co-polymer and excipient in controlled drug delivery (74 – 76) as drug carriers in tissue engineering scaffolds (77) as Hydrogels (78) and as solubility enhancers (79).

Santander-Ortega et al. (80) investigated the potential of starch nano-particles as a transdermal drug delivery system (TDDS). The challenge faced in delivering drug through these systems is that the skin acts as an effective barrier to drug passage and must therefore be overcome for effective drug delivery. Nano-particles were shown to facilitate drug delivery without interference to the skin's integrity. The method used to prepare the nano-particles was emulsification-diffusion due to its reproducibility, higher yields, ease of scale-up and control over size of particles and degree of polydispersity. Maize starch modified and un-modified (by the addition of propyl groups) was used as polymeric material to formulate 2 different types of nano-particles. The modified starch nano-particles were shown to be non-toxic using LDH (Lactose dehydrogenase) and MTT assay and resulted in particles of uniform size distribution while the nano- particles formulated from the native starch was not observable. Flufenamic acid, caffeine and testosterone were used as model drugs and their delivery across the skin was analyzed using excised skin from female Caucasian patients who had undergone abdominal plastic surgery. Permeation data obtained for caffeine and testosterone were similar for nano-encapsulated and free drugs while the delivery of flufenamic acid using the nano-particles was enhanced by about ten-fold.

Starch nano-particles have been employed to deliver insulin via non-invasive routes; Makham (81) investigated the use of chitosan cross linked starch polymers as carriers for

oral insulin delivery, manipulating the bio-adhesive and not so adhesive properties of chitosan and carboxymethyl starch to formulate hydrogels loaded with insulin. The authors however noted that, Insulin delivered by this method however faces the challenge of being broken down by proteases.

The nasal route can also be considered as an alternative to the subcutaneous route of administration because it is highly vascularised and is of great benefit in drug delivery as drugs given through this route are not subject to first-pass metabolism. However for effective delivery through this route, it is crucial that barriers to nasal drug delivery which include the lipophilic epithelium and muco-ciliary clearance must be overcome. Jain et al., (82) reports a size dependent insulin release in rats from starch nano-particles. Potato starch was used to prepare 2 differents types of nano-particles by cross-linking with epichlorohydrin and phosphoryl chloride ($POCl_3$) using both the gel and emulsion methods. These methods however led to the production of polydispersed nano-particles. There were statistically significant differences in mean sizes except in emulsion prepared epichlorohydrin cross linked particles which were smaller and of uniform distribution. In-vitro studies showed that drug release followed first order kinetics and was diffusion controlled along with burst effect, due to the presence of left-over insulin on the surface of the nanopartices after entrapment. Emulsion cross-linked particles released their drug faster than gel cross linked particles with 85-90% and 81% release in 12 hrs respectively. These differences were attributed to the diffusion path length of the drug within the particles. The smaller the particle size the less distance the drug will travel to be released. Tests carried out on the diabetes induced rats showed a 50-65% reduction in blood glucose by nano-particles compared to plain insulin formulation which served as control and this lasted for about 6hrs. Permeation enhancers modulated the hypoglycaemic effect and bioavailability of nano-particles, Plasma insulin levels of small sized nano-particles were also found to be significantly higher. Conclusions obtained from the study however recommend that further work would be needed in order to produce a more efficient carrier system.

Simi and Abraham (83) note that the presence of hydroxyl groups on starch enhances its hydrophilicity and confers on it low moisture resistance. This property poses a major constraint in drug delivery as a result of which it is often necessary to modify the polymer before it is made into nano particles as observed above. In their study, starch extracted from cassava tuber was modified by graft co-polymerization using long chain fatty acids before the resulting polymer was made into nano-particles (83). The nano-particles were prepared by dialysis and subsequently crosslinked using sodium tripolyphosphate. Oleic acid and stearic acid were both used as fatty acids while indomethacin was used as model drug. Findings showed that drug release from both types of nano-particles was effectively controlled. It is however not clear whether there was a significant difference between drug releases in both types of nano-particles. No attempts were also made to formulate the un-modified starch granules into nano-particles though this may have been due to results obtained from differential scanning calorimetry which showed that native starch was less processable than grafted starch.

In addition, magnetised iron-oxide nano-particles coated with starch were used by Cole et al. (84) as a means of targeting brain tumours. Magnetic resonance imaging and histological reports showed that surface modification with polyethylene oxide improved delivery to tumour cells resulting in a greater accumulation of particles in the glioma compared to the rest of the brain.

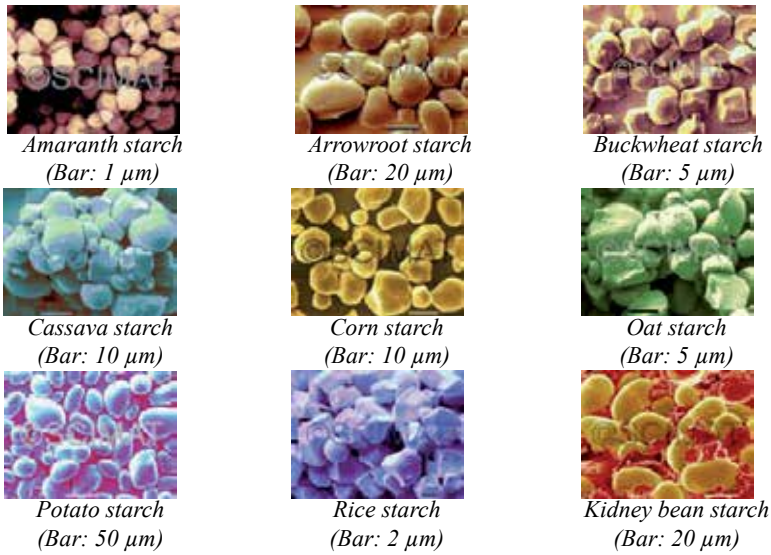


Figure 7. Granule structure of some common native starches

4.2. Chitosan

This polymer is obtained from the partial N-deacetylation of chitin found in the shells of crustacean. It is composed of glucosamine and N-acetyl glucosamine linked by β 1-4 glucosidic bonds and is one of the most widely studied natural polymers for nano-drug delivery. The deacetylation of chitin is both concentration and temperature dependent with optimal yields achieved at temperatures between 60^0C- 80^0C using 50%w/w alkali (85).

Park et al.,(86) in a review on chitosan describes its numerous applications in delivering low molecular weight drugs and summarises the reason for its choice being in its physiochemical and biological properties, enabling chemical modification and enhanced residence time respectively. It has been used as both a composite membrane with collagen (87) and a cross linked polymer for transdermal delivery of propranolol (88).

Nano-particles fabricated with chitosan as co-polymer was used to by Dev et al., (89) to investigate the controlled release of anti-retroviral drug, lamivudine. The nano-particles were prepared by emulsion and solvent evaporation technique and characterised using dynamic light scattering. The use of this method resulted in monodispersed particles with a

size range of 300-350nm. Two formulations with differences in percentage drug weight (3% and 6%) were made, of which drug release rate was higher from the nano-particles with higher drug loading, though both were able to control drug release fairly well. Drug release kinetics showed that the mechanism of drug release was by diffusion. Conclusions reached suggested that the nano-particles could be applied for gastrointestinal drug delivery because drug release was relatively slower at neutral pH compared to acidic pH and also slower in the acidic pH compare to the alkaline pH.

Chitosan combination was also used by Menon et al. (90) for therapeutic drug delivery. Nano-complexes of chitosan and polyoxometalates (POM) were tested as anti-cancer preparation. Since POM's though toxic have shown promise in being used as anti- viral and anti-tumour agent, the role of chitosan was to minimise the toxicity associated with POM, by modifying its surface properties. Mono-dispersed particles with size 200nm were produced using ionotropic gelation technique and the use of probe sonication was shown to control particle size and distribution compared to ultrasonication. In-vitro studies showed that the nano-complex was able to sustain drug release with enhanced anti-tumour activity at much lesser doses than the POM alone.

Similarly as with starch nano particles, Luo et al. (91) used chitosan oligosaccharides (COS) to coat lipid based carriers in order to enhance ocular drug delivery. This material is obtained from the decomposition of chitosan, but it is more soluble in water than chitin and chitosan. Drug introduced into the eye have minimal residence times as they are quickly washed away and have to be re-administered regularly. But in this study, COS enhanced permeation and adhesion of the cornea. There was a 7.7 fold and 2.8 fold retention of the model drug, flubiprofen by the COS coated nano lipid carriers compared to the phosphate buffer solution and uncoated nanolipid carriers which were attributed to the mucoadhesive properties of COS. The use of COS was also found to be non-irritating to the eye, a property which is of utmost importance in the choice of a suitable eye formulation.

4.3. Gelatin

Gelatin is obtained from the breakdown and hydrolysis of collagen, obtained from the connective tissues, bones and skins of animals. It is a known matrixing agent drug delivery. Bajpai and Shoubey (92) describes a process for the controlled release of sulphamethoxazole using 2 different gelatin nano-particles {Type A (porcine skin) and type B gelatin(bovine skin)} and cross linked with gluteraldehyde; Nano-particles of varying gelatin concentrations were prepared by solvent evaporation techniques and drug release kinetics evaluated using appropriate kinetic models. Findings from this system suggest that this system could be of use in targeted drug delivery such as colon drug delivery where pH is an important consideration. Drug release was found to increase following increased swelling of the nanoparticles. In addition, swelling was further enhanced by an increase in pH with greater drug release occuring at pH 7.5 than at pH 1.8. The nano particles were also not degraded in simulated gastric fluid thereby showing

their stability under acidic conditions. An increase in concentration of the cross linker led to an increase in swelling and drug release up until a certain concentration(10.6mM) when swelling began to decline. This relationship between the amount of cross linker and the polymer has also been reported by Das et al. (93); In their case, nano-particles composed of gelatin blended with montmorillonite (MMT) were loaded with the anti-cancer agent paclitaxel. These nano-particles were prepared by the same method of solvent evaporation and produced similar results. Increase in gluteraldehyde concentrations was reported to increase swelling and consequently drug release up until a certain point, when further increases in concentration of the cross linker led to decreased swelling and drug release. There was also a cumulative increase in drug release with increased pH. 80% of the drug was released within 8hrs at pH 7.4 while there was less than 44% drug release within 4 h at pH 1.2. Increasing concentration of the loaded drug also led to an increase in drug release.

The use of proteins as nano carriers is also employed in gene therapy. Viral and non-viral vectors are used for the transfection of DNA into cells, because, the injection of naked DNA into living tissue results in enzymatic degradation and reduced cellular uptake due to repulsion between the negatively charged DNA and cell membrane. In this domain, Coester et al., (94) used avidin modified gelatin nano-particles for the delivery of biotinylated PNA (Peptide nucleic acids) in other to investigate their use as anti-sense therapy. Zwiorek et al. (95) suggests that gelatin nano- particles have the potential to be used for effective non-viral gene delivery and are a safer alternative to the use of viral vectors. A 2 step desolvation process was used to prepare cationized particles of uniform size distribution and low polydispersity and comparisons between polyethyleneimine- DNA complexes and the gelatin particles showed that the latter is effective in facilitating gene expression, has less toxic and better tolerated.

Transfection with the aid of gelatin nanoparticles was also used by Xu et al. (96) for the delivery of DNA plasmids encoding for insulin growth like factor 1(IGF-1) into chrondrocytes. In order to incorporate the plasmids into the gelatin nano-particles, complex coacervation was employed because it is an easy, fast and particularly useful method for the incorporation of large molecules. The authors proved that, cationized gelatin particles were of smaller sizes than non- cationized particles, this they attributed to the condensation of the cationized particles. Fluorescence spectroscopy showed that the cationized gelatin nano-particles were successfully transfected and expressed the gene while the reverse was the case for the non-cationized gelatin particles. This is probably due to enhanced endocytosis, occurring as a result of interactions between the positive charge on the former and the negative charge on the cell membrane. A 5-fold increase in growth factor production was observed in cells containing these nano-particles. Findings also showed that over expression of the gene was maintained steadily for up to 2 weeks when they were grown in collagen (type II) -glycosaminoglycan scaffolds in 3D culture. Since a prolonged and localized release of IGF-1 was achieved in this study, and IGF-1 is known to promote growth in skeletal muscle, cartilage and bones and numerous other tissues in the body tissue, this approach shows potential applications in gene therapy and tissue engineering.

5. Properties of nanoparticles

Some of the properties of nanoparticles that are important for application in drug delivery include simple, affordable manufacturing process that is easy to scale up. The manufacturing process excludes organic solvents or potentially toxic ingredients. All the components of the formulation should be commercially available, safe, affordable, non-toxic and biodegradable. The nanoparticles should be stable with respect to size, surface morphology, size distribution and other important physical and chemical properties.

6. Preparation of nanoparticles

6.1. Nanosuspensions

Nanosuspension refers to production of sub-micron-sized particles by subjecting the combination of drug and a suitable emulsifier to the process of milling or high-pressure homogenization. Conventional milling and precipitation processes generally result in particles with sizes that are much greater than 1 mm. As such, a critical step in the nanosuspension preparation is the choice of the manufacturing procedure to ensure production of sub-micron particles. Nanosuspension formulations can be used to improve the solubility of poorly soluble drugs. A large number of new drug candidates emerging from drug discovery programs are water insoluble, and therefore poorly bioavailable, leading to abandoned development efforts. These can now be rescued by formulating them into crystalline nanosuspensions. Techniques such as media milling and high-pressure homogenization have been used commercially for producing nanosuspensions. The unique features of nanosuspensions have enabled their use in various dosage forms, including specialized delivery systems such as mucoadhesive hydrogels. Nanosuspensions can be delivered by parenteral, per- oral, ocular, and pulmonary routes. Currently, efforts are being directed to extending their applications in site-specific drug delivery. Various particle sizes of spironolactone, a model low solubility drug, have been formulated to yield micro- and nanosuspensions of the type solid lipid nanoparticles and DissoCubes. The DissoCubes nanosuspension yielded highly significant improvements in bioavailability. Particle size minimization is not the major determining factor in the bioavailability improvement. Rather, the type of surfactant used as stabilizer in the formulations is of greater importance. Improvement in drug solubility in the intestine as well as in dissolution rate of spironolactone is the most likely mechanisms responsible for the observed effect, although additional mechanisms such as permeability enhancement may also be involved.

Development of nanoparticle formulations for improved absorption of insoluble compounds and macromolecules enables improved bioavailability and release (97)

Particle size reduction to sizes below 1 mm is usually difficult due to possible particle aggregation and generation of high surface area materials. Milling techniques that have been used to generate nano-sized particles are ball milling or pearl milling that applies milling beads of sizes ranging from 0.4 to 3 mm and these beads may be composed of glass, ceramics or plastics (98). The time required for milling depends on the hardness and

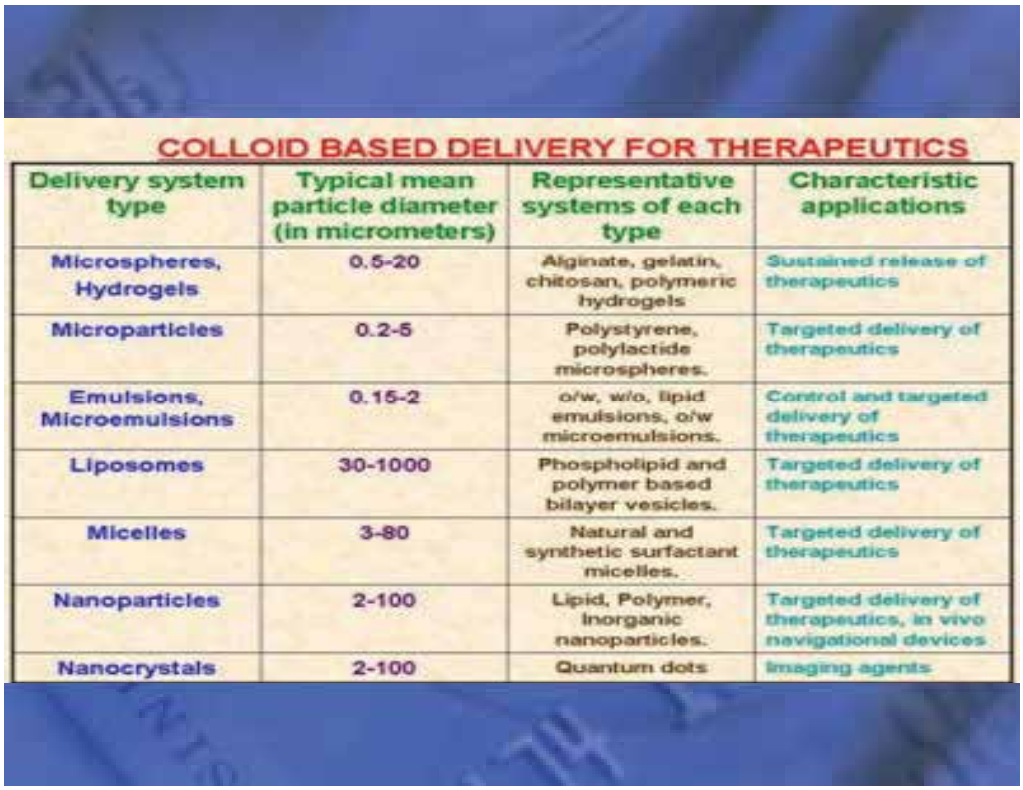
brittleness of the drug material in comparison to milling material and inertial forces set up within the mill. Some of the challenges that milling processes can pose in drug development are (i) undesirable erosion of the milling equipment components into the drug product; (ii) the process is usually time consuming, thereby prolonging drug development time; (iii) milling over a few days may bring the risk of microbiological problems or increases in the cost of production; also (iv) prolonged milling may induce the formation of amorphous domains in crystalline starting materials or may lead to changes in the polymorphic form of the drug. The generation of amorphous form of the drug is problematic because these forms may crystallize during the shelf life of the drug leading to changes in solubility and bioavailability of the drug. An example of the conversion of crystalline to amorphous form of the drug was observed in jet milling of albuterol sulfate. Also, the generation of high-energy surfaces that affected wettability was observed with acetylsalicylic acid. Some examples of nano-sized particles produced by milling as reviewed by Majuru and Oyewumi (70) are (i) naproxen nanoparticles approximately 200 nm in diameter and (ii) danazol particles of a mean size of 169 nm. The authors also reported that, there were four approved drug products in the USA that are based on NanoCrystal technology: (a) Rapamune (sirolimus) tablets by Wyeth; (b) Tricor (fenofibrate) tablets by Abbott; (c) Emend (aprepitant) capsules by Merck; and (d) Megace ES (megestrol) oral suspension by Par Pharmaceuticals (70). High pressure homogenization has also been recognized as an effective method of producing nanosuspensions (98). Again, Majuru and Oyewumi (70) reported that, high-pressure homogenization has been applied commercially with the development of some drug products, such as fenofibrate and paclitaxel. A typical procedure for preparing nanosuspension involves, preparing an aqueous suspension of drug in surfactant solution, this is then passed through a high pressure of typically 1500 bar at 3–20 homogenization cycles. The suspension is then passed through a small gap in the homogenizer of typical width 25 mm at 1500 bar. Due to built up cavitation forces that are created drug particles are broken down from micro to nanoparticles. An example is in the micro fluidization of atovaquone to obtain particles in the 100–300 nm size range Majuru and Oyewumi (70). It has been reported that, nanosuspension particles in most cases have an average size ranging from 40 to 500 nm with a small (0.1%) proportion of particles larger than 5 mm Majuru and Oyewumi (70). Experts have recognized that, a major challenge in the use of high-pressure homogenization is the possible changes in drug crystal structure that may cause batch-to-batch variation in crystallinity level, and have suggested that, application in drug delivery should include the desired specification by which the quality of each batch will be evaluated.

6.2. Polymeric nanoparticles

Polymeric nanoparticles can be identified as submicronic (size< 1μm) colloidal carriers. Compared to other colloidal carriers polymeric Nanoparticles hold significant promise for the advancement of treating diseases and disorders. They have attractive physicochemical properties such as size, surface potential, hydrophilic-hydrophobic balance and for this reason they have been recognized as potential drug carriers for bioactive ingredients such as

anticancer drugs, vaccines, oligonucleotides , peptides, etc. Their widespread use for oral delivery also aims at improving the bioavailability of drugs with poor absorption characteristic, reducing GI mucosa irritation caused by drugs and assuring stability of drugs in the GI tract. Thus, all these and many more such characteristics of nanoparticles qualify them as a promising candidate in drug-delivery technology. Although various biodegradable nanoparticles of natural polymers such as starch, chitosan, liposomes etc, are largely in use as drug carriers in con- trolled Drug-delivery technology (99). Many FDA-approved biodegradable and biocompatible polymers have been used in nanoparticle preparation. These include polylactide-polyglycolide copolymers, polyacrylates and polycaprolactones. Nanoparticles can be prepared from polymerization of monomers or from preformed polymer with the possibility of performing many chemical modifications. The polymerization reaction in these systems generally occurs in two steps: a nucleation phase followed by a growth phase and the process can be carried out in two ways either as emulsion polymerization or as interfacial polymerization. When nanoparticle preparation involves polymerization, it is undesirable to have residual monomers and initiators in the final nanoparticle formulation. A critical step of the process is the purification and removal of residual monomers. It is also very important to separate free drugs from the drug loaded nanoparticle suspension. A potential challenge for polymeric nanoparticles is associated with residues from organic solvents and polymer toxicity. If the drug to be incorporated in nanoparticles is hydrophobic, the drug is dissolved or dispersed into the polymer solution. The polymer solution is then added to an aqueous solution, followed by high-speed homogenization or sonication to form an oil-in-water emulsion. Nanoparticle preparation is usually facilitated and stabilized with the aid of an emulsifier or stabilizer. If the drug to be incorporated in nanoparticles is hydrophilic, the drug is added to the aqueous phase and entrapped into nanoparticles through a double emulsification method to form water-in-oil-in-water emulsion Majuru and Oyewumi (70). Residual organic solvent can be removed by evaporation or a decreased pressure or under a vacuum environment with or without the aid of inert gas flow. Solid nanoparticles are cured from the suspension by centrifugation, filtration or freeze drying. Another method is based on particle precipitation upon addition of a non-solvent to polymer solution under mechanical stirring. This method allows the formation of nanoparticles without prior emulsification. Nanoparticle formation and characteristics are dependent on the choice of the polymer/solvent/non-solvent system that will ensure mutual miscibility of the solvent and non-solvent of the polymer, Majuru and Oyewumi (70). Nanoparticles can also be prepared from natural macromolecules using methods such as thermal denaturation of proteins (such as albumin) or gelification process such as in alginates. In general, the controlling factors in the nanoparticle formulation process, which are adjustable for an ideal design, are the polymer type and its molecular weight, the copolymer blend ratio, the type of organic solvent, the drug loading level, the emulsifier/stabilizer and oil–water phase ratio, the mechanical strength of mixing, the temperature and the pH. These authors have opined that, in production of a drug product it is important to set a limit for residual solvent in the formulation that is based on the acceptable daily intake and to develop analytical methods for testing of the solvent levels in

the nanoparticles. Table 4 below shows some of the applications of polymeric materials in nanodrug delivery.



COLLOID BASED DELIVERY FOR THERAPEUTICS

Delivery system type	Typical mean particle diameter (in micrometers)	Representative systems of each type	Characteristic applications
Microspheres, Hydrogels	0.5-20	Alginate, gelatin, chitosan, polymeric hydrogels	Sustained release of therapeutics
Microparticles	0.2-5	Polystyrene, polylactide microspheres.	Targeted delivery of therapeutics
Emulsions, Microemulsions	0.15-2	o/w, w/o, lipid emulsions, o/w microemulsions.	Control and targeted delivery of therapeutics
Liposomes	30-1000	Phospholipid and polymer based bilayer vesicles.	Targeted delivery of therapeutics
Micelles	3-80	Natural and synthetic surfactant micelles.	Targeted delivery of therapeutics
Nanoparticles	2-100	Lipid, Polymer, Inorganic nanoparticles.	Targeted delivery of therapeutics, in vivo navigational devices
Nanocrystals	2-100	Quantum dots	Imaging agents

Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

Table 4. Polymer colloids for nanodrug delivery

6.3. Polymers for gene delivery

The delivery of nucleic acid into cells in vitro and in vivo is a critical technique for the study of genes and development of potential gene therapies. To fully utilize this potential, safer and more efficient vectors for delivery of genes are required. Current nucleic acid delivery falls into two major categories, viral and nonviral. In nonviral gene delivery, cationic lipids or polymers are used to both protect nucleic acids from degradation and facilitate entry into the target cells. The resulting complexes self-assemble via electrostatic interactions to form stable aggregates. Recent reports have discussed the promise of lipid-DNA (lipoplex) and polycation-DNA complexes (polyplexes) (77) as potential therapeutics, including recent efforts to incorporate bioresponsive chemistries for increased effectiveness. Successful gene transfer requires sufficient stability of DNA during the extracellular delivery phase,

transportation through cell membranes and cytoplasm, and eventual disassembly and nuclear delivery. A molecular architecture that achieves all the requirements will most likely consist of a virus like layered structure incorporating several components. Though nonviral gene vectors can be efficient in vitro and in vivo, their uncontrolled and often undefined interactions under physiological conditions still represent a major obstacle to their use in gene therapy. In particular, it has been shown that nonviral gene vectors or their constituents interact strongly with negatively charged serum proteins and other blood components. Such opsonization alters the physicochemical characteristics of vectors, may interfere with vector targeting, and is of concern if vectors are to be applied in humans. Consequently, one major objective in nonviral vector development is to devise vectors that are inert in the in vivo environment during the delivery phase. Poly (ethylene glycol) (PEG) has often been used to confer to these drug carriers the desired stability during the extracellular delivery phase. The incorporation of PEG to lipo- or polyplexes has been proven effective in reducing undesired effects such as immune response, unspecific interactions, and degradation. PEGylation can be implemented by using PEGylated components in the initial complex formation. Alternatively, PEG shielding can be applied to preformed complexes in a secondary processing step by using either electrostatic self-assembly or chemical grafting. While PEGylation is a necessity to improve extracellular stability and circulation half-life, it often decreases the transfection efficiency due to reduced specificity and inhibited cell association and uptake. Incorporating receptor targeting or using bioresponsive linkers to release PEG have proven useful to overcome these intracellular barriers to efficient delivery (100). Previous work (100) with a copolymer-protected gene vector (COPROG), consisting of a branched polyethylenimine (bPEI)/ DNA polyplex subsequently shielded with a copolymer consisting of both PEG and anionic peptides (P6YE5C), showed the presence of the copolymer, which provides steric stabilization, protection from opsonization, and allows freeze-drying of the vector with little loss of activity. COPROG particles have proven to be effective gene delivery vectors with decreased cellular toxicity without impairing gene transfer. The decreased toxicity of COPROG is likely a result of the removal of unbound polycation by the excess anionic copolymer emphasizing the potential role of binding stoichiometry in three-component complexes. Likely due to their stabilizing and opsonization- inhibiting properties, COPROGs have proven advantageous in promoting the tranfection capacity of polyplex-loaded sponges upon subcutaneous implantation, and when colyophilized with fibrinogen, are a simple means to achieve an injectable fibrin gene-activated matrix (100). At the level of research, many synthetic DNA particles have been prepared for transfection in cell cultures and in animal studies. However, several authors (70).are of the opinion that, certain issues must be addressed in the development of DNA particles with cationic polymers. These are (i) potential toxicity of cationic polymers especially when administered at high concentrations; (ii) instability of particles on storage; (iii) instability of DNA particle size and particle size distribution leading to undesirable particle aggregation; (iv) poor transfection efficiency; (v) poor stability in blood circulation; and (vi) high cost of scaling up the process to achieve reproducible product quality.

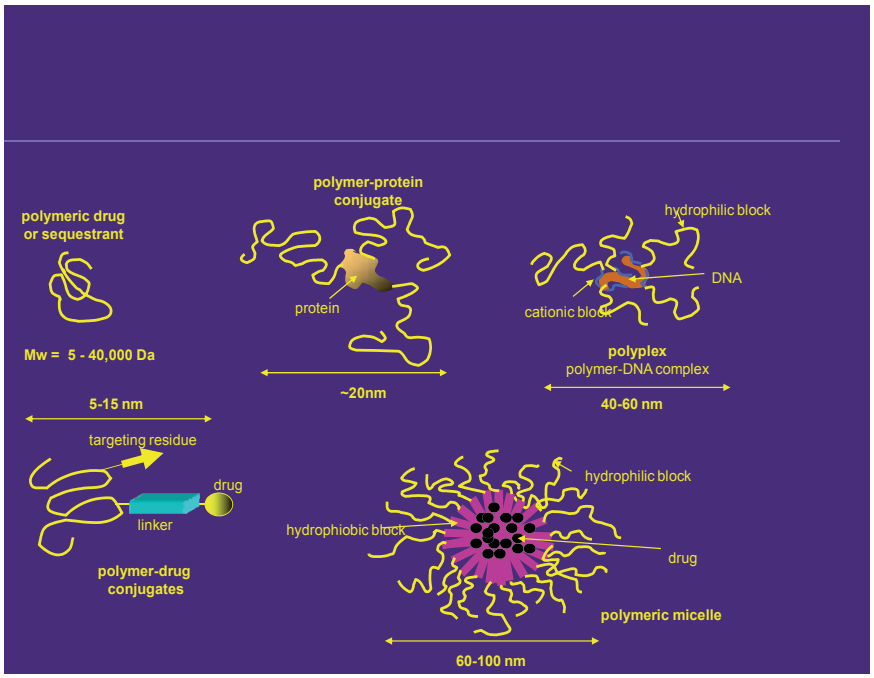


Figure 8. Nanosize medicines

6.4. Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are particles made from solid lipids with mean diameters ranging between 50–1000nm and represent an alternative to polymeric particulate carriers. The main advantage offered by lipid carriers in drug delivery is the use of physiological lipids or lipid molecules with a history of safe use in human medicine, which can decrease the danger of acute and chronic toxicity (101). Sufficient data are available for the use of drug-loaded lipid nano and microparticles for oral delivery, the main mechanism of lipid particulate materials translocation across the intestine being the uptake via Peyer's patches. Research reported in literature (102) shows that SLN constituted of stearic acid and phosphatidylcholine were evidenced in lymph and blood after duodenal administration to rats: the small diameters of SLN may facilitate their uptake by the lymphatics. Up until today, only a few methods are described in the literature for SLN preparation, including high pressure hot homogenization and cold homogenization techniques (101) microemulsion-based preparation and solvent emulsification/evaporation method. Particularly, the emulsification/evaporation method concerns the preparation of nanoparticles dispersions from O/W emulsions: the lipophilic material is dissolved in a water-immiscible organic solvent that is emulsified in an aqueous phase. Upon evaporation of the solvent, a nanoparticle dispersion is formed by precipitation of the lipid in the aqueous medium. Depending on the

composition and the concentration of the lipid in the organic phase, very low particle sizes can be obtained, ranging from 30–100nm, but a clear disadvantage of this method is the use of organic solvents, whose toxicity cannot always be neglected. Recently, an emulsification–diffusion technique was developed using non-toxic and physiologically compatible solvents and monoglycerides or waxes as components of the disperse phase of oil-in-water emulsions obtained at 50 °C (103). The solvent-in-water emulsion–diffusion technique was before described in the literature mostly for the obtainment of polymeric micro- and nanoparticles and only a few authors proposed its application in the production of SLN (101). According to the moderate water solubility of the solvents employed, the dilution of the emulsions determined the diffusion of the organic solvent from the droplets to the continuous phase with the consequent instant solidification of lipophilic material. The emulsion compositions and process parameters used were the results of a formulative study aimed to develop optimized nanosphere formulations, whose mean sizes were below 200nm. In a separate report (104), the possibility of incorporating a peptide drug such as insulin in the SLN obtained with the developed method was considered, aiming to protect it from chemical and enzymatic degradation, as it is well-known that the incorporation of peptides in polymeric or non-polymeric particles should exert a certain protection of the drug against the proteolytic enzymes present in the gastrointestinal tract (101). Indeed, the use of lipids as matrix materials for sustained-release formulations for peptides and proteins has been reported only by few authors (101), owing to the hydrophobic nature of the lipid matrix that can be more appropriate to incorporate lipophilic drugs rather than hydrophilic proteins. An adequately high solubility of the drug in the lipid melt is therefore the pre-requisite to obtain a sufficient SLN loading capacity. Considering that the solubility of insulin in most commonly employed solvents and lipids is quite low, a specific solvent medium of the peptide was required. Isobutyric acid, a partially water-miscible solvent with low toxicity, revealed a totally unexpected high insulin-solubilization capacity at 50 °C, further increasing when the solvent was water-saturated (104). Solid lipid insulin-loaded microparticles were therefore produced using isobutyric acid as a solvent. Preliminary analysis of microparticles content after processing showed an insulin-high encapsulation efficiency; moreover, insulin in SLN did not undergo any chemical modification and its in vitro release from the microparticles was very low, with an initial burst effect of 20% of the dose.

Of recent, SLN has become a popular drug delivery system for ophthalmic application. It is gaining prominence as promising approach to improve the poor ocular bioavailability of biomolecules. In particular, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), regarded as the first and second generation of lipid nanoparticles are currently being applied.(105, 106) NLC was developed due by combining the advantages of SLN and avoidance of their limitations such as low drug loading capacity, poor long-term stability and early drug expulsion caused by lipid polymorphism.(107, 108) NLC consist of a mixture of spacially different solid and liquid lipids molecules, resulting in a structure with more imperfections in crystal lattice to accommodate drugs.(109, 110) As

drug delivery devices, NLC show great promise for the eye, due to their better biocompatibility, modified drug release kinetics, reduction of drug leakage during storage, avoidance of organic solvents during production process and feasibility of large scale production.(111, 112)

To prepare particles using the homogenization method, the drug is dissolved or solubilized in the lipid that has been melted and heated to a temperature approximately 5–10 ^{o}C above its melting point. For the hot homogenization technique, the drug dissolved in the lipid melt is dispersed under stirring in a hot aqueous surfactant solution of identical temperature. The obtained pre-emulsion is homogenized to produce nanoemulsions that are subsequently cooled to room temperature. Solid lipid nanoparticles are obtained upon lipid recrystallization at room temperature. Some of the process variables that will affect the particle size of nanoparticles as well as drug loading are (i) the type of homogenization technique; (ii) speed of homogenization; and (iii) rate of cooling in hot homogenization. Cold homogenization is applied for highly temperature-sensitive drugs and hydrophilic drugs. For the cold homogenization technique the drug containing lipid melt is cooled and ground to obtain lipid particles. The lipid particles are dispersed in a cold surfactant solution that is homogenized at or below room temperature. The process avoids or minimizes the melting of lipids and therefore minimizing the loss of hydrophilic drugs to the water surface. Solid lipid nanoparticles can also be prepared by using microemulsions as precursors.

7. Characterization of nanoparticles

Measurement of nanoparticles can be carried out by photon correlation spectroscopy (PCS) or dynamic light scattering (DLS). Photon correlation spectroscopy determines the hydrodynamic diameter of the nanoparticles by Brownian motion. In addition to the size, other important properties of nanoparticles include (i) density, molecular weight and crystallinity which can affect drug release and degradation; (ii) surface charge and hydrophobicity which may significantly influence nanoparticle behavior after administration. Zeta potential is also one of the properties of nanoparticles, and this is usually employed to measure the cell-surface charge density. The surface and bulk morphology are also important in determining the drug release kinetics of nanoparticles. Nanoparticles are usually visualized by scanning electron microscope (SEM) and atomic force microscopy (AFM). Thermal profile of nanoparticles can be studied using the differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), while crystallinity can be monitored by x-ray crystallography. Other parameters which should normally be evaluated include; drug encapsulation efficiency; physical properties such as size, shape, stability of size, drug loading as well as drug release.

The common techniques for characterization as summarized by B. K. Nanjwade is shown in Tabs 5 – 8 below:

Morphology

Properties	Common Techniques
Size (primary particle)	TEM, SEM, AFM, XRD
Size (primary/aggregate/agglomerate)	TEM, SEM, AFM, DLS, FFF, AUC, CHDF, XDC, HPLC, DMA(1)
Size distribution	EM, SEM, AFM, DLS, AUC, FFF, HPLC, SMA
Molecular weight	SLS, AUC, GPC
Structure/Shape	TEM, SEM, AFM, NMR
Stability (3D structure)	DLS, AUC, FFF, SEM, TEM

Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

Table 5.

Surface

P roperties	Common Techniques
Surface area	BET
Surface charge	SPM, GE, Titration methods
Zeta potential	LDE, ESA, PALS
Surface coating composition	SPM, XPS, MS, RS, FTIR, NMR
Surface coating coverage	AFM, AUC, TGA
Surface reactivity	Varies with nanomaterial
Surface-core interaction	SPM, RS, ITC, AUC, GE
Topology	SEM, SPM, MS

Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

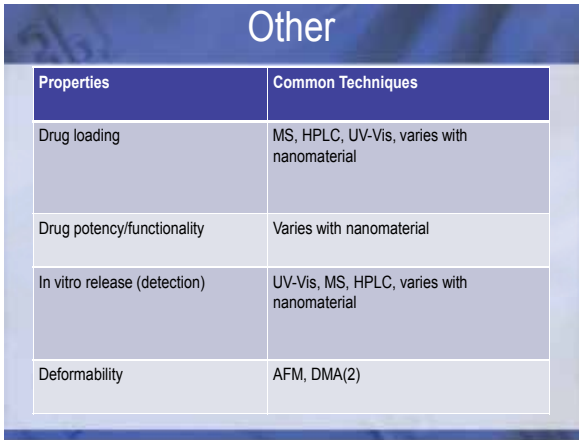
Table 6.

Chemical

Properties	Common Techniques
Chemical composition (core, surface)	XPS, MS, AAS, ICP-MS, RS, FTIR, NMR
Purity	ICP-MS, AAS, AUC, HPLC, DSC
Stability (chemical)	MS, HPLC, RS, FTIR
Solubility (chemical)	Varies with nanomaterial
Structure (chemical)	NMR, XRD
Crystallinity	XRD, DSC
Catalytic activity	Varies with nanomaterial

Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

Table 7.



Source: B. K. Nanjwade *api.ning.com/.../DevelopmentofNanotechnologyBasedDrugsanditsGu*

Table 8.

8. Nanoparticle application in non-parenteral applications

8.1. Oral administration

For many reasons, oral drug delivery continues to be the preferred route of drug administration. It is the oldest and the commonest mode of drug administration as it is safer, more convenient, does not need assistance, non-invasive, often painless, the medicament need not be sterile and so is cheaper (113). However, the oral route is not suitable for drugs that are poorly permeable or easily degradable in the gastrointestinal tracts (GIT). For instance, delivery of proteins and peptides via the oral route will be greatly impacted by barriers such as (i) epithelial cell lining; (ii) the mucus layer; (iii) proteolytic enzymes in the gut lumen (such as pepsin, trypsin and chymotrypsin); and (iv) proteolytic enzymes (endopeptidases), at the brush border membrane. Drug loaded in nanoparticles will be protected from the enzymatic degradation along the GIT providing the potential benefit of enhanced absorption. It has been reported (70) that, particulate absorption takes place mainly at the intestinal lymphatic tissues (the Peyer's patches). The epithelial cell layer overlying the Peyer's patches contains Mcells. The differences between absorptive enterocytes and M cells are expressed in that M cells have (a) underdeveloped microvillous and glycocalyx structures, (b) apical microfolds, (c) increased intracellular vacuolization and (d) absence of mucus. The follicle-associated epithelia (FAE) are made up of the M cells and absorptive enterocytes (70). It has been reported that, the FAE and Mcells are predominantly responsible for particle uptake along the GIT. In this regard, nanotechnology is reportedly gaining attention in the development of proteins, peptides and DNA delivery systems (70).

8.2. Pulmonary administration

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 interception of capillary bed in the lungs. If a drug is prepared as microspheres in the size range of 7–25 μm, the microspheres can be concentrated in lung through i.v. administration (114). This technique can improve pulmonary drug concentration to maximize its effectiveness against some pulmonary infections such as mycoplasmal pneumonia and minimize the adverse side effects. The final nanoparticulate formulation may be administered either as a nebulizer (metered dose inhalers) or dry powder inhalers. Local delivery of drugs to the lung is desirable for many clinical conditions such as asthma; chronic pulmonary infections, lung cancer and cystic fibrosis. For both local and/or systemic delivery, the effectiveness of drug delivery by inhalation may be greatly imparted by mucociliary clearance (70). Studies have shown that nanoparticles may facilitate transport of drugs to the epithelium while avoiding undesirable mucociliary clearance. Other benefits of nanoparticle-based formulations are in the suitability for (i) sustained drug effect due to possible prolonged residence of drug at the site of action or absorption; (ii) controlled or targeted drug delivery. The small size of nanoparticles makes them highly suitable for pulmonary delivery because they can easily be air borne and delivered to the alveolus. It is important that the components of the nanoparticle formulation are biodegradable to avoid accumulation in the lungs and that they do not cause irritation of the air ways and lung tissue. The control of the particle size of the formulation during manufacture and the entire shelf life of the drug product is also very important for an acceptable product (70).

8.3. Topical administration

As noted by Majuru and Oyewumi (70) the feasibility of applying nanoparticles in topical/cosmetic preparations has been a subject of several commentaries. In any case, this dosage form utilizes the advantages of nanoparticles such as (a) protection of labile compounds; (b) controlled release of incorporated drugs; (c) ability of solid lipid nanoparticles to act as occlusive to increase the water content of the skin; and (d) ability of nanoparticles to serve as physical barriers on the skin for blocking UV light and, as such, for use in sunscreen formulations.

9. Conclusions

Drug Delivery scientists are searching for the ideal nanovehicle for the ideal nanodrug delivery system; one that would dramatically reduce drug dosage, such that, there is improvement in the absorption of the drug, 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 desired site and limit or eliminate side effects. As it stands today, the scope of this emerging field seems to be limitless. However, considerable technological and financial obstacles still need to be properly addressed by both the private sector and governments before nanotechnology's full promise can be realized. Ranking highest among the challenges is the need to develop and perfect reliable

techniques to produce nanoscale particles that does not just have the desirable particle sizes, but also minimal structural defects and acceptable purity levels. This is because these attributes can drastically alter the anticipated behavior of the nanoscale particles. Moving today's promising nanotechnology-related developments from laboratory- and pilot-scale demonstrations to full-scale commercialization is still a big challenge and nanotechnology scientists must gear up to these challenges. Nanotechnology, deals with the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanometer scale. Two principal factors cause the properties of nanomaterials to differ significantly from other materials: increased relative surface area, and quantum effects. These factors can change or enhance properties such as reactivity, strength, electrical characteristics as well as most of the biomedical properties. Nanotechnology in biomedical sciences is expected to create innovations and play a vital role, not only in drug delivery and gene therapy, but also in molecular imaging, biomarkers and biosensors. Today the application of nanotechnology in drug delivery is widely expected to change the landscape of pharmaceutical and biotechnology industries for the foreseeable future. Target-specific drug therapy and methods for early diagnosis of pathologies are the priority research areas where nanotechnology would play a prominent role. Using nanotechnology, it may be possible to achieve (1) improved delivery of poorly water-soluble drugs; (2) targeted delivery of drugs in a cell- or tissue-specific manner; (3) transcytosis of drugs across tight epithelial and endothelial barriers; (4) delivery of large macromolecule drugs to intracellular sites of action; (5) co-delivery of two or more drugs or therapeutic modality for combination therapy; (6) visualization of sites of drug delivery by combining therapeutic agents with imaging modalities; and (7) real-time read on the in vivo efficacy of a therapeutic agent. For example, with more than 10 million new cases every year, cancer has become one of the most devastating diseases worldwide, yet, the most common cancer treatments are limited to chemotherapy, radiation, and surgery. Although conventional treatment options such as chemotherapy and radiation have experienced many advances over the past decades, cancer therapy is still far from optimal. Frequent challenges encountered by the current cancer therapies include nonspecific systemic distribution of antitumor agents, inadequate drug concentrations reaching the tumor, and the limited ability to monitor therapeutic responses. Poor drug delivery to the target site leads to significant complications, such as multidrug resistance, here nanodrug delivery holds great promise. A large number of nanocarriers have been designed for delivery of peptides via liposomes, noisome, polymeric nanoparticles, solid lipid nanoparticles etc. Polymeric based nanoparticles have taken much attention for safe and effective delivery of proteins. Nanoparticles prepared, particularly in the size range from 10 nm to 100 nm, are considered optimal for cancer therapeutics. Thus multifunctional nanoparticles combining different functionalities like targeting, imaging and therapy into one system can be used for effective cancer treatment. Multifunctional nanoparticles hold great promise for the future of cancer treatment because they can detect the early onset of cancer in each individual patient and deliver suitable therapeutic agents to enhance therapeutic efficacy. The combination of tumor-

targeted imaging and therapy in an all-in-one system provides a useful multimodal approach in the battle against debilitating health conditions like cancer. Despite the promise of many of the early nanotechnology-related breakthroughs, the ability to develop cost-effective, commercially and technically viable applications for these laboratory wonders will ultimately be predicated on the research community's ability to bridge the gap—some might say chasm—between the science involved and engineering required, particularly during scale up.

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Chapter 5

Lipid Nanoparticulate Drug Delivery Systems: A Revolution in Dosage Form Design and Development

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Additional information is available at the end of the chapter

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1. Introduction

Nanoparticulate drug delivery systems (DDS) have attracted a lot of attention because of their size-dependent properties. Among the array of nanoparticles being currently investigated by pharmaceutical scientists, lipid nanoparticles have taken the lead because of obvious advantages of higher degree of biocompatibility and versatility. These systems are commercially viable to formulate pharmaceuticals for topical, oral, pulmonary or parenteral delivery. Lipid nano formulations can be tailored to meet a wide range of product requirements dictated by disease condition, route of administration and considerations of cost, product stability, toxicity and efficacy. The proven safety and efficacy of lipid-based carriers make them attractive candidates for the formulation of pharmaceuticals, as well as vaccines, diagnostics and nutraceuticals [1].

The most frequent role of lipid-based formulations has traditionally been to improve the solubility of sparingly water soluble drugs especially Biopharmaceutics Classification System (BCS) Classes II & IV drugs. However, the spectrum of applications for lipid-based formulations has widened as the nature and type of active drugs under investigation vary. Lipid-based formulations may also protect active compounds from biological degradation or transformation, that in turn can lead to an enhancement of drug potency. In addition, lipid-based particulate DDS have been shown to reduce the toxicity of various drugs by changing the biodistribution of the drug away from sensitive organs. This reduction in toxicity may allow for more drug to be administered and forms the basis for the current success of several marketed lipid-based formulations of amphotericin B (Ambisome®, Abelcet®) and doxorubicin (Doxil®, Myocet®) [1].

Rapid advances in the ability to produce nanoparticles of uniform size, shape, and composition have started a revolution in science. The development of lipid-based drug carriers

has attracted increased attention over the last decade. Lipid nanoparticles (e.g. solid lipid nanoparticles, SLNs) are at the forefront of the rapidly developing field of nanotechnology with several potential applications in drug delivery, clinical medicine and research, as well as in other varied sciences. Due to their size-dependent properties, lipid nanoparticles offer the possibility to develop new therapeutics that could be used for secondary and tertiary level of drug targeting. Hence, lipid nanoparticles hold great promise for reaching the goal of controlled and site specific drug delivery and has attracted wide attention of researchers.

At the turn of the millennium, modifications of SLN, nanostructured lipid carriers (NLC) and lipid drug conjugate (LDC)-nanoparticles were introduced [2, 3] in addition to liquid crystal DDS. These carrier systems overcome observed limitations of conventional SLN and more fluid lipid DDS. Compared to liposomes and emulsions, solid particles possess some advantages, e.g. protection of incorporated active compounds against chemical degradation and more flexibility in modulating the release of the compound. This paper focuses on the different lipid based nano systems, their structure and associated features, stability, production methods, drug incorporation and other issues related to their formulation and use in drug delivery. The following advantages among others, could be ascribed to lipid based nanocarriers:

- ability to control and target drug release.
- ability to improve stability of pharmaceuticals.
- ability to encapsulate high drug content (compared to other carrier systems e.g. polymeric nanoparticles).
- the feasibility of carrying both lipophilic and hydrophilic drugs.
- most of the lipids used are biodegradable, and as such they have excellent biocompatibility, are non-toxic, non-allergenic and non-irritating.
- they can be formulated by water-based technologies and thus can avoid organic solvents.
- they are easy to scale-up and sterilize.
- they are less expensive than polymeric/surfactant based carriers.
- they are easy to validate.

2. Drug delivery systems

A drug delivery system is defined as a formulation or a device that enables the introduction of a therapeutic substance in the body and improves its efficacy and safety by controlling the rate, time, and place of release of drugs in the body. The process of drug delivery includes the administration of the therapeutic product, the release of the active ingredients by the product, and the subsequent transport of the active ingredients across the biological membranes to the site of action. DDS interface between the patient and the drug. It may be a formulation of the drug or a device used to deliver the drug [4].

3. Lipids

The carboxylic acid group of a fatty acid molecule provides a convenient place for linking the fatty acid to an alcohol, via an ester linkage. If the fatty acid becomes attached to an

alcohol with a long carbon chain, the resultant substance is called a wax. When glycerol and a fatty acid molecule are combined, the fatty acid portion of the resultant compound is called an acyl group, and the glycerol portion is referred to as a glyceride. A triacylglyceride thus has three fatty acids attached to a single glycerol molecule. Sometimes, this name is shortened to triglyceride. Triglyceride substances are commonly referred to as fats or oils, depending on whether they are solid or liquid at room temperature [5]. A lipid is thus a fatty or waxy organic compound that is readily soluble in non-polar solvents (e.g. ether), but not in polar solvent (e.g. water). Examples of lipids are waxes, oils, sterols, cholesterol, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides (fats), and phospholipids. Fatty acids (including fats) are a subgroup of lipids, hence, it will be inaccurate to consider the terms synonymous.

3.1. Classification of solid lipids for delivery of bioactives

Lipids can be grouped into the following categories based upon their chemical composition.

3.1.1. Homolipids

Homolipids are esters of fatty acids with alcohols. They are lipids containing only carbon (C), hydrogen (H), and oxygen (O), and as such are referred to as simple lipids. The principal materials of interest for oral delivery vehicle are long chain and medium chain fatty acids linked to a glycerol molecule, known as triacylglycerols. The long-chain fatty acids ranging from C14 to C24 appear widely in common fat while the medium chain fatty acids ranging from C6 to C12 are typical components of coconut oil or palm kernel oil [6]. Examples of homolipids include: cerides (waxes e.g. beeswax, carnauba wax etc.), glycerides (e.g. fats and oils) and sterides (e.g. the esters of cholesterol with fatty acids).

3.1.2. Heterolipids

Heterolipids are lipids containing nitrogen (N) and phosphorus (P) atoms in addition to the usual C, H and O e.g. phospholipids, glycolipids and sulfolipids. They are also known as compound lipids. The emphasis here will be on the phospholipids only. Two main classes of phospholipids occur naturally in qualities sufficient for pharmaceutical applications. These are the phosphoglycerides and phosphosphingolipids. Some phosphosphingolipids such as ceramide are used mainly in topical dosage forms. Phospholipids can be obtained from all types of biomass because they are essential structural components in all kinds of membranes of living organisms [6].

3.1.3. Complex lipids

The more complex lipids occur closely linked with proteins in cell membranes and subcellular particles. More active tissues generally have a higher complex lipid content. They may also contain phospholipids. Complex lipids in this context include lipoproteins, chylomicrons, etc. Lipoproteins are spherical lipid-protein complexes that are responsible

for the transport of cholesterol and other lipids within the body. Structurally, lipoprotein consists of an apolar core composed of cholesterol esters or triacylglycerols, surrounded by monolayer of phospholipid in which cholesterol and one or more specific apoproteins are embedded e.g. chylomicrons and lipoproteins

3.2. Lipid drug delivery systems

Lipid-based DDS are an accepted, proven, commercially viable strategy to formulate pharmaceuticals for topical, oral, pulmonary or parenteral delivery. Lipid formulations can be tailored to meet a wide range of product requirements. One of the earliest lipid DDS-liposomes have been used to improve drug solubility. Currently, some companies have established manufacturing processes for the preparation of large scale batches of sparingly soluble compounds, often at drug concentrations several orders of magnitude higher than the nominal aqueous solubility because of the introduction of novel lipid-based DDS [1].

3.3. Lipid nanoparticulate drug delivery systems

Lipid nanoparticles show interesting nanoscale properties necessary for therapeutic application. Lipid nanoparticles are attractive for medical purposes due to their important and unique features, such as their surface to mass ratio that is much larger than that of other colloidal particles and their ability to bind or adsorb and carry other compounds. Lipid nano formulations produce fine dispersions of poorly water soluble drugs and can reduce the inherent limitations of slow and incomplete dissolution of poorly water soluble drugs (e.g. BCS II & IV drugs), and facilitate formation of solubilised phases from which drug absorption occurs. In any vehicle mediated delivery system (whether the vehicle is an emulsion, liposome, noisome or other lipidic systems), the rate and mode of drug release from the system is important in relation to the movement of the delivery system *in vivo*.

Lipid particulate DDS abound depending on their architecture and particle size. Due to the large number of administration routes available, these delivery systems perform differently depending on the formulation type and route of administration. Figure 1 shows some of the different lipid particulate DDS available.

3.3.1. Solid lipid nanoparticles (SLN)

SLN are particulates structurally related to polymeric nanoparticles. However, in contrast to polymeric systems, SLN can be composed of biocompatible lipids that are physiologically well tolerated when administered *in vivo* and may also be prepared without organic solvents. The lipid matrices can be composed of fats or waxes (homolipids) that provide protection to the incorporated bioactive from chemical and physical degradation, in addition to modification of drug release profile. Typical formulations utilize lipids such as paraffin wax or biodegradable glycerides (e.g. Compritol 888 ATO) as the structural base of the particle [7].

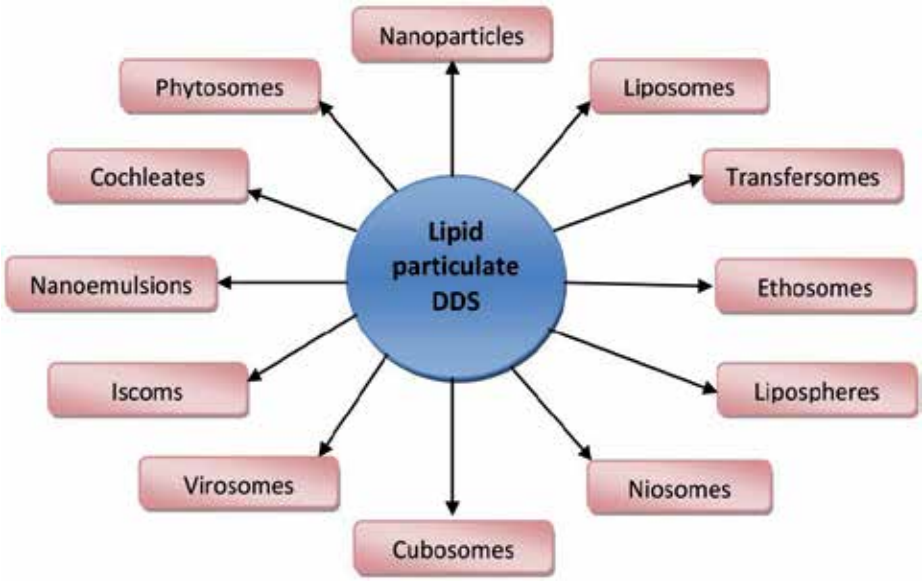

Figure 1. Lipid particulate drug delivery systems

SLN were developed in the 1990s as an alternative carrier system to the existing traditional carriers, such as emulsions, liposomes and polymeric nanoparticles. SLN are prepared either with physiological lipids or lipids with generally regarded as safe (GRAS) status. Under optimized conditions they can incorporate lipophilic or hydrophilic drugs and seem to fulfil the requirements for an optimum particulate carrier system [8]. SLN have a potential wide application spectrum- parenteral administration and brain delivery, ocular delivery, rectal delivery, oral delivery, topical delivery and vaccine delivery systems etc., in addition to improved bioavailability, protection of sensitive drug molecules from the outer environment and even controlled release characteristics. Common disadvantages of SLN are particle growth, unpredictable gelation tendency, unexpected dynamics of polymorphic transitions and inherent low incorporation rate due to the crystalline structure of the solid lipid [9].

3.3.2. 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 [10]. 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 are being developed.

3.3.3. Lipid drug conjugates (LDC)-nanoparticles

A major problem of SLN is the low capacity to load hydrophilic drugs due to partitioning effects during the production process. Only highly potent low dose hydrophilic drugs may be suitably incorporated in the solid lipid matrix [11]. In order to overcome this limitation, LDC nanoparticles with drug loading capacities of up to 33% were developed [8]. 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. to ester or ethers). The obtained LDC is then processed with an aqueous surfactant solution to nanoparticle formulation by high pressure homogenization (HPH). Such nanoparticles may have potential application in brain targeting of hydrophilic drugs in serious protozoal infections [12].

3.3.4. Liposomes

Liposomes are closed vesicular structures formed by bilayers of hydrated phospholipids. The bilayers are separated from one another by aqueous domains and enclose an aqueous core. As a consequence of this alternating hydrophilic and hydrophobic structure, liposomes have the capacity to entrap compounds of different solubilities. Additionally, the basic liposome structure of hydrated phospholipid bilayers is amenable to extensive modification or 'tailoring' with respect to the physical and chemical composition of the vesicle. This versatility has resulted in extensive investigation into the use of liposomes for various applications such as in radiology, cosmetology and vaccinology.

Liposomes used in drug delivery typically range from 25 nm to several micrometers and are usually dispersed in an aqueous medium. There are various nomenclatures for defining liposome subtypes based either on structural parameters or the method of vesicle preparation. These classification systems are not particularly rigid and a variation exists in use the of these terms, particularly with respect to size ranges. Liposomes are often distinguished according to their number of lamellae and size. Small unilamellar vesicles (SUV), large unilamellar vesicles and large multilamellar vesicles or multivesicular vesicles are differentiated. SUVs with low particle sizes in the nanometer range are of interest as liposomal nanocarriers for drug and antigen delivery [13, 14].

3.3.5. Transfersomes

Transfersome technology was developed with the intention of providing a vehicle to allow delivery of bioactive molecules through the dermal barrier. Transfersomes are essentially ultra-deformable liposomes, composed of phospholipids and additional 'edge active' amphiphiles such as bile salts that enable extreme distortion of the vesicle shape. The vesicle

diameter is in the order of 100 nm when dispersed in buffer [15]. These flexible vesicles are thought to permeate intact through the intact dermis under the forces of the hydrostatic gradient that exists in the skin [16]. Drug or antigen may be incorporated into these vesicles in a manner similar to liposomes.

3.3.6. Niosomes

Niosomes are vesicles composed mainly of non-ionic bilayer forming surfactants [17]. They are structurally analogous to liposomes, but the synthetic surfactants used have advantages over phospholipids in that they are significantly less costly and have higher chemical stability than their naturally occurring phospholipid counterparts [18]. Niosomes are obtained on hydration of synthetic non-ionic surfactants, with or without incorporation of cholesterol or other lipids. Niosomes are similar to liposomes in functionality and also increase the bioavailability of the drug and reduce the clearance like liposomes. Niosomes can also be used for targeted drug delivery, similar to liposomes. As with liposomes, the properties of the niosomes depend both on the composition of the bilayer and the method of production. Antigen and small molecules have also been delivered using niosomes [19, 20].

3.3.7. Liquid crystal drug delivery systems

The spontaneous self assembly of some lipids to form liquid crystalline structures offers a potential new class of sustained release matrix. The nanostructured liquid crystalline materials are highly stable to dilution. This means that they can persist as a reservoir for slow drug release in excess fluids such as the gastrointestinal tract (GIT) or subcutaneous space, or be dispersed into nanoparticle form, while retaining the 'parent' liquid crystalline structure. The rate of drug release is directly related to the nanostructure of the matrix. Lyotropic liquid crystal systems that commonly consist of amphiphilic molecules and solvents can be classified into lamellar (L_α), cubic, hexagonal mesophases, etc. In recent years, lyotropic liquid crystal systems have received considerable attention because of their excellent potential as drug delivery vehicles [21]. Among these systems, reversed cubic (Q_{II}) and hexagonal mesophases (H_{II}) are the most important and have been extensively investigated for their ability to sustain the release of a wide range of bioactives from low molecular weight drugs to proteins, peptides and nucleic acids.

3.3.8. Nanoemulsions

Lipid-based formulations present a large range of optional delivery systems such as solutions, suspensions, self-emulsifying systems and nanoemulsions. Among these approaches, oral nanoemulsions offer a very good alternative because nanoemulsions can improve the bioavailability by increasing the solubility of hydrophobic drugs and are now widely used for the administration of BCS class II and class IV drugs. Oral nanoemulsions use safe edible materials (e.g., food-grade oils and GRAS-grade excipients) for formulation of the delivery system. Nanoemulsions possess outstanding ability to encapsulate active

compounds due to their small droplet size and high kinetic stability [22, 23]. Nanoemulsions have sizes below 1 μm and have been extensively investigated as novel lipid based DDS [22] together with microemulsions.

3.4. Functional properties of lipids used in formulating lipid drug delivery systems

3.4.1. Crystallinity and polymorphism of lipids

Many pharmaceutical solids exist in different physical forms. It is well recognised that drug substances and excipients can be amorphous, crystalline or anhydrous, at various degrees of hydration or solvated with other entrapped solvent molecules, as well as varying in crystal hardness, shape and size. Amorphous solids consist of disordered arrangements of molecules and do not possess a distinguishable crystal lattice. In the crystalline state (polymorphs, solvates/hydrates, co-crystals), the constituent molecules are arranged into a fixed repeating array built of unit cells, which is known as lattice. Possession of adequate crystallinity is a prerequisite for a good lipid particulate DDS.

Triglycerides, which are mainly used as lipid matrices crystallize in different polymorphic forms. The most important forms are the α and β forms. Since the formulation of lipid particulate DDS may involve melting at some point, recrystallization from the melt results in the metastable α-polymorph, which subsequently undergoes a polymorphic transition into the stable β-form via a metastable intermediate form (β') [24]. The β-polymorph especially consists of a highly ordered, rigid structure with low loading capacity for drugs. The formation of all these polymorphic forms has been proved amongst solid triglyceride nanoparticles [25].

3.4.2. Melting characteristics of lipid matrices

A pure triacylglycerol has a single melting point that occurs at a specific temperature. Nevertheless, certain lipids contain a wide variety of different triacylglycerols, with different melting points and as a result, they melt over a wide range of temperatures, producing a wide endothermic transition in differential scanning calorimeter. High purity lipids with sharp melting transitions exclude drugs on recrystallization. In addition to the solidity or melting point of each individual triglyceride, in drug delivery, we are interested and concerned with the combination of triglycerides throughout the fat mixture. This impacts the plasticity and the melting point range. In the development of lipid nanoparticles, lipids with melting points well above the body temperature are preferred. This will enable among others, sustained release of the encapsulated drug.

3.5. Crystallinity and polymorphism vs drug loading capacity and drug release

Crystallinity and polymorphism have a lot of influence on some properties of lipid matrices used in lipid DDS. Parameters like drug loading capacity and drug release depend highly on the crystallinity and the polymorphic form of the lipids. The crystalline order and density

increase from α to β forms and are highest for the β-forms of polymorphic lipids [24]. An increasing crystalline order has a great impact on the drug loading capacity, since an increase in order reduces the ability to incorporate different molecules including drugs [24]. Hence, the drug loading capacity of the poorly organized polymorphic forms is high [26]. However, this advantage goes along with the particles being in a metastable form which are able to transform into the stable β-polymorph upon storage. As a consequence of this transformation, often drug expulsion occurs. The increasing order of the matrix also reduces the diffusion rate of a drug molecule within the particle and hence reduces the rate of drug release.

3.6. Strategies to improve drug loading in lipid particulate drug delivery systems

The high crystallinity of SLN leads to a rather low drug loading capacity for many drugs, a problem still being addressed. However, for lipophilic drugs the incorporation into the particles is much easier and often results in rather high drug loading. In order to overcome the disadvantage of low loading capacity, many investigations have been done. Müller *et al.* [10] introduced NLC. In these formulations, lipids of highly ordered crystalline structure are combined with chemically different lipids of amorphous structure, giving rise to structured matrices that accommodate more drug.

Friedrich *et al* [27] reported a different method to increase the drug payload by incorporating amphiphilic phospholipids into the lipid matrix. This resulted in a much higher solubility of the drug in the matrix, which was attributed to the formation of a solidified reverse micellar solution within the matrix. In this case, the nanoparticles were prepared by cold homogenization which may have prevented a redistribution of the lecithin to the surface of the particles or into the aqueous phase. Such a behaviour could be observed for a similar system after high pressure homogenization of the molten lipids [28].

Another mechanism of increasing the drug loading capacity of SLN has been recently developed [29-32]. In these works, the researchers used mixtures of solid lipids of natural origin possessing fatty acids of different chain lengths. In the analytical characterization of the lipid mixtures using differential scanning calorimetry (DSC), X-ray diffraction and isothermal microcalorimetery, it was observed that the mixtures were able to form matrices of imperfect structure composed of mixed crystals and mixtures of crystals, which enhanced drug incorporation compared with the single lipids.

3.7. Ingredients used in the formulation of lipid based particulate drug delivery systems

3.7.1. Emulsifiers

Emulsifiers are essential to stabilize lipid nanoparticle dispersions and prevent particle agglomeration. The choice of the ideal surfactant for a particular lipid matrix is based on the surfactant properties such as charge, molecular weight, chemical structure, and respective hydrophile-lipophile balance (HLB). The HLB of an emulsifier is given by the balance

between the size and strength of the hydrophilic and the lipophilic groups. Table 1 shows some of the emulsifiers employed in the production of lipid nanoparticles. The choice of the emulsifiers depends on the route of administration of the formulation, for e.g. for parenteral formulations, there are limits of the emulsifiers to be used [33]. For topical and ocular route the issue of skin sensitization has to be considered, while for oral route, the emulsifier should not produce any physiological effect at the use concentration. Emulsifiers could be used in combination to produce synergistic effect and better stabilize the formulation [22].

Emulsifiers/coemulsifiers	HLB	References
Lecithin	4-9	[9]
Poloxamer 188	29	[34]
Poloxamer 407	21.5	[35]
Polysorbate 20	16.7	[36]
Polysorbate 65	10.5	[7]
Polysorbate 80	15	[9]
Cremophor EL	12-14	[37]
Solutol HS 15	15	[28]

Table 1. Some emulsifiers used for the production of lipid nanoparticles

3.7.2. Lipids

The matrices for lipid nanoparticle preparation are natural, semi-synthetic or synthetic lipids which can be biodegradable, including triglyceride (tri-stearic acid, tri-palmitic acid, tri-lauric acid and long-chain fatty acid), steroid and waxes (e.g. beeswax, carnauba wax, etc) and phospholipids. They could be used singly or in combination. Lipids for the production of nanoparticles may be grouped into two: bilayer and non-bilayer lipids.

3.7.3. Bilayer lipids used in drug delivery

Some lipids are capable of adopting a certain orientation depending on the processing condition. Compounds that have approximately equal-sized heads and tails e.g. phospholipids (Figures 2 and 3) tend to form bilayers instead of micelles in aqueous system. In these structures, two monolayers of lipid molecules associate tail to tail, thus minimizing the contact of the hydrophobic portions with water and maximizing hydrophilic interactions [38] (Figure 3). The phospholipid molecules can move about in their half the bilayer, but there is a significant energy barrier preventing migration to the other side of the bilayer. Cholesterol can insert into the bilayer, and this helps to regulate the fluidity of the membrane. The self-assembled nature of lipid bilayers implies that they are normally in a tension-less state.

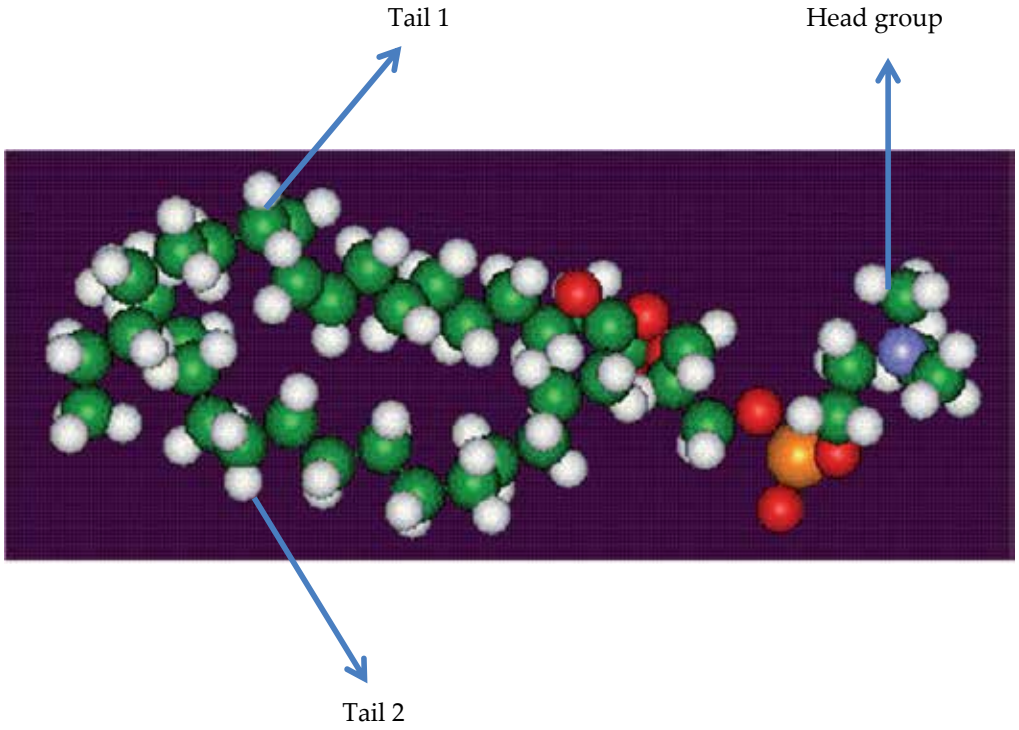

Figure 2. Structure of phospholipid (phosphatidylcholine) [Ref. 39].

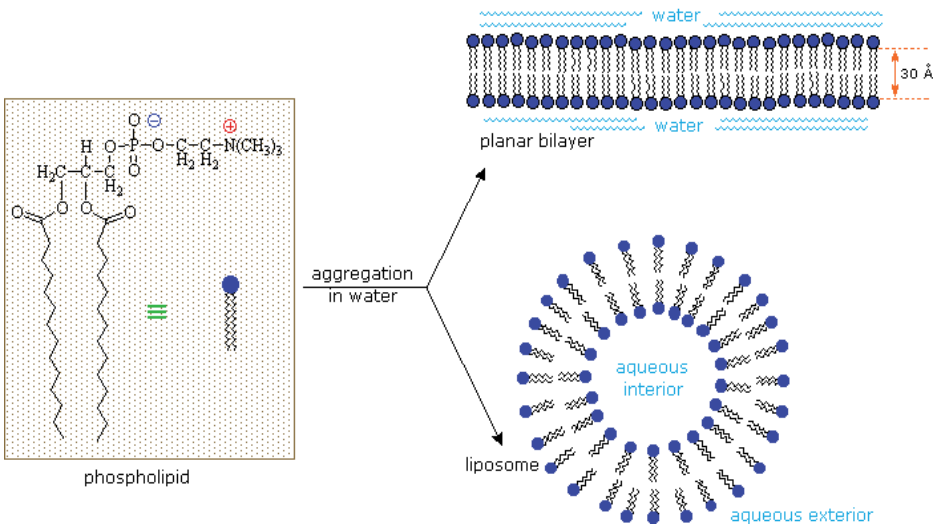

Figure 3. Interaction of phospholipid with water [38].

Phospholipids with certain head groups e.g. phosphatidylcholine (Figure 2) [39], can alter the surface chemistry of a lipid particle. The packing of phospholipid chains within the surface of the particle also affects its mechanical properties, including swelling, stretching,

bending and deformability. Many of these properties have been taken advantage of in the design of novel lipid particulate DDS such as surface modification for improved drug loading capacity [31, 32, 40]. Deformability has been utilized in the development of ultra-deformable liposomes termed transfersomal DDS (transfersomes). Cholesterol strengthens the bilayer but decrease its permeability. Bilayer lipids when present in lipid particulate DDS may define the boundaries of the particle and its environment (aqueous), and are often involved in many complex processes occurring at the interface.

3.7.4. Non-bilayer lipids used in drug delivery

In many biological systems, the major lipids are non-bilayer lipids, which in purified form cannot be arranged in a lamellar structure in the presence of aqueous systems. The structural and functional roles of these lipids in drug delivery are mainly in their utilization as matrix-forming lipids. They include such lipids as homolipids e.g. triglycerides and waxes. Their functional properties in lipid nanotechnology differs depending partly on their melting points, crystallinity and polymorphic characteristics. However, they may have absorption promoting properties especially for lipophilic drugs. Table 2 shows some of the non-bilayer lipids used in the formulation of lipid micro- and nanoparticles.

Hard fats e.g.	***Natural hard fats e.g.***
Stearic acid [Ref. 41] Palmitic acid [Ref. 42] Behenic acid [Ref. 43]	Goat fat [Ref. 45] Theobroma oil [Ref. 53]
Triglycerides e.g.	**Waxes e.g.**
Trimyristin (Dynasan 114) [Ref. 44] Tripalmitin (Dynasan 116) [Ref. 45] Tristearin (Dynasan 118) [Ref. 44] Trilaurin [Ref. 46]	Beeswax [Ref. 9] Cetyl palmitate [Ref. 47] Carnauba wax [Ref. 89]
Mono, di and triglycerides mixtures e.g.	
Witepsol bases [Ref. 48] Glyceryl monostearate (Imwitor 900) [Ref. 49] Glyceryl behenate (Compritol 888 ATO) [Ref. 50] Glyceryl palmitostearate (Precirol ATO 5) [Ref. 51] Softisan 142 and Softisan 154 [Refs. 52, 27]	

Table 2. Some non-bilayer lipids used in the formulation of lipid nanoparticles

3.8. Characterization and selection of lipids for particulate drug delivery systems

There is an increasing interest in lipid-based DDS due to factors such as better characterization of lipidic excipients and formulation versatility and the choice of different DDS. Apart from the fatty acid profile of previously undefined lipids, many different

analytical procedures are used to characterize lipids. These technologies provide different scales of analysis and may be used in combination to select the appropriate lipid matrix for use in formulation. DSC is the most widely used thermo-analytic technique for studying fats, oils and their mixtures. It gives information about the temperatures and energy associated with their fusion and crystallization, phase behaviour, polymorphic transformations, and data to estimate solid fat contents. DSC reports the destruction of structures in recordings obtained in a permanent out-of-equilibrium state. X-ray diffraction (small angle X-ray diffraction, SAXD and wide angle X-ray diffraction, WAXD) is also an essential tool for elucidating properties of fats and their mixtures. However, it complements DSC. XRD recordings provide both short and long spacings at a given temperature at which the sample is supposed to be in equilibrium. Since lipid systems are quite sensitive to their preparation history, only simultaneous recordings of SAXD, WAXD, and DSC circumvent the problem of reproducibility and guarantee identical conditions for all three measurements whatever may be the thermal treatment of the sample. Polarized light microscopy (PLM) is an analytical technique used in characterization of fats to observe the microstructures of the various polymorphic forms of fats. With a hot stage coupled, it is used to observe the microstructural changes in fats during melting, as the lipid passes from crystalline phase to isotropic phase, to visualize crystallization from isotropic melts and to visually detect undissolved drug crystals in the lipid matrix.

Isothermal microcalorimetry (IMC) is a recent and an important tool in studying the time dependent crystallization of lipids and lipid matrices. It has been applied in both pure and mixed systems. Isothermal crystallization kinetics studies of mixtures of lipids using IMC also address the question of how the crystallinity of one component affects the crystallization behaviour of the other [29, 52, 53]. Atomic force microscopy (AFM) can provide invaluable information about the physicochemical characteristics of the carriers that play an important role in determining the performance of the DDS. A lot of this information cannot be obtained from other characterization techniques due to the unique ability of the atomic force microscope to probe nanometer scale features at the molecular level.

3.9. Preparation of lipid nanoparticulate drug delivery systems

There many methods for the preparation of lipid nanoparticulate DDS. The method used is dictated by the type of drug especially its solubility and stability, the lipid matrix, route of administration, etc. Liposomal preparation follows a different method as described by Mozafari [54]. In this section, emphasis was laid on the production of SLN, NLC and LDC-nanoparticles, with methods that can also be applied to the formulation of liquid crystal DDS.

3.9.1. High pressure homogenization

High pressure homogenisation (HPH) is a suitable method for the preparation of SLN, NLC and LDC-nanoparticles and can be performed at elevated temperature (hot HPH technique)

or at and below room temperature (cold HPH technique) [11, 55]. The particle size is decreased by impact, shear, cavitation and turbulence. Briefly, for the hot HPH, the lipid and drug are melted (approximately 10 °C above the melting point of the lipid) and combined with an aqueous surfactant solution at the same temperature. A hot pre-emulsion is formed by homogenisation (e.g. using Ultra-Turrax). The hot pre-emulsion is then processed in a temperature-controlled high pressure homogenizer at 500 bar (or more) and predetermined number of cycles. The obtained nanoemulsion recrystallizes upon cooling down to room temperature forming SLN, NLC or LDC-nanoparticles. The cold HPH is a suitable technique for processing heat-labile drugs or hydrophilic drugs. Here, lipid and drug are melted together and then rapidly ground under liquid nitrogen forming solid lipid microparticles. A pre-suspension is formed by homogenisation of the particles in a cold surfactant solution. This pre-suspension is then further homogenised in a HPH at or below room temperature at predetermined homogenisation conditions to produce SLN, NLC or LDC-nanoparticles. The possibility of a significant increase in temperature during cold homogenisation should be borne in mind. Both HPH techniques are suitable for processing lipid concentrations of up to 40% and generally yield very narrow particle size distributions [56]. A schematic representation of HPH method of lipid particle preparation is shown in Figure 4.

3.9.2. Production of SLN via microemulsions

Gasco [57] developed and optimised a suitable method for the preparation of SLN via microemulsions. In a typical process, a warm microemulsion is prepared and thereafter, dispersed under stirring in excess cold water (typical ratio about 1:50) using a specially developed thermostated syringe. The excess water is removed either by ultra-filtration or by lyophilisation in order to increase the particle concentration. Experimental process parameters such as microemulsion composition, dispersing device, effect of temperature and lyophilisation on size and structure of the obtained SLN should be optimized. The removal of excess water from the prepared SLN dispersion is a difficult task with regard to the particle size. Also, high concentrations of surfactants and cosurfactants (e.g. butanol) are necessary for the formulation, but less desirable with respect to regulatory purposes and application.

3.9.3. SLN prepared by solvent emulsification/evaporation

For the production of nanoparticle dispersions by solvent emulsification/evaporation, the lipophilic material is dissolved in water immiscible organic solvent (e.g. cyclohexane) that is emulsified in an aqueous phase [58]. Upon evaporation of the solvent, nanoparticle dispersion is formed by precipitation of the lipid in the aqueous medium. Siekmann and Westesen [59] produced cholesterol acetate nanoparticles with a mean size of 29 nm using solvent emulsification/evaporation technique.

Other methods of lipid nanoparticle preparation include phase inversion and supercritical fluid (SCF) technology.

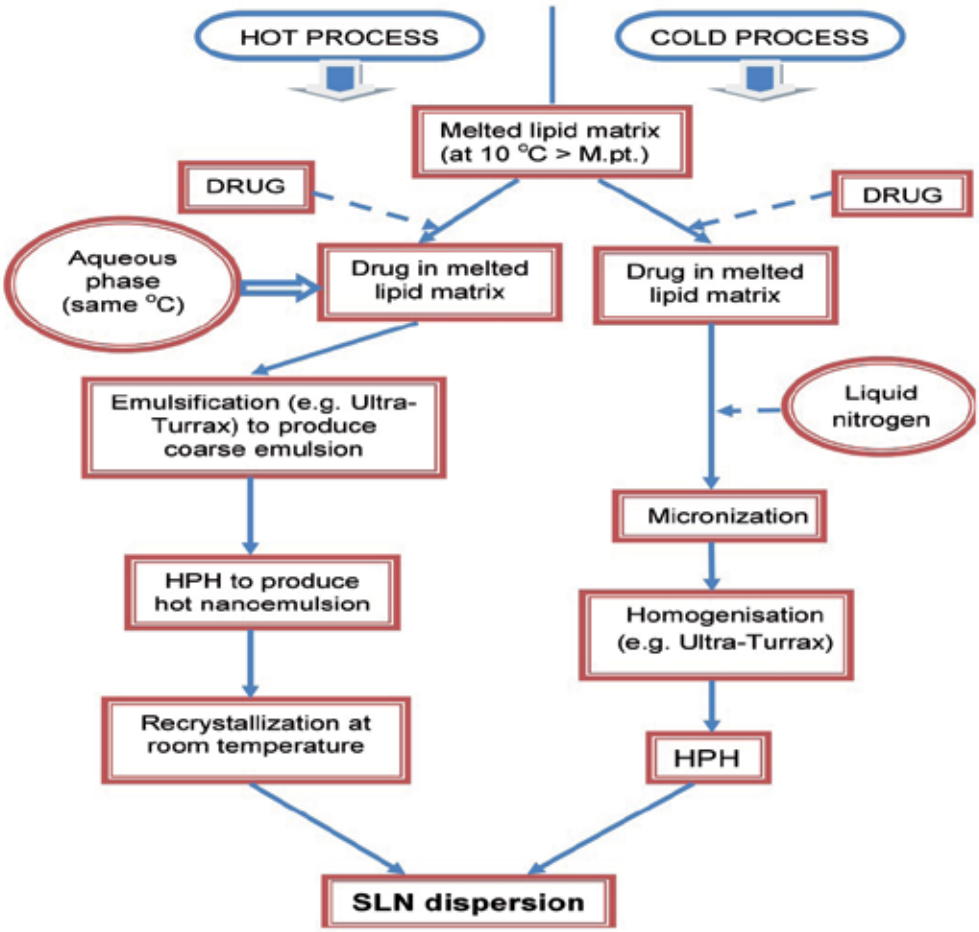

Figure 4. Production of lipid nanoparticles (SLN) by high pressure homogenisation (HPH)

3.10. Characterization of lipid nanoparticle quality

Quantitative analysis of particle characteristics such as morphological features can be very informative as biophysical properties are known to influence biological activity, biodistribution and toxicity. Several techniques are often used to assess nanoparticle characteristics such as lamellarity, size, shape and polydispersity [60]. Adequate and proper characterization of the lipid nanoparticles is necessary for quality control. However, characterization of lipid nanoparticles is a serious challenge due to the colloidal size of the particles and the complexity and dynamic nature of the delivery system.

The important parameters which need to be evaluated for the lipid nanoparticles are particle size, size distribution kinetics (zeta potential), degree of crystallinity and lipid modification (polymorphism), coexistence of additional colloidal structures (micelles, liposome, super cooled melts, drug nanoparticles), time scale of distribution processes, drug content

(encapsulation efficiency and loading capacity), *in vitro* drug release and surface morphology.

Particle size and size-distribution may be studied using photon correlation spectroscopy (PCS) otherwise known as dynamic light scattering (DLS), static light scattering (SLS), transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), scanning tunneling microscopy (STM), freeze fracture electron microscopy (FFEM) or cryoelectron microscopy (Cryo-EM). These microscopy techniques are also used to study the morphology of nanoparticles.

Among the imaging techniques, AFM has been widely applied to obtain the size, shape and surface morphological information on nanoparticles. It is capable of resolving surface details down to 0.01 nm and producing a contrasted and three-dimensional image of the sample. X-ray diffraction and differential scanning calorimetric analysis give information on the crystalline state and polymorphic changes in the nanoparticles. Confocal laser scanning microscopy (CLSM) gives information on interaction of nanoparticles with cells. Nuclear magnetic resonance **(**NMR) can be used to determine both the size and the qualitative nature of nanoparticles. The selectivity afforded by chemical shift complements the sensitivity to molecular mobility to provide information on the physicochemical status of components within the nanoparticle.

An important characterization technique for lipid nanoparticles is determination of solid state properties. This is very important in order to detect the possible modifications in the physicochemical properties of the drug incorporated into the lipid nanoparticles or the lipid matrix. It has been proven that although particles were produced from crystalline raw materials, the presence of emulsifiers, preparation method and high shear encountered (e.g. HPH) may result in changes in the crystallinity of the matrix constituents compared with bulk materials. This may lead to liquid, amorphous or only partially crystallized metastable systems [61]. Polymorphic transformations may cause chemical and physical changes (e.g. shape, solubility, melting point) in the active and auxiliary substances. The solid state analysis of lipid nanoparticles is usually carried out using the following procedures: DSC, X-ray diffraction, hot stage microscopy, Raman spectroscopy and Fourier-transform infrared spectroscopy.

3.11. Drug incorporation and loading capacity

Many different drugs have been incorporated in lipid nanoparticles. A very important point to judge the suitability of a drug carrier system is its loading capacity. The loading capacity is generally expressed in percent related to the lipid phase (matrix lipid and drug). Westesen *et al*. [61] studied the incorporation of drugs using loading capacities of typically 1 - 5%, but for ubidecarenone loading capacities of up to 50% were reported. Different loading capacities were obtained for other drugs [62]. Factors determining the loading capacity of a drug in the lipid include among others, solubility of drug in melted lipid; miscibility of drug melt and lipid melt; chemical and physical structure of solid lipid matrix; and polymorphic state of lipid material.

The prerequisite to obtain a sufficient loading capacity is a sufficiently high solubility of the drug in the lipid melt. This is why it is necessary to determine the solubility of a drug in the lipid matrix as a preformulation strategy. Typically, the solubility should be higher than required because it decreases when cooling the melt and might even be lower in the solid lipid. Solubility of a drug in the lipid melt could be enhanced by the addition of solubilizers.

3.12. Drug release from lipid nanoparticles

There have been many studies dealing with drug incorporation, however, there are distinctly less data available about drug release, especially information about the release mechanisms. Drug release from lipid nanoparticles could be conducted using different models and biorelevant media. Generally, artificial membranes, tissue constructs or excised skin are used as barriers. A major problem encountered with lipid nanoparticles is the burst release observed with these systems, however, a prolonged drug release could also be obtained with these systems. It is possible to modify the release profiles as a function of lipid matrix, surfactant concentration and production parameters (e.g. temperature) and by surface modification [40].

The release profiles of drugs from lipid nanoparticles could be modulated to obtain prolonged release without burst effect, or to generate systems with tailored burst, followed by prolonged release. The burst can be exploited to deliver an initial dose when desired. It is important to note that the release profiles are not or only slightly affected by the particle size. Predominant factors for the shape of the release profiles are the production parameters (surfactant concentration, temperature) and the nature of the lipid matrix. During particle production of lipid nanoparticles by the hot homogenization technique, drug partitions from the liquid oil phase to the aqueous water phase. The amount of drug partitioning to the water phase will increase with the solubility of the drug in the water phase, that means with increasing temperature of the aqueous phase and increasing surfactant concentration as these two parameters directly affect the solubility of drugs in aqueous system.

It was reported that there was a decrease in release with decrease in temperature for a drug encapsulated in lipid nanocapsule [63]. This decrease in drug release with decreasing temperature was attributed to an increased microviscosity of the oil delaying the drug diffusion out of lipid nanocapsule core into the aqueous release medium. The effect of ambient temperature on viscosity, however, was not limited to the internal oily core material but applies also to the external aqueous phase.

Investigation of drug release from different wax matrix pellets using theophylline as a lipophilic drug showed that as the hydrophobicity of the wax increased, the drug release rate decreased. The more hydrophilic is the wax, the more it is susceptible to hydration by the release medium and therefore the faster the drug release. The drug release pattern was also highly dependent on the drug aqueous solubility. The release process was mainly affected by the relative affinity of the drug to the wax and the aqueous release medium [63]. Therefore, wax could be modified with compatible hydrophilic material to moderate drug release from lipid-based wax matrices. It was also found that minor temperature changes around 37 ^{o}C will not significantly influence the drug release from these lipid nanocapsules,

which excluded the option of a temperature-dependent release under *in vivo* conditions. zur Mühlen *et al*. [64] reported that interactions between drug and lipid molecules plays important role in controlling the drug release. These interactions could affect the viscosity of the solid lipid matrix leading to different release rates for different drugs although having similar lipophilic properties. They demonstrated that the development of sustained release SLN is possible by the proper choice of the drug and the lipid and their degree of interaction.

3.13. Challenges of lipid nanoparticle drug delivery system

One major problem with the intravenous administration of colloidal particles is their interaction with the reticulo-endothelial system (RES). Nanoparticles for medical applications are frequently given via parenteral administration. As with any foreign material, the body mounts a biological response to an administered nanoparticle. This response is the result of a complex interplay of factors, not just the intrinsic characteristics of the nanoparticle. In particular, most materials, upon contact with biological matrices, are immediately coated by proteins, leading to a protein "corona" [65]. Protein coronas are complex and variable. Certain components of the nanoparticle corona, called opsonins, may enhance uptake of the coated material by cells of the RES. The presence of opsonins on the particle surface creates a "molecular signature" which is recognized by immune cells and determines the route of particle internalization. The route of internalization affects the eventual fate of the nanoparticle in the body (i.e. its rate of clearance from the bloodstream, volume of distribution, organ disposition, and rate and route of clearance from the body). The cells of RES are capable of mopping up particles that they do not recognise as self i.e. particles they recognise as foreign. The negative effect can however, be remedied by linking of polyethylene glycol molecules to the lipid nanoparticles, thus increasing their hydrophilicity and the residence time of these particles in circulation. Alternatively, the lipid nanoparticles could be engineered to evade these RES cells by limiting the particle sizes to about 200 nm or less. It is believed that these cells do not recognise low nanometer-sized particles as foreign. Several other varied and unrelated challenges are encountered in lipid nanoparticle technology. These challenges constitute a serious research question which current strategies are targeted to address.

3.13.1. Formation of perfect crystalline structure during storage

Triglycerides crystallize in different polymorphic forms such as α, γ, β', and β- forms. Recrystallization from the melt results in the metastable α-polymorph which subsequently undergoes a polymorphic transition into the stable β-form via a metastable intermediate. The β-polymorph especially consists of a highly ordered, rigid structure with low loading capacity of drugs. Transition to the β-form via a metastable intermediate form leads to drug expulsion and inability to protect or prolong the release of the encapsulated drug.

3.13.2. Physical stability

The stability of lipid particulate systems is influenced by the size of the nanoparticles. Lipid nanoparticles are prevented from sedimentation by Brownian motion, but other instabilities

like Ostwald ripening and aggregation may occur. Since nanoparticles have a large specific surface area, stabilization of the surface with sufficient amounts of emulsifier(s) is necessary. The formulation of the colloidal carriers themselves is a difficult task due to many problems that arise from their colloidal state and specific pharmaceutical demands on such formulations. Stability in a pharmaceutical sense refers to a shelf life of usually 3-5 years. Shorter shelf lives will only be accepted in very special cases. However, for most systems of pharmaceutical interest the colloidal state is at its best metastable. The colloidal state may cause several additional instabilities, for example, due to the presence of large interfaces (adsorption-desorption processes, interactions in the stabilizer layer, higher risk of chemical instabilities, etc.). Since many colloidal administration systems are intended for intravenous use, stability is very crucial. Ability to be sterilized is also an added advantage.

3.13.3. Gel formation

The change in morphology of lipid nanoparticles from spheres to platelets is responsible for the gelation of solid lipid nanoparticle dispersions [66]. Depending on the composition, especially of the emulsifier(s) and the amount of lipid matrix, a gelation of the normally liquid dispersions can be observed on storage [66, 67]. By means of TEM and synchrotron measurements, the reason for the gelation was found. The gelation may derive from a reversible self-association of the particles due to a stacking of the platelets [68, 69]. This gel-like feature of highly concentrated dispersions favours application as dermal drug delivery system since the viscoelastic features resemble those of semisolid creams [56]. In contrast, the lipid nanoparticle formulations for intravenous and ocular administration have to remain fluid. Phase transitions that would lead to an unusual lamellar gel phase (L_β) should be investigated for in parenteral formulations.

3.13.4. High water content of dispersions

The high water content of lipid nanoparticles (70-95%) could lead to drug degradation and high cost of energy input during lyophilisation. During lyophilisation, the integrity of the nanoparticles could be affected if adequate croprotectant or lyoprotectant was not included in the formulation. Water free nanoparticles could be used in tablet production or the nanoparticle dispersion used as granulating fluid during production. SLN can be transformed into a powder by spray-drying. In any case, it is beneficial to have a higher solid content to avoid the need of having to remove too much water. For cost reasons, spray drying might be the preferred method for transforming SLN dispersions into powders, with the previous addition of a protectant [26].

3.13.5. Dosing problems

Selection of appropriate dosage form for lipid nanoparticle may be a problem. Outside injectables, there is need to package lipid nanoparticles in appropriate dosage units e.g. dispersible powders or hard/soft gelatine capsules especially for oral administration. Since the stomach acidic environment and its high ionic strength favour particle aggregation, aqueous dispersions of lipid nanoparticles might not be suitable to be administered orally as

a dosage form. In addition, the presence of food will also have a high impact on their performance [70]. Packaging of SLN in a sachet for redispersion in water or juice prior to administration will allow an individual dosing by volume of the reconstituted SLN. This means additional step in packaging, which might result in introduction of additional technology and increase in the cost of the product.

3.13.6. Coexistence of other colloidal structures in the system (e.g. liposome and vesicles in SLN and NLC containing phospholipids)

Considerable amounts of emulsifiers are needed for the stabilization of lipid nanoparticles. If the emulsifiers redistribute from the particles into the aqueous phase, they can form colloidal structures like micelles or liposomes or other vesicular structures by self-assembly. Drugs can be solubilized within these structures, affecting drug release as well as drug loading capacity [70]. Hence the formation of additional colloidal structures has to be investigated for each formulation to enable further precautions to be taken to avoided it. The coexistence of liposomes and oil droplets was detected in an intravenous o/w nanoemulsion [71]. In solid lipid nanoparticle dispersion, additional liposomes were observed by means of cryo-TEM, although the amount was lower than in a corresponding emulsion [72]. In contrast to this, Schubert *et al.* [73] performed NMR, TEM and small angle X-ray scattering (SAXS) measurements and showed that no additional colloidal structures were formed. Drug nanocrystals could also be formed when the amount of the drug present far exceeds its solubility limit in the lipid matrix.

3.13.7. Supercooling of nanoparticles

Lipid nanoparticles prepared from triglycerides which are solid at room temperature may not necessarily crystallize on cooling to common storage temperatures. The particles can remain liquid for several months without crystallization (supercooled melt) [74]. Dispersions with lower melting points, in particular, monoacid triglycerides such as trilaurin or trimyristin, do not display melting transitions upon heating in differential scanning calorimeter or reflections due to crystalline nature in X-ray diffractometer after storage at room temperature. As confirmed by studies with quantitative ^{1}H NMR spectroscopy, the matrix of the dispersed particles consists of liquid triglycerides in such particles. The particles have a high tendency toward supercooling. The critical crystallization temperature is mainly dependent on the composition of the triglyceride matrix and can also be modified by incorporated drugs. The degree of supercooling is much higher in the nanoparticles than for the bulk triglyceride. This supercooling could be taken advantage of and utilized as a delivery system of its own but this has to be planned for *ab initio*.

3.14. Applications of lipid particulate drug delivery systems

During the last decade, different substances have been entrapped into lipid nanoparticles ranging from lipophilic to hydrophilic molecules and including difficult compounds such as proteins and peptides.

3.14.1. Lipid nanoparticles as carriers for oral drug delivery

Lipid nanoparticles such as SLN can be administered orally as dispersion, SLN-based tablet, pellets or capsules [70] or even as lyophilized unit dose powders for reconstitution for oral delivery. The stability of the particles in the GIT has to be thoroughly tested, since low pH and high ionic strength in the GIT may result in aggregation of the particles. In order to prove this, an investigation of the effect of artificial gastric fluids on different lipidic nanoparticle formulations was performed. The authors showed that a zeta potential of at least 8-9 mV in combination with a steric stabilization hinders aggregation under these conditions [75]. Additionally, for oral drug delivery, a release upon enzymatic degradation has to be taken into account [3, 76].

The routes for particle uptake after oral application are transcellular (via the M cells in the Peyer's patches or enterocytes) or paracellular (diffusion between the cells). However, the uptake via M cells is the major pathway, resulting in the transport of the particles to the lymph [77]. Uptake into the lymph and the blood was demonstrated by means of TEM and gamma counting of labelled SLN. It was found that uptake to the lymph was considerably higher than to the plasma [78], and as such, a reduced first pass effect concludes, as the transport via the portal vein to the liver is bypassed [79].

SLN containing the antituberculosis drugs rifampicin, isoniazid and pyrazinamide have been studied in animals model and it was found that administration every 10 days could be successful for the management of tuberculosis [80].

3.14.2. Lipid nanoparticles for parenteral drug delivery

Lipid nanoparticles can be formulated for subcutaneous, intramuscular or intravenous administration. For intravenous administration, the small particle size is a prerequisite as passage through the needle and possibility of embolism should be considered. SLN offer the opportunity of a controlled drug release and the possibility to incorporate poorly soluble drugs. Additionally, especially for intravenous application, drug targeting via modification of the particle surface is possible, and for SLN formulation with a controlled release, higher plasma concentrations over a prolonged period of time can be obtained. Such systems form an intravenous depot. Further studies with different drugs such as idarubicin, doxorubicin, tobramycin, clozapine or temozolomide also showed a sustained release as described in a review paper by Harms and Müller-Goymann [81].

3.14.3. Lipid nanoparticles as carriers for peptides and proteins drugs

Lipid nanoparticles have been extensively studied for the delivery of proteins and peptides [82]. Therapeutic application of peptides and proteins is restricted by their high molecular weight, hydrophilic character and limited chemical stability, which cause low bioavailability, poor transfer across biological membranes and low stability in the bloodstream. Most of the available peptides and proteins are delivered by injection, but their short half life demands repeated doses that are costly, painful and not well tolerated by patients. Lipid nanoparticles could be useful for peptide and protein delivery due to the

stabilizing effect of lipids and to the absorption promoting effect of the lipidic material that constitute this kind of nanoparticles [83]. The use of niosomes and liposomes as adjuvants for the delivery of Newcastle disease virus to chicks has been reported [14].

3.14.4. Lipid nanocarriers for nasal vaccination

The use of lipid nanocarriers provides a suitable way for the nasal delivery of antigenic molecules. Besides improved protection and facilitated transport of the antigen, nanoparticulate delivery systems could also provide more effective antigen recognition by immune cells. These represent key factors in the optimal processing and presentation of the antigen, and therefore in the subsequent development of a suitable immune response. In this sense, the design of optimized vaccine nanocarriers offers a promising way for nasal mucosal vaccination [82].

3.14.5. Lipid nanoparticles as carriers in cosmetic and dermal preparation

Lipid nanoparticles can be incorporated into a cream, hydrogel or ointment to obtain semisolid systems for dermal applications. Another possibility is to increase the amount of lipid matrix in the formulation above a critical concentration, resulting in semisolid formulations [84]. The substances used for the preparation of dermal SLN are rather innocuous since they are mostly rated as GRAS and many of them are used in conventional dermal formulations. This resulted in the first dermal formulations containing SLN for cosmetic purposes entering the market [85].

Due to the adhesiveness of small particles, SLN adhere to the stratum corneum forming a film as this films have been shown to possess occlusive properties [86]. It was shown that the degree of crystallinity has a great impact on the extent of occlusion by the formulation. With increasing crystallinity the occlusion factor increases as well [87]. This explains why liquid nanoemulsions in contrast to SLN do not show an occlusive effect and why the extent of occlusion by NLC compared to SLN is reduced. Other parameters influencing the occlusion factor are the particle size and the number of particles. Whilst with increasing size the factor decreases, an increase in number results in an increase in the extent occlusion [88]. The occlusive effect leads to reduced water loss and increased skin hydration. Highly crystalline SLN can be used for physical sun protection due to scattering and reflection of the 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 [89]. 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 decyl oleate [90].

3.14.6. Lipid nanoparticles for ocular application

The eye possesses unique challenges with respect to drug delivery especially with respect to the posterior segment and treating vision threatening diseases. Poor bioavailability of drugs from ocular dosage form is mainly due to the pre-corneal loss factors which include tear

dynamics, non-productive absorption, transient residence time in the cul-de-sac, and relative impermeability of the corneal epithelial membrane. Due to the adhesive nature of the small nanoparticles, these negative effects can be reduced. For ocularly administered SLN an increase in bioavailability was observed in rabbits by Cavalli *et al.* [91] using tobramycin ion pair as the model drug. Various *in vitro* studies show a prolonged and enhanced permeation when the drug is incorporated in lipid nanoparticles [39]. For systems containing phospholipids, a further improved permeation of diclofenac sodium was observed [31].

3.14.7. Pulmonary application of lipid nanoparticles

Pulmonary drug application offers the advantage of minimizing toxic side effects if a local impact is intended. Systemic delivery can be achieved through pulmonary delivery, offering the advantage of bypassing the first pass effect, as well as offering a large absorptive area, extensive vasculature, easily permeable membrane and low extracellular and intracellular enzyme activities [92]. A problem of this method of administration is the low bioavailability. SLN can easily be nebulized to form an aerosol of liquid droplets containing nanoparticles for inhalation [82]. *In vivo* studies showed that the administered drugs (rifampicin, isoniazid and pyrazinamide for the treatment of tuberculosis) resulted in a prolonged mean residence time and a higher bioavailability than the free drug [93, 94].

3.14.8. Application of liquid crystal drug delivery systems

The spontaneous self assembly of some lipids to form liquid crystalline structures offers a potential new class of sustained release matrix. The nanostructured liquid crystalline materials are highly stable to dilution. This means that they can persist as a reservoir for slow drug release in excess fluids such as the GIT or subcutaneous space, or be dispersed into nanoparticle form, while retaining the parent liquid crystalline structure. The rate of drug release is directly related to the nanostructure of the matrix. The particular geometry into which the lipids assemble can be manipulated through either the use of additives to modify the assembly process, or through modifying conditions such as temperature, thereby providing a means to control drug release.

Liquid crystal depot could be injected as a low-viscosity solution. Once in the body, it self-assembles and encapsulates the drug in a nanostructured, viscous liquid crystal gel. The drug substance is then released from the liquid crystal matrix over a time period, which can be tuned from days to months. The liquid crystal depot system is capable of providing *in vivo* sustained release of a wide range of therapeutic agents over controlled periods of time. Liquid crystal nanoparticles can be combined with controlled-release and targeting functionalities. The particles are designed to form *in situ* at a controlled rate, which enables an effective *in vivo* distribution of the drug. The system has been shown to give more stable plasma levels of peptides in comparison to competing microsphere and conventional oil-depot technologies [21, 95].

Oral liquid crystal DDS are designed to address the varied challenges in oral delivery of numerous promising compounds including poor aqueous solubility, poor absorption, and

large molecular size. Compared with conventional lipid or non-lipid carriers, these show high drug carrier capacity for a range of sparingly water-soluble drugs. For drugs susceptible to *in vivo* degradation, such as peptides and proteins, liquid crystal nanoparticles protect the sensitive drug from enzymatic degradation. The system also addresses permeability limitations by exploiting the lipid-mediated absorption mechanism. For water-soluble peptides, typical bioavailability enhancements range from twenty to more than one hundred times. In an alternative application large proteins have been encapsulated for local activity in the GIT. Liquid crystal nanoparticle systems can be designed to be released at different absorption sites (e.g., in the upper or lower intestine) which is important for drugs that have narrow regional absorption windows.

With regards to topical application, liquid crystal systems form a thin surface film at mucosal surfaces consisting of a liquid crystal matrix, whose nanostructure can be controlled for achieving an optimal delivery profile. The system also provides good temporary protection for sore and sensitive skin. Their unique solubilizing, encapsulating, transporting, and protecting capacity is advantageously exploited in liquid and gel products used to increase transdermal and nasal bioavailability of small molecules and peptides.

3.15. Lipid nanoparticles in drug targeting

Nanoscale lipid materials containing drugs and diagnostics are being developed to image the distribution of tumour cells in the body, target and attach to cancerous cells, destroy unwanted cells via ablation or interference with cellular functions. Drug targeting might overcome the problem of repeated administration by facilitating the efficacy of drug administered systemically and attenuating side effects on healthy tissues. Ultimately, lipid nanoparticles represent versatile DDS, with the ability to overcome physiological barriers and guide the drug to specific cells or intracellular compartments by means of passive or ligand-mediated targeting mechanisms.

There are many research reports dealing with targeting of drugs to certain cellular targets in the organs using lipid nanoparticles. The knowledge of the pathophysiological/passive targeting approaches used in cancer chemotherapy is used to develop nanocarriers for targeting drug to appropriate cancer cells in the body. Small molecules administered systemically are easily eliminated through the kidney into the urine and distributed equally not only in the target tissue but also in other healthy tissues, resulting in less efficacy and more possible side effects [96]. On the other hand, large molecules have longer half-life in the blood and tend to be passively delivered to the lesions with highly permeable vessels in neovascularization and inflammation. This tendency is remarkable especially in solid tumors because of high density of neovascular vessels and immature lymph systems, and is termed enhanced permeability and retention (EPR) effect. Because of the successes being recorded from *in vitro* experiments, lipid nanoparticles have been subject of further investigation for drug targeting to the brain, several types of cancer, posterior segments of the eye, otic (inner ear) diseases, and delivery of nucleic acids and genes, utilizing the both active and passive targeting opportunities [97].

3.16. Scale up issues and ultimate dosage form development

Large scale production of lipid nanoparticles is possible using lines available in pharmaceutical plants. In spite of intensive research on colloidal drug carrier systems, in some cases for several decades (e.g., with lipid emulsions or liposomes), remarkably few have been introduced into the market [98, 99] as presented in Table 3 indicating that there seem to be problems either with the underlying concepts or with the formulation of adequate colloidal carriers, or both. Most of the products are cosmetic products. In this context, it has, of course, to be taken into account that the development and registration of a new type of administration system may take as long for a new drug or novel formulation thereof; that means up to 10 years or even more. Therefore, products will appear on the market only after considerable period of time when the concept has been successfully realized.

Product name	**Producer**	**Market entry date**	**Main active ingredients**
Cutanova Cream Nano Repair Q10	Dr. Rimpler	10/2005	Q 10, polypeptide, Hibiscus extract, ginger extract, ketosugar
Intensive Serum NanoRepair Q10		10/2005	Q 10, polypeptide, mafane extract
Cutanova Cream NanoVital Q10		06/2006	Q 10, TiO_2, polypeptide, ursolic acid, oleanolic acid, sunflower seed extract
SURMER Crème Legère Nano-Protection SURMER Crème Riche Nano-Restructurante SURMER Elixir du Beauté Nano- Vitalisant SURMER Masque Crème Nano-Hydratant	Isabelle Lancray	11/2006	Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
NanoLipid Restore CLR	Chemisches Laboratorium Dr. Kurt Richter, (CLR)	04/2006	Black currant seed oil containing ω-3 and ω-6 unsaturated fatty acids
Nanolipid Q10 CLR		07/2006	coenzyme Q10 and black currant seed oil
Nanolipid Basic CLR		07/2006	caprylic/capric triglycerides
NanoLipid Repair CLR		02/2007	black currant seed oil and manuka oil
IOPE SuperVital - Cream - Serum - Eye cream	Amore Pacific	09/2006	coenzyme Q10, ω-3 und ω-6 unsaturated fatty acids

Product name	Producer	Market entry date	Main active ingredients
- Extra moist softener - Extra moist emulsion			
NLC Deep Effect Eye Serum NLC Deep Effect Repair Cream NLC Deep Effect Reconstruction Cream NLC Deep Effect Reconstruction Serum	Beate Johnen	12/2006	coenzyme Q10, highly active oligo saccharides Q10, TiO_2, highly active oligo saccharides Q10, Acetyl Hexapeptide-3, micronized plant collagen, high active oligosaccharides in polysaccharide matrix
Regenerationscreme Intensiv	Scholl	6/2007	Macadamia Ternifolia seed oil, Avocado oil, Urea, Black currant seed oil
Swiss Cellular White Illuminating Eye Essence Swiss Cellular White Intensive Ampoules	la prairie	1/2007	Glycoprotiens, Panax ginseng root extract, Equisetum Arvense extract, Camellia Sinensis leaf extract, Viola Tricolor Extract
SURMER Creme Contour Des Yeux Nano-Remodelante	Isabelle Lancray	03/2008	Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, hydrolized wheet protien
Olivenöl Anti Falten Pflegekonzentrat Olivenöl Augenpflegebalsam	Dr. Theiss	02/2008	Olea Europaea Oil, Panthenol, Acacia Senegal, Tocopheryl Acetate Olea Europaea Oil, Prunus Amygdalus Dulcis Oil, Hydrolized Milk Protein, Tocopheryl Acetate, Rhodiola Rosea Root Extract, Caffeine

Table 3. Examples of cosmetic products currently on the market containing lipid nanoparticles (Adapted from [98, 99]).

4. Future prospects of novel lipid particulate drug delivery systems

Novel lipid based nanoparticles offer a highly versatile platform that should be considered when working with drugs that present solubility and/or bioavailability challenges. An increasing number of drugs under development are poorly water soluble and therefore have poor bioavailability. These are designated BCS class II and class IV drugs. Creative formulation techniques are required to produce finished drug products of these drugs that have acceptable pharmacokinetics. A common formulation approach with such compounds

is to focus on creating and stabilizing very small particles of the drug in an attempt to increase the surface area available for dissolution *in vivo*, and hence the rate of dissolution, and consequently plasma or tissue levels of drug. Shelf-life stability and enzymatic degradation are two main areas of concern, and formulation design focuses on stabilizing the drug in storage and protecting it from endogenous enzyme degradation until it reaches its therapeutic target. These novel DDS are well suited for the formulation of these bioactives.

Lipid nanoparticle DDS can be employed for the delivery of phytomedicines intended for oral and topical administration, which are the main routes of administration of phytomedicinals. This application holds great promise in the development and use of phytomedicines considering the difficulties of their delivery owing to their physicochemical properties. Since many phytomedicines usually possess different pharmacological activities, the delivery can be targeted to specific part(s) of the body where action is desired by means of lipid nanoparticle technology [60]. Thus, undesired effects and wastage of materials would be avoided. The implication is that with efficiency of preparation, only required quantity is utilized for formulation and adequate dose is absorbed and successfully delivered to the target for the desired activity. Lipid nanoparticle formulation of phytomedicinals would find useful applications in nanomedicine especially where targeted delivery is important such as in the treatment of cancer.

5. Conclusions

The use of lipid particles as drug carrier systems has been favoured recently as result of the GRAS status of the excipients and their traditional use in other food and pharmaceutical products. Lipids and lipid nanoparticles are promising delivery systems for oral administration of small molecule drugs, proteins and peptides. Lipid formulations of drugs are able to control the release of drugs and reduce absorption variability. The oral administration of lipid nanoparticles is possible as aqueous dispersion or alternatively transformed into a traditional dosage forms such as tablets, pellets, capsules, or powders in sachets. The ability to incorporate drugs into lipid nanocarriers offers a new prototype in drug delivery that could be used for passive and active drug targeting. Lipid nanoparticles for topical application could be formulated with high content of lipid matrix or dispersed in creams or gels to give it 'body'. With the development and interest in lipid particulate drug delivery systems shown by pharmaceutical formulation scientists, a future full of lipid nanoparticle products in the market is envisaged.

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Chapter 6

Niosomes as Carrier in Dermal Drug Delivery

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Additional information is available at the end of the chapter

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1. Introduction

Colloidal vesicular carriers such as liposomes or niosomes have been extensively applied in drug delivery systems due to unique advantages. These vesicles can act as drug reservoirs and the rate of drug release can be modified by changing of their composition. These lipid carriers can encapsulate both hydrophilic drugs (by loading in inner space) and hydrophobic drugs (in lipid area). Because of their potential to carry a variety of drugs, these lipid vesicles have been widely used in various drug delivery systems like drug targeting, controlled release and permeation enhancement of drugs (Akhilesh et al., 2011). Dermal (topical) delivery defines a targeting to the pathological sites within the skin with the least systemic absorption. Drug localization in this case is a crucial issue in the treatment of dermatological problems such as skin cancer, psoriasis, alopecia and acne, where the origin of disease is located in the skin (Brown et al., 2006). Topical drug administration has been initiated since long time to accomplish several functions on different skin levels (skin surface, epidermis, dermis and hypodermis). But, several limitations have been associated with the conventional topical preparations e.g. low percutaneous penetration because of the barrier function of the stratum corneum, the outermost layer of the skin, (Rubio et al., 2011) and absorption to the systemic circulation (Dubey et al., 2012). The scientific reports nowadays offer several systems that can be able to deliver drugs through the skin (Higaki et al., 2005). Recently niosomes are becoming popular in the field of topical drug delivery due to its outstanding characteristics like enhancing the penetration of drugs, providing a sustained pattern of drug release and ability to carry both hydrophilic and lipophilic drugs (Sathali et al., 2010). This chapter deals with the potential of niosome in topical delivery system focusing on its clinical approach. Skin represents a multilayered effective barricade to the penetration of drugs. The outer layer of skin, stratum corneum, provides a rate limiting step during percutaneous absorption of drug. Drug transfer across the stratum corneum is mainly a passive process, and thus the physicochemical properties of a permeant have a key role in its capability to penetrate and diffuse across the membrane. Compounds can penetrate

through the stratum corneum via three routes: intercellular, transcellular (paracellular), and transappendageal (figure 1). Once it has transferred through the epidermis, a compound may be carried away by the dermal blood circulation or to be transported to deeper tissues. The relative significance of these penetration pathways will be largely dependent on the physicochemical characteristics of the drug molecules, particularly the partition and diffusion coefficients into the protein or lipid regions (Barry, 1991).

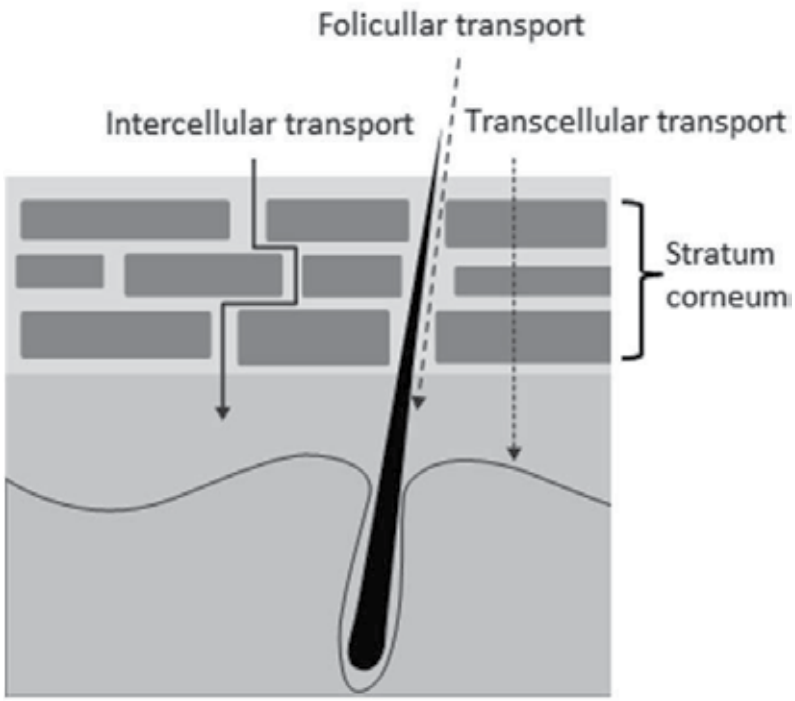

Figure 1. Possible pathways for delivery of compounds across the stratum corneum adapted from reference (Gašperlin et al., 2011).

For successful dermal or transdermal delivery, a reversible overcoming of skin barrier is required. Therefore, many strategies have been assessed to overcome the barrier function of the stratum corneum and to improve drug transport into the skin (Ghafourian et al., 2004; Rahimpour et al., 2012). Targeting of topically administered drugs to the different skin layers and appendages is becoming a main center of interest for many pharmaceutical research groups studying in dermatology (Nounou et al., 2008). The carrier is one of the most important entities required for successful targeted drug delivery (figure 2). Carriers will enable a drug to reach the desired pharmacological site of action at a controlled rate and to have a sustained duration of action. Consequently, as a vehicle for active substances and targeting to skin layers, surfactant based carriers such as niosomal systems are gaining more interest.

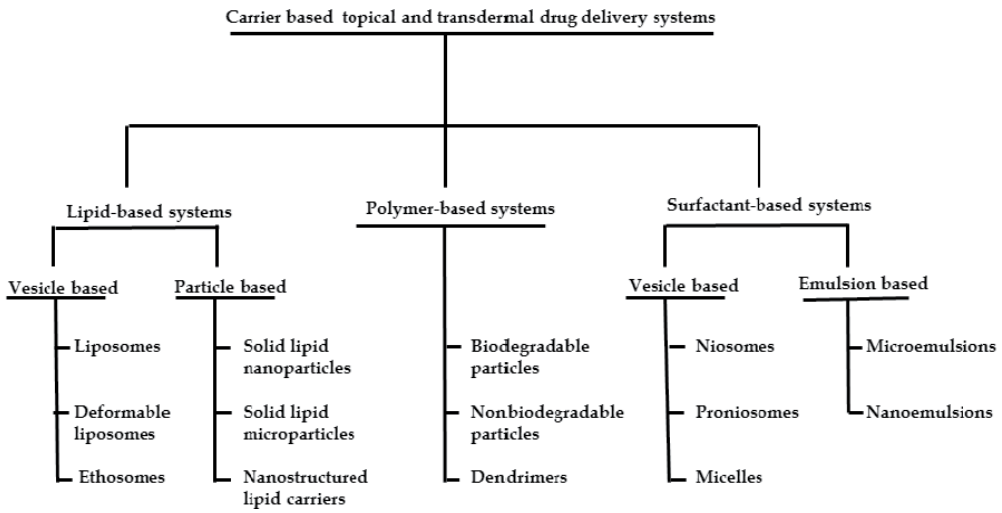

Figure 2. Carriers for topical and transdermal drug delivery adapted from reference (Venuganti et al., 2009).

2. Niosome

Niosomes are microscopic lamellar structures composed of non-ionic surfactants and cholesterol. The niosomes have amphiphillic bilayer structure in a way that polar region is oriented outside and inside the vesicles where the hydrophilic drug will be entrapped and non-polar region is formed within the bilayer where hydrophobic drug can be entrapped (Khan et al., 2011). The formation of vesicular system based on hydration of mixture of a single-alkyl chain nonionic surfactant and cholesterol was firstly reported in 1979 (Handjani-Vila et al., 1979). Niosomes might be produced by various types of nonionic surfactants including polyglycerol alkyl ethers, crown ethers, ester-linked surfactants, glucosyldialkyl ethers, polyoxyethylene alkyl ethers, Brij, Tweens and Spans. Nonionic surfactants used to prepare niosomes carry no charge and are relatively nontoxic and mild to use (Azeem et al., 2009).

2.1. Types of niosomes

2.1.1. Proniosomes

Proniosome is a dry granular product that can be able to form a niosome suspension after hydration (Hu et al., 2000). Proniosomes are developed to overcome the disadvantage of vesicular system. Proniosome are prepared based on the simple scheme that a mixture of surfactant/alcohol/aqueous phase can be used to prepare a concentrated proniosome gel, which can instinctively be converted to a stable niosomal dispersion by dilution with excess aqueous phase (Perrett et al., 1991).

Carrier + surfactant = Proniosomes

Proniosomes + water = Niosomes

2.1.2. Aspasomes

Mixture of acorbyl palmitate, cholesterol and highly charged lipid diacetyl phosphate leads to the construction of vesicles named aspasomes. Aspasomes are first hydrated with water/aqueous solution and then sonicated to attain the niosomes. Aspasomes are suggested to improve the transdermal permeation of drugs. Aspasomes have also been used to reduce disorders caused by reactive oxygen species due to its intrinsic antioxidant property (Gopinath et al., 2004; Rajera et al., 2011).

2.1.3. Vesicles in water and oil system (v/w/o)

In this system suspension of aqueous niosomes (v/w) are emulsified into the oily phase at 60°C to form vesicle in water in oil emulsion (v/w/o) (Yoshida et al., 1992; Hu et al., 2000). Cooling to room temperature forms vesicle in water in oil gel (v/w/o gel) (Yoshioka et al., 1992). The prepared v/w/o gel can entrap the hydrophilic active ingredients which are susceptible for enzymatic degradation such as proteins/proteinous drugs and also provide a controlled release pattern in drug delivery.

2.1.4. Deformable niosomes

Elastic niosomes are prepared of nonionic surfactants, ethanol and water. They show superior to conventional niosomes due to their capability to increase penetration efficiency of a compound through intact skin by passing through pores in the stratum corneum, which are smaller than the vesicles (figure 3). The flexibility of their structure allows them to pass through pores that are less than one-tenth of these vesicles (Cevc, 1996; Cevc et al., 1996).

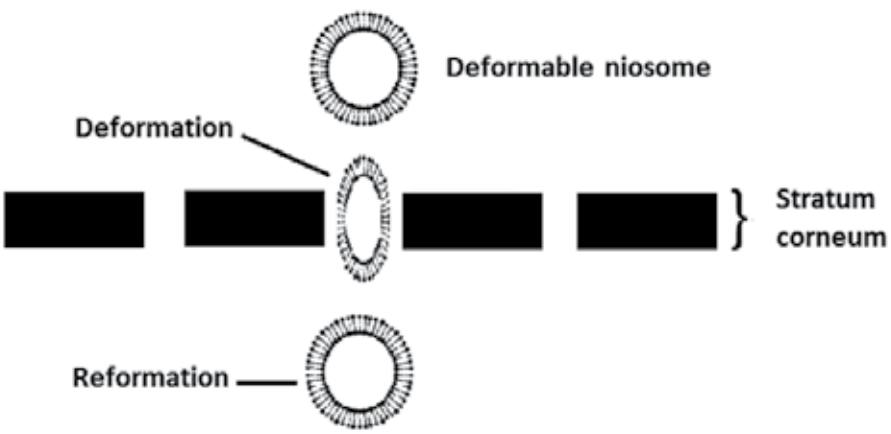

Figure 3. Mechanism of skin permeation of deformable niosome adapted from reference (Kumar et al., 2012)

2.2. Advantages of noisome

The major advantages of niosomes can be mentioned as: i) niosomes are biodegradable, biocompatible, non-toxic and non-immunogenic (Azeem et al., 2009); ii) niosomes are able to

encapsulate large amount of materials in a small volume of vesicles (Nasr et al., 2008); iii) niosomes have better patient adherence and satisfication and also better effectiveness than conventional oily formulations (Jain et al., 2006); iv) niosomes can entrap wide range of chemicals (hydrophilic, lipophilic and amphiphilic drugs) due to its unique structure (Raja Naresh et al., 1994); v) the characteristics of niosome such as shape, fluidity and size can be easily controlled by changing in structural composition and method of production (Bayindir et al., 2010); vi) niosomes can be prescribed via different administration routes such as oral, parenteral and topical, etc. with different dosage forms such as semisolids, powders, suspensions (Malhotra et al., 1994) and vii) due to chemical stability of structural composition, the storage of the niosome is easy (Jain et al., 2006). In spite of several above mentioned merits, niosomes show some disadvantages. The aqueous suspensions of niosomes may undergo fusion, aggregation, leaking of entrapped drugs, and hydrolysis of encapsulated drugs, which lead to limited shelf life. The methods of preparation of multilamellar vesicles such as extrusion, sonication, are time consuming and may require specialized equipments for processing (Khan et al., 2011)

2.3. Application of niosome

Niosomes have been successfully used in drug targeting to various organs such as skin (Agarwal et al., 2001), brain (Abdelkader et al., 2012), liver (Baillie et al., 1986; Lala et al., 2004), lung (Desai et al., 2002; Abd-Elbary et al., 2008) ocular systems (Pugeat et al., 1991; Abdelkader et al., 2012), tumor (Ruckmani et al., 2000; Shi et al., 2005) etc. Niosomes show a higher bioavailability than conventional dosage forms (Raja Naresh et al., 1994). Controlled and sustained release of drugs have been achieved by niosomes (Bayindir et al., 2010). Permeation of drugs through the skin has been enhanced by niosomes (Pardakhty et al., 2007). Noisome, itself improves the stratum corneum properties both by reducing transepidermal water loss and skin condition by increasing smoothness via reloading lost skin lipids (Firthouse et al.). Niosomes can be applied for drug protection from biological enzymes and acid thereby increasing the drug stability (Jain et al., 2006).

2.4. Niosomes in comparison with liposomes

Niosomes and liposomes have similar application in drug delivery but chemically differ in structure units. Niosomes constitute of non-ionic surfactant whereas liposomes comprise of phospholipids (Khan et al., 2011). They are functionally the same, have the same physical properties and act as amphiphilic vesicules. Both can be used in targeted and sustained drug delivery system. Property of both depends upon composition of the bilayer and methods of their preparation (Verma et al., 2012). Studies have also shown that the function of niosomes in vivo is similar to that of liposomes (Hofland et al., 1994). In spite of these comparable characteristics, niosomes offer several advantages over liposomes: intrinsic skin penetration enhancing properties, higher chemical stability and lower costs (Nasr et al., 2008). Both of the last features make the niosome more attractive for industrial manufacturing (Hu et al., 2000). Also, niosomes do not require special conditions such as low temperature or inert atmosphere during preparation and storage (Verma et al., 2012). Although the niosome

shows better chemical stability, the physical instability during dispersion may be comparable to that of the liposome. Both niosomes and liposomes are at risk of aggregation, fusion, drug leakage, or hydrolysis of entrapped drugs during storage (Hu et al., 2000; Rahimpour et al., 2012).

3. Mechanisms of niosomal skin delivery

Several mechanisms have been suggested to describe the ability of niosomes in transdermal and dermal drug delivery: i) niosomes diffuse from the stratum corneum layer of skin as a whole; ii) new smaller vesicles are formed in skin (re-formation of noisome vesicles). The water content of skin is crucial issue for interpreting and establishing this mechanism. Smaller diameter of lipid lamellar spaces of the stratum corneum than noisome vesicles makes this mechanism more meaningful (Sahin, 2007). iii) noisomes interact with stratum corneum with aggregation, fusion and adhesion to the cell surface which causes a high thermodynamic activity gradient of the drug at the vesicle-stratum corneum surface, which is the driving force for the penetration of lipophilic drugs across the stratum corneum (Ogiso et al., 1996). Scanning electron microscopy confirmed the fusion of niosome vesicles of estradiol on the surface of skin (Schreier et al., 1994) iv) niosomes may modify stratum corneum structure which makes the intercellular lipid barrier of stratum corneum looser and more permeable (Fang et al., 2001); v) non-ionic surfactant itself, the composing ingredient of niosome, acts as a permeation enhancer and might partly contribute to the improvement of drug permeation from niosomes (Javadzadeh et al., 2010). The type of surfactant plays an important role in modification of permeation using niosome vehicles. Niosomes fabricated from polyoxyethylene stearyl ether and existing in the gel state did not enhance estradiol permeation, and those prepared from polyoxyethylene lauryl ether and polyoxyethylene oleyl ether, both existing as liquid crystalline vesicles, considerably improved transport (Hofland et al., 1991).

4. Toxicity of niosomes

Surfactants are suspected to show toxicity but there are virtually not enough research about toxicity of niosomes (Yadav et al., 2011). A study on the toxicity effect of surfactant type of niosomal formulations on human keratocytes showed that the ester type surfactants are less toxic than ether type due to enzymatic degradation of ester bounds (Hofland et al., 1991). Hofland et al. studied the toxicity of CxEOy surfactants via ciliotoxicity model on nasal mucosa (which is important for intranasal administration) and on human kerationocytes (which is important for the transdermal application of vesicles). The results showed that increase in alkyl chain length of surfactant causes a reduction in toxicity, while, increase in the polyoxyethylne chain length enhances ciliotoxicity. The study supposed that ciliotoxicity related to liquid state formation with increasing in polyoxyethylene chain length, while increase in alkyl chain length of surfactant leads to formation of gel which is more safe than liquid state (Hofland et al., 1992). In another study, vincristine, a potent anti-tumor agent, was loaded into niosomes and administered intravenously. Result showed a significant

increase in vincristine anti-tumor activity in S-180 sarcoma and Erlich ascites mouse models followed by reduction of its side effect such as diarrhea, neurological toxicity and alopecia compared with free drug (Parthasarathi et al., 1994). Toxicity effect of niosomes should be considered according to the intended route of delivery. For example, hemocompatibility studies should be carried out when the niosomes are meant to be delivered by intravenous route to evaluate their toxic potential (Uchegbu et al., 1997). The first in vivo experiment on drug delivery by means of synthetic non-ionic surfactant vesicles were carried out by Azmin et al. and reported that no unfavorable effects were observed in the performed experiment (Azmin et al., 1985). Rogerson et al. reported in vivo experiment over 70 male BALB/C mice and stated that no fatalities were related to niosomes. The drug associated toxicity were also reduced (Rogerson et al., 1987). Niosomes which have been prepared with Bola-surfactants showed a certain and encouraging safety and tolerability both in vitro on human keratinocyte cells up to an incubation time of 72 h for the different concentrations studied (0.01-10 μM) and in vivo on human volunteers that showed no skin erythema when topically treated with the drug free Bola-niosome formulation (Paolino et al., 2007).

5. Indications for niosomes as drug carriers in dermatology

Niosomes were formulated and patented for the dermatological purposes in 1975 for the first time and since then many of products were developed based on this technology and appeared in the market such as Lancome Noisome Plus as an anti-ageing formulation (Azeem et al., 2009). Recently, the topical delivery of certain drugs using niosome has been developed. Here, current attempts with a focus on clinical application were reviewed to prove the ability of niosomes as the superior topical carrier.

5.1. Local anesthesia

Dermatologists take benefit of topical anesthetics for decreasing pain relief prior to cutaneous procedures, pain associated with laser pulses, or soft tissue augmentation. Inefficient formulations of local anesthetics resulted in severe dermatitis, systemic toxicity, or inadequate local analgesia. Carafa et al. fabricated lidocaine and lidocaine hydrochloride-loaded non-ionic surfactant vesicles using Tween 20™ and cholesterol. The ability of drug to diffuse through a model lipophilic membrane (Silastic™) and through mouse skin were studied and compared with classical liposomes and Tween 20 micelles. Dicetylphosphate and N-cetylpyridinium chloride were also used to prepare negatively and positively charged vesicles, respectively to study the effect of vesicle charge on drug encapsulation efficiency. Diffusion experiments indicated that the flux of charged lidocaine (lidocaine hydrochloride) through Silastic™ membrane was possible only after the vesicle encapsulation. Permeation of lidocaine hydrochloride-loaded vesicles through mouse abdominal skin presented a higher flux and a shorter lag time with respect to classical liposome formulations, whereas lidocaine permeation rate was quite similar for niosomes and liposome formulations. Furthermore fluorescence quenching study showed that positive and negative charged vesicles had negligible entrapment efficiency of drug, at pH

5.5 compare with neutral vesicles (Tween 20 and cholesterol). The importance of non-ionic surfactant vesicles in dermal delivery of charged local anesthetics is concluded in this study (Carafa et al., 2002).

5.2. Psoriasis

Psoriasis is a chronic inflammatory condition of the skin which may drastically undermined the patient quality of life. Psoriasis, a T-lymphocyte-mediated autoimmune disease of the dermis and epidermis, is characterized by leukocyte infiltration into the skin and localized deregulated skin growth, which leads to the development of scaling erythematous plaques (Dubey et al., 2007). Although psoriasis is rarely life-threatening, it causes an unpleasant appearance that makes the patients to miss their confidence and suffer from itching, painful and disfiguring skin lesions. Methotrexate, anthralin, corticosteroids, coal tar, vitamin D3 analogs, tacrolimus and retinoids are administered topically for psoriasis treatment (Su et al., 2008). Topical therapy is the commonly used in patients, even though the use of topical formulations based on conventional excipients shows some disadvantages that limit considerably their use in therapy (Puglia et al., 2012). With the use of niosomes as carrier for topical drug delivery, the possibility to improve efficacy and safety of the topical products has improved manifold. Dithranol, one of the key medicines in the topical treatment of psoriasis, has staining, burning, irritating and necrotizing effects on the normal and also the diseased skin. Entrapment of dithranol in niosomal and liposomal systems could be achieved after optimizing the various process and formulation variables. These systems presented size stability and improved drug permeation properties. Although the in vitro study using laca mice abdominal skin shows higher skin penetration for vesicular systems compared with the cream base but niosomal vesicular systems had three times less percutaneous permeation of dithranol compared with liposomal formulation (Agarwal et al., 2001). Methotrexate is an antifolate class of anti-neoplastic medicine which is commonly used in the psoriasis therapy. The systemic administration of this drug causes several side effects such as hepatic toxicity. Dermal delivery of methotrexate offers a valuable alternative way to reduce its adverse effects (Javadzadeh et al., 2011). The double-blind placebo-controlled study of methotrexate-loaded niosomal vesicles in chitosan gel on healthy human volunteers and psoriasis patients carried out and its efficacy compared with marketed methotrexate gel. The irritation and skin sensitivity of formulations were assessed via human repeated insult patch test (HRIPT). The HRIPT method did not yield any significant sensitization or irritation on healthy human volunteers. The severity of the lesions of psoriasis was assessed via Psoriasis Area Severity Index (PASI). The global score used to assess the outcome of therapy (on a scale of 0-5), where score of 5 indicates the worsening of lesion and score 0 indicates complete clearance of lesion. There was a three times reduction in PASI scores after 12 weeks of niosomal methotrexate gel. The results of study offer niosomal formulation of methotrexate for dermal treatment of psoriasis due to its better clinical efficacy, tolerability and patient compliance (Lakshmi et al., 2007). Urea is an emollient topically used for the non-surgical removal of dystrophic nails in cases of fungal infections for decades and used as a penetration enhancer for the topical corticosteroid

treatment of hyperkeratotic psoriatic plaques (Draelos, 2008). Urea may increase ability of skin to retain water due to hygroscopic effect. The same group in another study encapsulated urea into niosomes and prepared a gel using chitosan polymer, to test the same on healthy human volunteers to check the irritation on the skin and to study its clinical effectives on psoriasis patients. Niosomes prepared using span 60 showed a better entrapment than other spans. Better diffusion of drug through the human skin and skin drug deposition were concluded from niosomal gel in comparison to plain gel. The niosomal urea gel and plain gel did not produce any irritation of the human skin. The gels were assessed on psoriasis patients with less than 25% severity of any types of psoriasis. The niosomal gel showed a significant decrease in the lesion ($p<0.05$) than plain urea gel. It is suggested that chitosan gel can be used as an adjuvant in the treatment of psoriasis due to antifungal and anti-inflammatory effect of chitosan that supported the action of urea (Lakshmi et al., 2011).

5.3. Whitening effect

The attractiveness of pigment-lightening cosmeceuticals comes from the desire to not only fade pigmentation but also to even lighten skin tone (Choi et al., 2006). N-acetyl glucosamine (NAG), which is an amino sugar that occurs widely in nature and essential component of dermal tissues, is well-known for its role as a precursor of hyaluronic acid, a key structural composition of skin. NAG shown to inhibit melanin production in melanocyte culture, thus has a potential to reduce hyperpigmentation by topical administration (Bissett et al., 2007). To improve NAG penetration into the skin Shatalebi et al. encapsulated it into niosomes and investigated its flux across excised rat skin using Franz diffusion cells. All formulations significantly enhanced the drug localization in the skin, as compared to NAG hydroalcoholic solution. Application of negatively charged dicetyl phosphate was suggested the reason for relatively high amount of entrapment of the very water soluble NAG. This study showed the potential of niosomes for improved NAG localization in the skin, as needed in hyperpigmentation disorders (Shatalebi et al., 2010). Ellagic acid, a polyphenol widely found in plants such as pomegranates, inhibits tyrosinase by its copper chelation. It may selectively inhibit melanin synthesis only in UV-activated melanocytes (Choi et al., 2006). A niosomal formulation of ellagic acid was developed from the mixture of Span 60 and Tween 60 for its dermal delivery. Skin distribution study revealed that the ellagic acid-loaded niosomes showed more efficient delivery of ellagic acid through human epidermis and dermis than ellagic acid solution. The results pointed out that the Span 60 and Tween 60 niosomes may be a potential carrier for dermal delivery of ellagic acid (Junyaprasert et al., 2011).

5.4. Vitiligo

Vitiligo is an acquired idiopathic, dermatological disorder described by well-circumscribed milky white macules in which melanocytes in the skin are damaged. Although vitiligo is not a life threatening issue but it might have an important negative

impaction on the quality of life, even leading to attempted suicide in some cases (Nogueira et al., 2009; Bhawna et al., 2010). Application of novel dermal drug delivery systems can play a key role in vitiligo treatment because side-effects or poor efficacy of conventional topical dosage forms affect their utility and patient compliance (Bhawna et al., 2010). Human tyrosinase gene is responsible for the production of tyrosinase, an enzyme implicates in melanogenesis. The defect of tyrosinase gene is one of the reasons for depigmented skin or vitiligo (Zhang et al., 2005; Kingo et al., 2007). Manosroi et al. prepared elastic cationic niosomes (Tween 61/Cholesterol/ dimethyl dioctadecyl ammonium bromide at 1:1:0.5 molar ratio) for the effective dermal delivery of pMEL34 (tyrosinase encoding plasmid). Percutaneous absorption of formulation was investigated through exercised rat skin by Franz diffusion cells during 6 hours. The flux of pMEL34-loaded elastic niosomes was more than non-elastic niosomes in viable epidermis and dermis, while only pMEL34 loaded in elastic cationic noisome was observed in the receiver solution. By application of pMEL34-loaded elastic cationic niosomes in melanoma cell lines showed about four times higher tyrosinase gene expression than the free and the loaded plasmid in nonelastic niosomes means higher tyrosinase activity for efficient topical delivery in vitiligo therapy (Manosroi et al., 2010). In another study, luciferase plasmid (pLuc) was encapsulated in non-elastic and elastic cationic nanovesicles (niosomes and liposomes) and its transdermal absorption was investigated through rat skin follow by stratum corneum stripping or iontophoresis techniques. Free pLuc with or without the stratum corneum stripping and iontophoresis techniques and pLuc-loaded in nonelastic vesicles without the application techniques could not penetrate skin. Though, the elastic vesicles even without any application techniques can increase the transdermal absorption of pLuc. In case of elastic vesicles, the pLuc-loaded niosomes provided higher pLuc flux than that in liposomes. After 6 hours, pLuc encapsulated in nanovesicles with the iontophoresis application was still intact, but the free pLuc was degraded. Result of this study has showed the superior delivery of pLuc thorough skin by loading in niosomes, together with the application of iontophoresis, which can be used as a novel method to deliver genetic materials via dermal administration in gene therapy. Specifically, elastic cationic niosomes appeared to be a more promising approach since no additional equipment is required (Manosroi et al., 2009). Both mentioned studies demonstrated the potential application of elastic cationic niosomes as an efficient topical delivery for the purpose of gene therapy.

5.5. Cutaneous inflammation

5.5.1. Non - steroidal anti-inflammatory drugs (NSAIDs)

Celecoxib, a selective COX–2 inhibitor and most commonly used drug in the treatment of arthritis was embedded in niosomal gel for the purpose of sustained and site-specific delivery. In vitro, ex-vivo and in vivo studies were carried out through albino rats to compare the efficiency of formulation with carbopol gel as control formulation. Niosomal gel showed 6.5 times higher drug deposition in deep skin layer and muscle compare with carbopol gel indicating better drug localization with niosomal gel. A significant reduction of

rat paw edema was resulted after administration of niosomal formulation compared to that after application of conventional gel confirming better skin permeation and deposition of celecoxib from niosomes. The authors concluded that niosomal gel formulation has great potential for improving site specific delivery, skin accumulation and prolonging release of celecoxib (Kaur et al., 2007). Multilamellar liposomes and niosomes of aceclofenac, a potent analgesic, anti-pyretic and anti-inflammatory agent were prepared and a comparative study was done between them through evaluation of entrapment efficiency, particle size, shape, differential scanning calorimetry, in vitro drug release and 3 months stability study. Results proved that niosomes have better stability than liposomes. Both vesicular systems showed considerable sustained anti-inflammatory activity compared with the commercial product, however niosomes being superior to liposomes as clearly showed by both oedema rate and inhibition rate percentages assessed by the rat paw oedema technique (Nasr et al., 2008). Proniosomal formulation of gugulipid (anti-inflamatory agent) was fabricated and characterized through in vitro drug release study and invivo anti-inflammatory activity via carrageenan-induced rat hind-paw method. In vitro study of proniosomal gel throught semi-permeable membrane exhibit the initial faster release followed by slow sustained release of gugulipid for 8 h. Gugulipid incoporated proniosomal gel showed good anti-inflammatory activity but not as good as commercial product diclofenac (Voveran®Emulgel). The authors state that proniosome formulation improve anti-inflammatory activity of gugulipids comparable to topical NSAIDs (Goyal et al., 2011). A niosome based transdermal drug delivery of nimesulide was prepared by lipid film hydration technique using different nonionic surfactants, Tweens® and Spans® and optimized for highest percent drug entrapment. Formulations were extensively characterized and evaluated in-vitro performance followed by in-vivo evaluation in rats by carrageenan induced rat paw edema method. The results were compared with plain drug gel, niosomally entrapment drug in carbopol gel base and marketed formulation. The percentage of edema inhibition was the highest for niosomal nimesulide gel after 24 hours more than five and three times than plain drug gel and market formulation, respectively. The results of this research suggest that niosomal formulation can offer consistent and prolonged anti-inflammatory effect and may improve therapeutic index of the formulation and is also expected to minimize the side effect due to drug localization at the site of action (Shahiwala et al., 2002).

5.5.2. Glucocorticosteroids

Topical corticosteroids are administered for the variety of dermatological disorders. Application of corticosteroid in proper carrier may help to prolonged action, subsequently less frequent administration and reduction of adverse effects. In a study, clobetasol propionate, a potent corticosteroid acts as an anti-inflammatory and anti-pruritic, was loaded in niosomal gel and its performance was compare with marketed gel and pure drug in term of in vitro drug release and in vivo pharmacodynamic studies. The results showed that the niosomal gel formulation provided a prolonged action due to the entrapment of clobetasol in vesicles (Lingan et al., 2011).

5.5.3. Antihistamines

Hence antihistamines are frequently administered as first line treatment to reduce the symptoms of urticaria pigmentosa because histamine is the single most important mediator involved (Osvaldo et al., 2010). The conventional oral administration of hydroxyzine hydrochloride (an antihistamine) causes CNS sedation, dry mouth and tachycardia whereas topical use in the form of semisolid dosage forms would lead to systemic side effects (Elzainy et al., 2003). A modified proniosomal gel of hydroxyzine hydrochloride was prepared by coacervation phase separation technique with different combination of non-ionic surfactants (Tweens and Spans) with phospholipids. Statistical experimental design was applied to optimize the various formulation variables. The optimized formulations were evaluated in vitro, ex vivo permeation, skin deposition, skin irritation and stability studies. The three months stability at refrigeration temperature and quite high encapsulation efficiency (95%) and drug deposition in the stratum corneum in 24 h (90%) were found for optimized formulation. The results indicated that modified proniosomal formulations of hydroxyzine hydrochloride were appropriate for topical drug delivery system for the treatment of localized urticarial (Rita et al., 2012). This formulation potentially improves drug penetration into the stratum corneum and localizes the drug within the dermoepidermal layers which would offer prompt onset and prolonged duration of action due to maintenance of effective concentrations in the skin, while systemic serum concentrations would be low (Carafa et al., 1998; Kirjavainen et al., 1999; Elzainy et al., 2003). Chlorpheniramine maleate is one of the most commonly used antihistamines and topically administered for skin disorders such as sunburns, urticaria, angioedema, pruritus, and insect bites. The proniosomes containing Span 40/lecithin/cholestrol formulated by ethanol showed optimum stability, loading efficiency, and particle size and appropriate release kinetic for percutaneous delivery of chlorpheniramine maleate. Ease of preparation and use of proniosomes were introduced as the greatest advantages provided by these types of dosage forms (Varshosaz et al., 2005).

5.6. Hair loss - Alopecia

The pilosebaceous unit including sebaceous gland, hair follicle and hair shaft has a unique biochemistry, metabolism and immunology. Targeted drug delivery may improve current therapeutic approaches to treat diseases of follicular origin (Weiner, 1998). In an increasing amount of topical studies, niosomes have been revealed to target drug delivery to the pilosebaceous unit. Androgenic alopecia (male pattern hair loss) is the most common reason of hair loss in men and characterized by the progressive hair thinning in genetically susceptible men (Jung et al., 2006). Semi-purified fraction of Oryza sativa contains the unsaturated fatty acids such as gamma-linolenic acid, linoleic acid and oleic acid have been proved to have anti-hair loss activity by inhibition of 5a-reductase type 1 (a key enzyme of androgenic alopecia) in DU-145 cell lines (Ruksiriwanich et al., 2011). Manosroi et al. prepared cationic niosomes (for higher stability) encapsulated with this extract and investigated physicochemical characteristics and transfollicular penetration of niosomes through porcine skin using follicular closing technique by Franz diffusion cells. The result of

this study confirmed efficient transfollicular delivery of unsaturated fatty acids using cationic niosomes as well as the advantage of low systemic effect than the neutral niosomes (Manosroi et al., 2012). Thai Lanna medicinal plants have been reported to improve hair growth and reduce hair loss owing to increase the blood circulation, anti-fungal, anti-bacterial, and anti-oxidation of the hair roots. But, the extracts of these plants are naturally not stable and are problematic to be absorbed through hair follicles. Niosomal formulation containing the Thai Lanna plant extracts, such as turmeric, chili, ginger and Tong-Pan-Chang extract incorporated in gel was fabricated and tested in 20 human volunteers (9 males and 11 females). Results showed a significant increase in hair density and a decrease in hair loss without any irritation in all subjects from the 8-week application ($p<0.01$) in non-heredity alopecia volunteers, with non-differences in male and female volunteers. This in vivo study confirms potential of niosomes carrier for topical application of Thai Lanna medicinal plants for anti-hair loss (Manosroi et al., 2008). The enzyme 5a-reductase, responsible for production of dihydrotestosterone from testosterone in hair follicles, which is supposed to be the reason of androgenetic alopecia, can be inhibited by finasteride. Finasteride shows several unwanted systemic adverse effects which will be reduced if it acts locally in the hair follicles (Javadzadeh et al., 2010). Tabbakhian et al. studied dermal application of finasteride-containing vesicles (niosomes and liposomes) for increasing drug concentration at the pilosebaceous units, as compared with finasteride hydroalcoholic solution. Both in vitro permeation of 3H-finasteride through hamster flank skin and in vivo deposition studies in hamster ear demonstrated the potentials of liquid-state niosomes and liposomes for successful delivery of finasteride to the pilosebaceous units compared with hydroalcoholic solution while optimized niosomal formulation also showed higher targeting ration than liposomal vesicles (Tabbakhian et al., 2006). Minoxidil, the most commonly used medicine for the treatment of androgenic alopecia with unknown mechanism of action on hair follicles (Messenger et al., 2004) were encapsulated into noisome and liposome vesicles. The percutaneous absorption study was carried out in vitro using vertical diffusion Franz cells using human skin and the results compared with dissolved minoxidil in propylene glycol–water–ethanol solution as a control. Although penetration of niosomal minoxidil in epidermal and dermal layers was greater than control solution but lower than liposomal formulations. These differences suggested to be attributed to the smaller size and the greater potential targeting to skin and skin appendages of liposomal carriers, which enhanced globally the skin drug delivery. This work generally suggests that niosomes and liposomes have a proper potential for drug dermal targeting and could be considered as a reasonable and practicable therapeutic approach to skin diseases such as hair loss (Mura et al., 2007). In a another study the effects of niosomal minoxidil formulation on the drug penetration in hairless mouse skin were investigated by in vitro permeation experiments, and the results compared with control minoxidil hydroalcoholic solution and a leading commercial topical formulation "Minoxyl^{TM}" containing 5% minoxidil and 1% dexpentanol. The result of study showed niosomal formulations increased the percentage of dose accumulated in the skin (1.03±0.18 to 19.41±4.04%) compared with control and commercial formulations (0.48±0.17 and 0.11±0.03 %, respectively). Physical stability of niosomal formulations which was evaluated for three

month at refrigerator temperature (2–8 °C) have shown a fairly high retention of minoxidil inside the vesicles (80%). The authors finally proposed that these niosomal formulations could be used as a feasible cargo carrier for the dermal delivery of minoxidil and promising approach in treatment alopecia (Balakrishnan et al., 2009). Recently minoxidil-loaded niosomes were prepared with series of surfactant and cholesterol in different molar ratio by ethanol injection method. Surfactant screening exhibited that only Span 20, Span 60 and Tween 20 with cholesterol have ability of nano size vesicle formation and Span 60 was shown to be a better surfactant for niosomal stable form with maximum entrapment efficiency (31.27±1.5 %) and minimal particle size (approx. 219 nm). The in vitro skin permeation and deposition study of minoxidil-loaded niosomal gel formulation prepared with 1:2 ratio of Span 60 and cholesterol showed significant improvement in skin accumulation (more than eightfold) as compared with plain minoxidil gel (17.21±3.2 and 2.26±1.3 %, respectively). Result of study demonstrates an increase in cholesterol concentration in niosome vesicles enhance minoxidil skin retention and effect on entrapment efficiency as well as size of niosomes to better cutaneous treatment. It was also concluded that the developed niosomal formulation could be a suitable option for cutaneous targeting of minoxidil (Mali et al., 2012).

5.7. Acne

Acne is the most common skin disease of multifactorial origin with an incidence of 70 - 80% in adolescence. While topical therapy has an important role in acne treatment, side effects associated with several topical antiacne agents affect their efficacy and patient satisfaction. Niosomes, capable of great features for skin administration, can play an essential role in improving the dermal delivery of antiacne agents by increasing their topical localization with an associated reduction in their adverse effects (Maibach et al., 2005). Benzoyl peroxide is a macrolide antibiotic generally applied for the management of acne either alone or in combination with other antiacne agents. Dermal administration of benzoyl peroxide causes side effects such as skin redness, itching, irritation and edema which lead to discomfort and ignorance of therapy and outcomes in no profit or emergence of resistant to microorganisms. In a study, niosomal benzoyl peroxide incorporated into HPMC gel designed and optimized by partial factorial design. Ex vivo release study on human cadaver skin showed increase in drug skin retention, extended drug release and improved permeation of drug across the skin which in turn will reduce the toxicity of drug and enhance the therapeutic efficacy (Vyas et al., 2011). Gallidermin is mainly promising for the dermal and cosmetic treatment of acne in human medicines because of its greatly active against *Propionibacterium acnes* and treatment of multidrug resistant *Staphylococcus Aureus* strains, which is a snowballing problem especially in the hospitals (Kempf et al., 1999). Gallidermin has limited absorption through skin and chemical instability due to its large molecular structure and peptide nature, respectively (Manosroi et al., 2005). Anionic niosomal gallidermin composed of tween 61/cholesterol /dicetylphosphate prepared and antibacterial activity of formulations against *Propionibacterium acnes* and *Staphylococcus Aureus* assayed with macrodilution method to determine the minimal concentration of the

sample necessary to inhibit or kill the microorganisms. Result of study demonstrates gallidermin loaded in niosomes offered lower antibacterial activity against tested microeorganisms compare with unloaded gallidermin due to the niosomes protecting role and making a sustained release of drug. Niosomal furmulation of gallidermin in gel showed more chemically stable at high temperatures and two fold higher cumulative amounts in viable epidermis and dermis of rat skin than aqueous solution of gallidermin. This study suggests that anionic niosomes of gallidermin can be considered as a superior topical antibacterial formulation because of chemical stability and high skin localization with no threat of systemic effect (Manosroi et al., 2010). Trans-retinoic acid or tretinoin, a vitamin A metabolite, is widely used in the topical treatment of various skin diseases such as acne, psoriasis and photoaging. But, high chemical instability and skin irritation strongly limited its administration for topical delivery (Ridolfi et al., 2011). Transdermal delivery of tretinoin-loaded niosomal and liposomal formulation was studied through the newborn pig skin using Franz diffusion cells and compared with commercial formulation (RetinA®). The effect of charge on the performance niosomes was studied by the preparation of niosomes with either stearylamine and dicetylphosphate as a positive and negative charge inducer, respectively. Small unilamellar negatively charged niosomal formulations saturated with tretinoin showed more upper cutaneous drug retention than both commercial and liposomal formulation. Result of this study demonstrates cutaneous delivery of tretinoin is powerfully affected by vesicle morphology, composition and thermodynamic activity of the drug (Manconi et al., 2006). The ability of niosomes as topical carriers capable of improving the stability of photosensitive drugs was studied by comparing the chemical stability of tretinoin in methanol and in vesicular suspensions exposed both to UV and artificial daylight conditions. Liposomes were also prepared and compared with niosomes. In order to evaluate the influence of vesicle structure on the photostability of tretinoin, tretinoin-loaded vesicles were prepared by the film hydration method, extrusion technique and sonication. Methanol dissolved tretinoin degraded immediately after UV irradiation while the loaded drug into vesicles showed a considerable reduction of the photodegradation process. The photoprotection provided by vesicles varied depending on the vesicle structure and composition. In addition, unilamellar vesicles showed a higher protection of tretinoin than the multilamellar ones. Unilamellar niosomes made from Brij®30 were the formulations with the highest protection of tretinoin (Manconi et al., 2003). In another study, the antiacne activity of the mixture of aromatic volatile oil extracted from Thai medicinal plants enhanced with encapsulating into nano sized niosomal vesicles (Manosroi et al., 2008). Slow penetration of drug through skin is the main disadvantage of transdermal route of delivery. An increase in the penetration rate has been achieved by transdermal delivery of erythromycin incorporated in niosomes for acne therapy (Jayaraman et al., 1996).

6. Conclusion

Niosomal drug delivery systems have been demonstrated to be promising controlled drug delivery systems for percutaneous administration. Niosomes also offer successful drug

localization in skin which are relatively non-toxic and stable. This advantage of niosomes has the potential of strengthening the efficacy of the drug accompanying with reducing its adverse effects associated with drug systemic absorption. Drug-associated challenges such as physical and chemical instability is also can be protected by vesicular carriers. Niosomes appeared to be a well preferred drug delivery system over liposome as niosomes being stable and cost-effective. Hence, many topical drugs may be developed using niosomal systems. But there are still some challenges in this area. Although some new approaches have been developed to overcome the problem of drug loading, it is still remain to be addressed. The researchers should be more alert in the selection of suitable surfactant for noisome preparation due to this fact that the type of surfactant is the main parameter affecting the formation of the vesicles, their toxicity and stability.

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Chapter 7

Magnetic Nanoparticles: Synthesis, Surface Modifications and Application in Drug Delivery

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Additional information is available at the end of the chapter

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1. Introduction

Magnetic nanoparticles (MNP) have gained a lot of attention in biomedical and industrial applications due to their biocompatibility, easy of surface modification and magnetic properties. Magnetic nanoparticles can be utilized in versatile ways, very similar to those of nanoparticles in general. However, the magnetic properties of these particles add a new dimension where they can be manipulated upon application of an external magnetic field. This property opens up new applications where drugs that are attached to a magnetic particle to be targeted in the body using a magnetic field. Often, targeting is achieved by attaching a molecule that recognizes another molecule that is specific to the desired target area. This often requires a chemical recognition mechanism and does not succeed as designed. Therefore, magnetic nanoparticles can offer a solution to carry drugs to the desired areas in the body.

Magnetic nanoparticles, although may contain other elements, are often iron oxides. Most common iron oxides are magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), hematite (α-Fe_2O_3) and geotite. Depending on the experimental conditions, one or more of the iron oxide phases may form. It is very important to carefully control the experimental conditions to ensure the presence of a single-phase.

Frequently encountered iron oxide nanoparticles in applications are superparamagnetic. Superparamagnetism is a form of magnetism, which is observed with small ferromagnetic or ferrimagnetic nanoparticles. In small enough nanoparticles, magnetization can randomly flip direction of nanoparticle under the influence of temperature. However, the magnetic susceptibility of superparamagnetic nanoparticles is much larger than the paramagnetic ones. Superparamagnetism occurs in nanoparticles that have single-domain, i.e. composed of a single magnetic domain. In this condition, it is considered that the magnetization of the nanoparticles is a single-giant magnetic moment, the sum of all the individual magnetic

moments carried by the atoms of the nanoparticle. When an external magnetic field is applied to the superparamagnetic nanoparticles, they tend to align along the magnetic field, leading to a net magnetization. In the absence of an external magnetic field, however, the dipoles are randomly oriented and there is no net magnetization. The size dependence of magnetic properties of Fe_3O_4 nanoparticles synthesized from non-aqueous homogeneous solutions of polyols has been recently investigated (Caruntu et al., 2007). Out of the previously mentioned iron oxides, magnetite and maghemite are superparamagnetic and studies where these are used as magnetic nanoparticles will predominantly be focused in this summary.

Recent toxicity studies on magnetic nanoparticles are summarized to show the biocompatibility of these particles. Research on targeting drugs using MNPs show to be very promising and some examples are given. Hyperthermia, which is a complementary treatment for tumors that uses magnetic field to increase temperature and cause cell death, can be achieved using MNPs and some recent advances in this field are presented. At the end, a table summaries different types of MNP matrixes used for drug delivery applications.

2. Toxicity

One of the main reasons that made magnetic nanoparticles interesting for biomedical applications is their biocompatibility. As these particles are being used as drug delivery vehicles, their cytotoxicity should be investigated in detail. These particles have been shown to have low toxicity in human body by several *in vitro* and *in vivo* studies.

Ferric iron is normally transported by means of transferrin, which can bind the cell-surface localized transferrin receptor. Within the cell cytoplasm, the majority of the cytoplasmic iron pool is stored in specialized proteins called ferritin. Due to the physiological relevance of iron, MNPs were initially considered to be non-cytotoxic. MNPs can naturally be broken down resulting in the release of ferric iron which can then participate in the normal iron metabolism. It has, however, been recognized that the small size of MNPs might pose an additional hazard as the particles can reach high local concentrations within the cells and are generally more difficult to be efficiently cleared from the body (Rivera et al., 2010; Chan et al., 2002). Furthermore, free iron has been associated with the formation of free radicals, which would be particularly harmful to neural tissues already weakened by pathological processes (Winer et al., 2011).

It is important to note that in almost all the studies, the toxicity is shown to increase significantly above a certain administration level. Although high loadings (>100 μg/mL) of MNPs cause cytotoxicity, the concentrations needed for drug delivery applications are often below the toxic level for suitably coated MNPs (Karlsson et al., 2008).

Toxicity is often a result of serum proteins binding to the surface of the MNPs, altering the composition of the cell medium to which the cells are exposed (Mahmoudi et al., 2009). Coated nanoparticles induce lower toxicity not only due to the presence of the biocompatible coating, but also due to the lower adsorption sites for proteins, ions and other components in the medium (Mahmoudi et al., 2010).

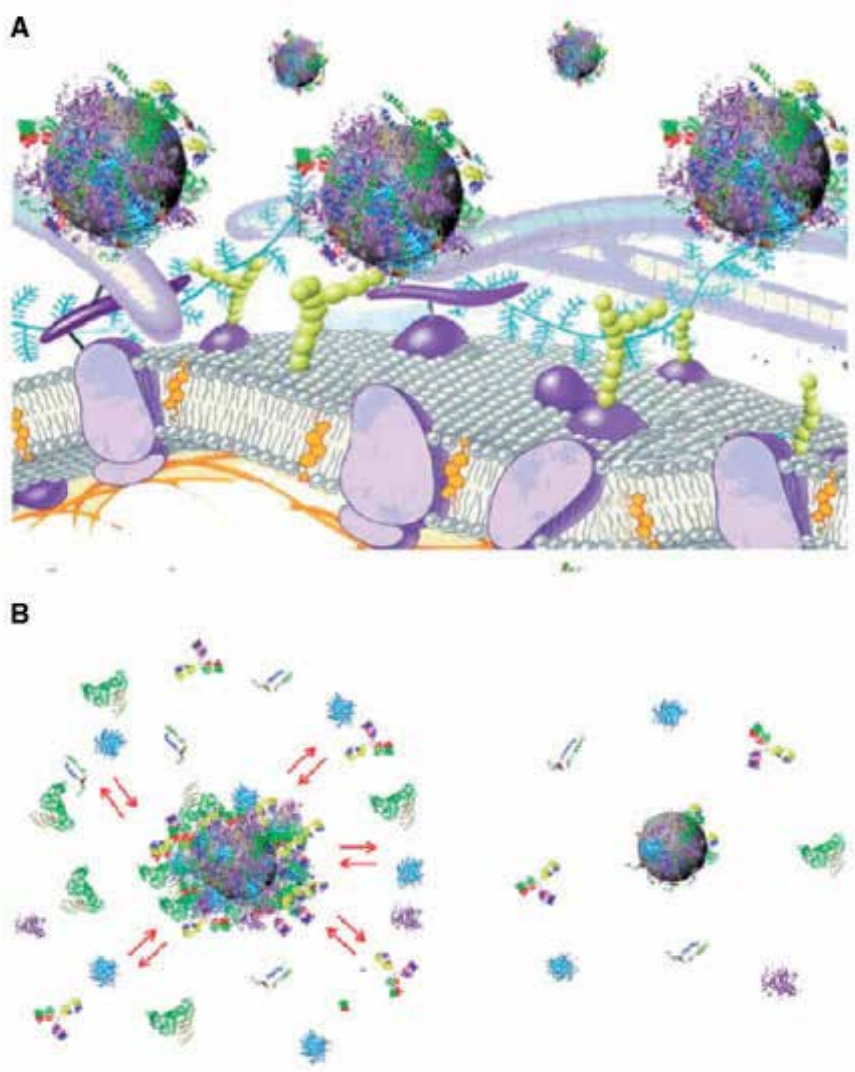

Figure 1. (A) Schematic representation of the possible exchange/interaction scenarios at the bionanointerface at the cellular level. (B) Schematic drawing of the structure of protein–nanoparticle in blood plasma confirming the existence of various protein binding (e.g. an outerweakly interacting layer of protein (full red arrows) and a hard slowly exchanging corona of proteins (right) (Mahmoudi et al., 2011)

Magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) can show different cellular responses because of their ability to undergo oxidation/reduction reactions. In fact, magnetite has been shown to cause higher levels of oxidative DNA lesions (using comet assay) in A549 human lung epithelial cell line in the absence of decreased cell viability as compared to maghemite owing to its potential to undergo oxidation (Karlsson et al., 2009; Karlsson et al., 2008).

One of the most sensitive parameters in toxicity is the surface coating of the nanoparticles. The degree of surface coverage has been postulated to be the main parameter in cellular

uptake as incomplete surface coverage was shown to promote opsonization and rapid endocytosis whereas fully coated MNPs escaped opsonization which, as a result, prolonged plasma half-life (Jung et al., 1995). The negatively charged uncoated MNPs have been shown to exhibit cytotoxicity above a certain threshold amount. Uncoated MNPs also have low solubilities which result in their precipitation in aqueous media impeding blood vessels in *in vivo* studies. In order to reduce the toxicity of MNPs, different coatings have been used. Häfeli at al. (Häfeli et al., 2009) have coated MNPs with polyethylene oxide (PEO) triblock copolymers (PEO-COOH-PEO) and found that the PEO tail block length inversely correlates with toxicity. PEO tail lengths above 2 kDa were suggested to be suitable for *in vivo* applications. Mahmoudi et al. (Mahmoudi et al., 2009) showed that uncoated particles induce greater toxicity than polyvinyl alcohol (PVA) coated magnetite particles. They also have shown that the toxicity of uncoated particles may significantly be reduced by substitution with surface-saturated uncoated particles. Coating maghemite particles with dimercaptosuccinic acid (DMSA) were shown to almost eliminate the toxicity of these particles (Auffan et al., 2006) by preventing direct contact between the particle and human dermal fibroblasts. However, in a different study using DMSA coated maghemite particles, a quantifiable model cell system is developed and showed that intercellular delivery of even moderate levels of MNPs may adversely affect cell function (Pisanic et al., 2007). Maghemite particles were coated with polyethylene imine (PEI)-g-polyethylene glycol (PEG) and their toxicity was compared with branched PEI coatings (Schweiger et al., 2011). Introduction of PEG was shown to have a shielding effect and resulted in lower toxicity Lee et al. (Lee et al., 2011) used ethylene glycol double layer stabilized maghemite nanoparticles and showed these to be non-toxic. PEG coating of magnetite particles also were shown to reduce the toxicity (Zhou et al., 2011).

When MNPs are embedded in chitosan to obtain magnetic chitosan particles, they have shown to exhibit relatively low cytotoxicity (Park et al., 2005) due to complete coverage of MNPs with chitosan.

Although dextran is a complex branched glucose that is often used in medical applications, dextran coated magnetite particles caused cell death as much as uncoated magnetite particles (Berry et al., 2003). Conversely, in a comparative study, uncoated magnetite, uncoated maghemite, dextran coated magnetite and dextran coated maghemite were investigated for cytotoxicity and neither of the samples exhibited cytotoxicity below 100 mg/mL and the only samples that demonstrated genotoxicity was the dextran coated maghemite (Singh et al., 2012). In a more extensive study, Ding et al. (Ding et al., 2010) showed that the cytotoxicity of dextran hybridized magnetite nanoparticles is cell-specific. This result suggests that the related cells should be concerned for the cytotoxicity evaluation.

A range of secondary surfactants around magnetic particles have been tested for toxicity *in vivo*. Citric and alginic acid surfactants were found to be significantly less toxic than starch, decanoic acid and PEG. This study shows the importance of optimizing surface coating to minimize toxicity (Kuznetsov et al., 1999).

In an *in vivo* study, albumin coated magnetite microspheres were shown to be well tolerated (Kuznetsov et al., 1999) Magnetite albumin microspheres bearing adriamycin (an anti-cancer drug) showed reduced toxicity to animal organs or cells compared to a single dose of adriamycin which reduces the side effects remarkably (Ma et al.,2000). However, in another study, albumin derivatized MNPs were found to cause membrane disruption, possibly due to the interaction of the protein with membrane fatty acids and phospholipids (Berry et al., 2003).

Low cytotoxicity compared to uncoated magnetite particles was evaluated of Fe_3O_4–PLLA–PEG–PLLA (PLLA: poly L-lactic acid) particles at the cellular level. They also create low genotoxic and immuntoxic at the molecular level. Acute toxicity tests showed quite a low toxicity which makes them have great potential for use in biomedical applications (Chen et al., 2012). Magnetite encapsulated in micelles of MPEG-PLGA (PLGA: poly (lactic-co-glycolic acid) exhibited no cytoxicity (Ding et al., 2012).

More recently, particle size as opposed to coating degree has been suggested to exert chief influence on the rates of uptake by macrophages (Raynal et al., 2004). In *in vivo* studies, MNPs of 50 nm (dextran coated) and 4 μm (polystyrene coated) were used and shown to be safe for intraocular applications (Raju et al., 2011). Oral, intravenous and intraperitoneal administration of MNPs of about 20 nm did not exhibit toxicity (Zefeng et al., 2005). MNPs with 1,6 hexanediamine were shown to be safe after being administered by intracerebral or intraarteral inoculation to rats (Muldoon et al., 2005). MNPs of 40 nm were shown to be non-toxic to mES cells (Shundo et al., 2012).

Under the application of magnetic field, MNPs were shown to exhibit higher toxicities which lead to cell death (Simioni et al., 2007; Bae et al., 2011). This is the basis of a tumor treatment, hyperthermia, which will be summarized in detail in the later sections.

Despite such routine use of MNPs, the long-term effects and potential neurotoxicity have, as yet, not been evaluated extensively (Yildirimer et al., 2011).

The ability to use magnetic nanoparticles in biomedical applications due to their low cytotoxicity, stirred a big interest in the scientific community to use these particles as drug carriers. In drug delivery, there are mainly two goals; first is the targeting of the drug to the desired area in the body to reduce the side effects to other organs and second is the controlled release of the drug to avoid the classical overdosing/underdosing cycle. Magnetic nanoparticles may provide a solution to both these goals. The coating around the magnetic nanoparticle is optimized to carry and release the drug in the desired fashion, like in the case of most nanoparticles. However, the unique property of these particles is that they are magnetic, allowing being manipulated using an external magnetic field. This forms the basis of magnetic targeting where the drug-carrying magnetic particle is directed to a specific area upon application of a magnetic field.

3. Magnetic targeting

In order to investigate the magnetic targeting *in vitro*, an experimental setup that models a branched artery supplying a tumor region with parameters close to the real system has been

constructed. The targeting of the particles was achieved and found to be dependent on the magnetic volume force in the branch point (Gitter et al., 2011). Using the same set-up, a novel quantitative targeting map that combines magnetic volume forces at characteristic points, the magnet position and quantitative data was constructed. Up to 97% of the nanoparticles were successfully targeted into the chosen branch (Gitter et al., 2011).

A device for magnetically targeted drug delivery system (MT-DDS), which can allow to navigate and to accumulate the drug at the local diseased part inside the body by controlling to magnetic field strength and/or gradient generated by the superconducting magnets was developed. Mn-Zn ferrite particles are injected to an experimental apparatus as a vein model of the Y-shaped glass tubes using multiple bulk superconductor magnets. This is a basic technology for magnetically targeted drug delivery system that provides the drug navigation in the blood vessel of the circulatory organs system, which shows the usefulness of the medicine transportation methodology for MT-DDS (Mishima et al., 2007).

To test seeding MNP in blood vessels and targeting the injected ones to these specific sites, experimental and computational models are constructed. To create strong and localized field gradients, microfluidic channels embedded with magnetic anchors were constructed using modified soft lithographic techniques to analyze the trapping process. Qualitative results from experimental investigations confirmed the legitimacy of the approach. It is demonstrated that capturing and aggregating magnetic microspheres at specified points in the vascular system is possible (Forbes et al., 2003).

Locally targeted drug delivery using two magnetic sources was theoretically modeled and experimentally demonstrated as a new method for optimizing the delivery of magnetic carriers in high concentration to specific sites in the human body. Experimental results have demonstrated that capturing superparamagnetic beads of both micrometer and sub-micrometer diameter at reasonably high concentrations is possible in flow conditions consistent with the dimensions and flow velocity occurring in the coronary artery in the human body. The same experiments performed with non-magnetic mesh resulted in no significant capture, indicating that the implant is responsible for providing the necessary magnetic field gradients and forces to capture the injected beads (Yellen et al., 2005).

There are several *in vivo* studies on magnetic targeting. Magnetic chitosan nanoparticles, were successfully targeted to tumor tissue for photodynamic therapy, resulting in low accumulation in skin and hepatic tissue (Sun et al., 2009).

Magnetic carbon nanotubes (MNT) with a layer of magnetite nanoparticles on their inner surface were prepared where the chemotherapeutic agents were incorporated into the pores. By using an externally placed magnet to guide the drug matrix to the regional lymph nodes, the MNTs are shown to be retained in the draining targeted lymph node for several days and continuously release chemotherapeutic drugs (Yang et al., 2008).

In an *in vitro* study, magnetic poly(ethyl-2-cyanoacrylate) (PECA) nanoparticles containing anti-cancer drugs were shown to release drug and have magnetic mobility under external magnetic field (Yang et al., 2006).

Intra-caroid administration of polyethyleneimine (PEI) modified magnetic nanoparticles in conjunction with magnetic targeting resulted in 30 fold increase in tumor entrapment of particles compared to that seen with intravenous administration (Chertok et al., 2010).

Magnetite-dextan composite particles were employed to deliver mitoxantrone *in vivo*. Mitoxantrone concentration in tumor tissue was found to be always significantly higher with magnetic targeting and the plasma iron concentrations fell after the application of the magnet, indicating the effectiveness of magnetic targeting (Krukemeyer et al., 2012).

In another study, mitoxantrone was bound to superparamagnetic Fe_3O_4-nanoparticles and the drug loaded nanoparticles were given through the femoral artery close to the tumor. The magnetic nanoparticles were attracted to the tumors by a focused external magnetic field during the application. Results from HPLC-biodistribution experiments showed that magnetic drug targeting allows to enrich the therapeutic agent up to 50 times higher in the desired body compartment (i.e. the tumor region) compared to the commonly used systemic application (Alexiou et al., 2011).

Magnetic nanoparticle seeds composed of magnetite carboxyl modified polydivinylbenzene and containing magnetite were studied *in vitro* for use as an implant in implant assisted-magnetic drug targeting (IA-MDT). In the presence of a 70mT external magnetic field, the MNP seeds were captured first from a fluid stream passing through a 70% porous polymer scaff old that was designed to mimic capillary tissue. This is then used to capture magnetic drug carrier particles (MDCPs) with the same magnetic field (Mangual et al., 2011).

Poly-[aniline-co-N-(1-one-butyric acid) aniline] (SPAnH) coated Fe_3O_4 particles with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Bound-BCNU-3 could be concentrated at targeted sites *in vitro* and *in vivo* using an externally applied magnet. When applied to brain tumors, magnetic targeting was found to increase the concentration and retention of bound-BCNU-3 (Hua et al., 2011).

The accumulation of superparamagnetic nanoparticles with starch coating in gliosarcomas were enhanced by magnetic targeting and quantified by MR imaging (Chertok et al., 2008).

PEG-modified cross linked starch coated magnetite particles for magnetic targeting studies *in vivo*. Selective, enhanced brain tumor targeting of intravenously administered PEG-MNPs was confirmed in a 9L-glioma rat model. Tumor targeting results, were promising and warranted both the further development of drug-loaded PEG-MNPs and concurrent optimization of the magnetic targeting strategy utilized (Cole et al., 2011).

Super high-magnetization nanocarriers (SHMNCs) comprising of a magnetic Fe_3O_4 (SHMNPs) core and a shell of aqueous stable self-doped poly[N-(1-onebutyric acid))aniline (SPAnH), which have a high drug loading capacity (27.1 wt%) of doxorubicin (DOX) were prepared. These nanocarriers enhanced the drug's thermal stability and maximized the efficiency with which it is delivered by magnetic targeting therapy to MGH-U1 bladder cancer cells, in part by avoiding the effects of p-glycoprotein (P-gp) pumps to enhance the intracellular concentration of DOX (Hua et al., 2011).

Magnetic particles are also targeted to tumor area so tumors can be imaged. Iron oxides particles are often used as contrast agents for MRI. In fact, magnetite is an FDA approved contrast agent. In this summary, magnetic particles used for MRI will not be covered as the focus of this study is to make a comprehensive summary on magnetic drug delivery.

As seen in the abovementioned studies, magnetic targeting is an efficient way to target drugs to the desired area, commonly to tumors. However, in some studies along with magnetic targeting, targeting ligands are also used. In the absence of magnetic targeting, targeting is achieved using ligands on drug carriers that specifically bind to receptors in the targeted area. A common ligand used for this purpose is folate (or folic acid). Folate has a high affinity for the folate receptor protein which is commonly expressed on the surface of many human cancers. If folate is tagged to a drug carrying nanoparticle, the folate binds to the folate receptor on the surface of cancer cell and the conjugate is uptaken via endocytosis, completing the targeted drug delivery. A schematic representation of a magnetic particle with targeting ligands is shown in Figure 2.

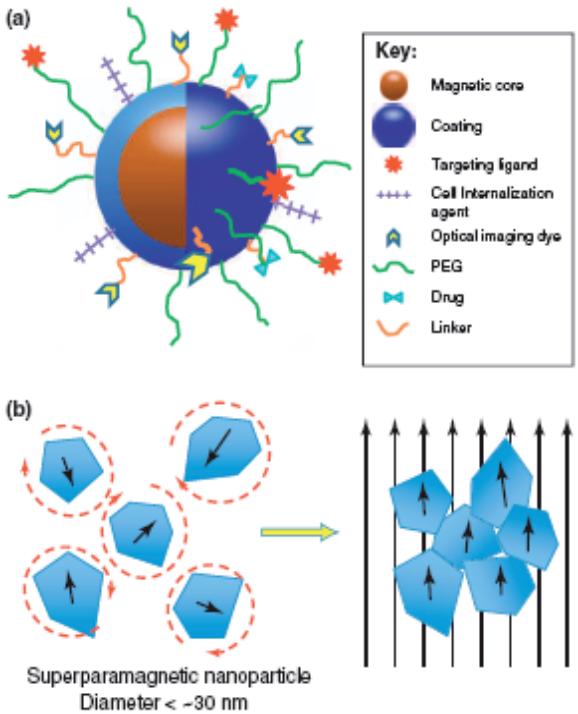

Figure 2. (a) Schematic representation of the ''core–shell'' structure of MNPs and multi-functional surface decoration. MNPs consist of a magnetic iron oxide core coated with a biocompatible material (e.g. polysaccharide, lipid, protein, small silane linkers, etc.). Functional groups on the surface of coatings are often used to link ligands for molecular targeting, cellular internalization, optical imaging, enhanced plasma residence and/or therapy. The variety of moieties that decorate the MNP surface imparts the nanoparticle with its multi-functional, theranostic character. (b) Illustration of superparamagnetic MNP response to applied magnetic fields. MNPs comprise rotating crystals that align with the direction of an applied magnetic field. Crystal reorientation provides the high magnetic

susceptibility and saturation magnetization observed for this material. The circular dashed lines around the superparamagnetic nanoparticles on the left illustrate the randomization of their orientation, due to temperature effects, in the absence of a magnetic field. (Cole et al.2011).

Magnetic nanocarriers were synthesized based on superparamagnetic iron oxide particles with biocompatible Pluronic F127 and poly(dl-lactic acid) (F127-PLA) copolymer chemically conjugated with folic acid (FA), carrying DOX. Magnetic particles were guided to targeted site by the aid of external magnetic field, and correspondingly the therapeutic efficacy of anti-tumor drug can be improved. These qualitative results were carried out with simply statistical analysis, which suggested that the dual targeting mechanisms can lead to better therapeutic results (Huang et al., 2012).

Superparamagnetic iron oxide nanocrystals and DOX are co-encapsulated into PLGA/polymeric liposome core–shell nanocarriers withcholesterol with or without folate. The folate-targeting DOX loaded magnetic core–shell nanocarriers were shown to have better targeting effect to the Hela cells *in vitro* than their non-folate targeting counterparts (Wang et al., 2012).

Thermosensitive magnetic liposomes with DPPC:cholesterol:DSPE-PEG2000:DSPE-PEG2000-Folate (DPPC: Dipalmitoylphosphatidylcholine; DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) at 80:20:4.5:0.5 molar ratio were prepared containing DOX. This carrier, when physically targeted to tumor cells in culture by a permanent magnetic field yielded a substantial increase in cellular uptake of DOX as compared to Caelyx® (a commercially available liposomal doxorubicin preparation), non-magnetic folate-targeted liposomes (FolDox) and free DOX in folate receptor expressing tumor cell lines (KB and HeLa cells) (Pradhan et al., 2010).

Magnetic nanoparticles with mesoporous core-shell structure of silica were prepared and successfully modified with a fluorescent polymer chain as a labeling segment and folic acid as the cancer targeting moiety and loaded with a drug for directional release. The drug carrier was shown to be able to drill into the cell membranes and obtain a sustained release of the anticancer drug into the cytoplasm. The *in vitro* cellular uptake of the drug demonstrated that the drug-loaded nanocomposites could effectively target the tumor cells (Chen et al., 2010).

Nanoparticles of Fe_3O_4 core with fluorescent SiO_2 shell were synthesized and grafted with hyperbranched polyglycerol (HPG-grafted Fe_3O_4@SiO_2 nanoparticles) conjugated with folic acid. Significant preferential uptake of the folic acid-conjugated nanoparticles by human ovarian carcinoma cells (SKOV-3) as compared to macrophages and fibroblasts were shown by *in vitro* studies (Wang et al., 2011).

Magnetite nanoparticles are decorated through the adsorption of a polymeric layer (carboxymethly chitosan) around the particle surface and are conjugated with fluorescent dye, targeting ligand, and drug molecules for improvement of target specific diagnostic and possible therapeutics applications. Acrylic acid, folic acid, particles (Fe_3O_4-CMC-AA-FA) and DOX was loaded into the shell of the MNPs and release study was carried out at

different pH. The Fe_3O_4-CMC-AA-FA-DOX NPs showed a significant growth inhibition for HeLa cells in a dose dependent manner in comparison to NIH3T3 cells. This study indicates that Fe_3O_4-CMC-AA-FA is able to provide a single nanoscale construct, which is capable of tumor cell-targeting, imaging, and drug delivery functions. This is the first description of a chitosan based MNPs system possessing all of the above mentioned capabilities (Sahu et al., 2012).

Other ligands than folate have also been used for active targeting of nanoparticles. DOX on 5-carboxylfluorescein (FAM) labeled AGKGTPSLETTP peptide (A54) coupled starch-coated iron oxide nanoparticles demonstrated the specificity of DOX-loaded A54-SIONs (SION: superparamagnetic iron oxide) to BEL-7402 cells *in vitro*. The microscopy images proved that DOX-loaded A54-SIONs were successfully targeted to tumor tissue of nude mice with an external magnetic field *in vivo* (Yang et al., 2009).

Ligand-modified CPT-SAIO@SiO_2 nanocarriers were used for the delivery of an anticancer agent (encapsulated camptothecin (CPT)). It was found that the modified nanocarriers showed reasonably high drug load efficiency for CPT and a high uptake rate by cancer cells overexpressing EGFR through clathrin-mediated endocytosis. The intracellular release of the CPT molecules via an external magnetic stimulus proved to be technically successful and ensured much higher therapeutic efficacy than that obtained with the free drug (Tung et al., 2011).

Cetuximab-immuno micelles in which the anti-EGFR (Epidermal growth factor receptor) (EGFR), monoclonal antibody was linked to poly(ethylene glycol)-block-poly(ε-caprolactone) (PEG–PCL) These micelles were loaded with DOX and Fe_3O_4 superparamagnetic iron oxide. It was demonstrated that the immunomicelles inhibited cell proliferation more effectively than their nontargeting counterparts. Cetuximab-immunomicelles bind more efficiently to the cancer cells that overexpress epidermal growth factor receptor, leading to a higher quantity of superparamagnetic iron oxide and DOX being transported into these cells (Liao et al., 2011).

An anticancer drug was conjugated onto the PEGylated SPIO (SPIO: superparamagnetic iron oxide) nanocarriers via pH-sensitive bonds. Tumor-targeting ligands, cyclo(Arg-Gly-Asp-D-Phe-Cys) (c(RGDfC)) peptides, and PET 64Cu chelators, macrocyclic 1,4,7-triazacyclononane-N, N0, N00-triacetic acid (NOTA), were conjugated onto the distal ends of the PEG arms. cRGD-conjugated SPIO nanocarriers exhibited a higher level of cellular uptake than cRGD-free ones *in vitro*. These nanocarriers demonstrated promising properties for combined targeted anticancer drug delivery and PET/MRI dual-modality imaging of tumors (Yang et al., 2011).

Polymeric liposomes (PEG/RGD-MPLs); composed of amphiphilic polymer octadecyl-quaternized modified poly (γ-glutamic acid) (OQPGA), PEGylated OQPGA, RGD peptide grafted OQPGA and magnetic nanoparticles. It provided a possibility to responded to external permanent magnet with superparamagnetic characteristics, when was used for magnetic tissue targeting *in vivo*. The cell uptake results suggested that the PEG/RGDMPLs

(with RGD and magnetic particles) exhibited more drug cellular uptake than non RGD and non magnetism carriers in MCF-7 cells (Su et al., 2012).

All these studies show that magnetic targeting is an efficient way to target drugs to tumor area. Coupled with active targeting using appropriate ligands, ligand-modified drug loaded magnetic nanoparticles, upon application of an external magnetic field provide excellent systems for effective drug targeting. Once targeting of magnetic particles to the desired area takes place, one of the most frequently used tumor treatments is hyperthermia. When magnetic nanoparticles are in the vicinity of the tumor and are subjected to an alternating magnetic field, dissipate heat and raise the temperature of the tumor, resulting in tumor cell death.

4. Hyperthermia treatment

Temperatures between 40°C and 45°C are generally being referred to as hyperthermia. Temperatures up to 42°C can render cancer cells more susceptible to the effect of irradiation and cause a certain degree of apoptosis, whereas temperatures >45°C are termed thermoablation and cause direct cell killing (necrosis) (Elsherbini et al., 2011).

In clinical applications of magnetic nanoparticle hyperthermia for cancer treatment it is very important to ensure a maximum damage to the tumor while protecting the normal tissue (Salloum et al., 2009). Although magnetic nanoparticle hyperthermia in cancer treatment holds great potential, it is severely limited by the fact that the anticipated heating distribution is difficult to control, and it leads to uneven and inadequate temperature elevation in tumor tissue. Transport of particles in tissue involves processes including extracellular transport of the carrier solution, transport of particles in the carrier solutions, and interaction between the particles and cell surface. The extracellular transport of nanoparticles in tumors is not well understood (Salloum et al., 2008).

Hyperthermia is almost always used with other forms of cancer therapy, such as radiation therapy and chemotherapy. Hyperthermia may make some cancer cells more sensitive to radiation or harm other cancer cells that radiation cannot damage. When hyperthermia and radiation therapy are combined, they are often given within an hour of each other. Hyperthermia can also enhance the effects of certain anticancer drugs (Van der Zee, 2002; Wust et al., 2002).

Numerous clinical trials have studied hyperthermia in combination with radiation therapy and/or chemotherapy. These studies have focused on the treatment of many types of cancer, including sarcoma, melanoma, and cancers of the head and neck, brain, lung, esophagus, breast, bladder, rectum, liver, appendix, cervix, and peritoneal lining (mesothelioma) (Falk et al., 2001; Feldman et al., 2003; Chang et al., 2001). Many of these studies, but not all, have shown a significant reduction in tumor size when hyperthermia is combined with other treatments. However, not all of these studies have shown increased survival in patients receiving the combined treatments (Van der Zee, 2002; Wust et al., 2002).Unique advantages of magnetic nanomaterials for hyperthermia based and combined therapies are schematically shown in Figure 3.

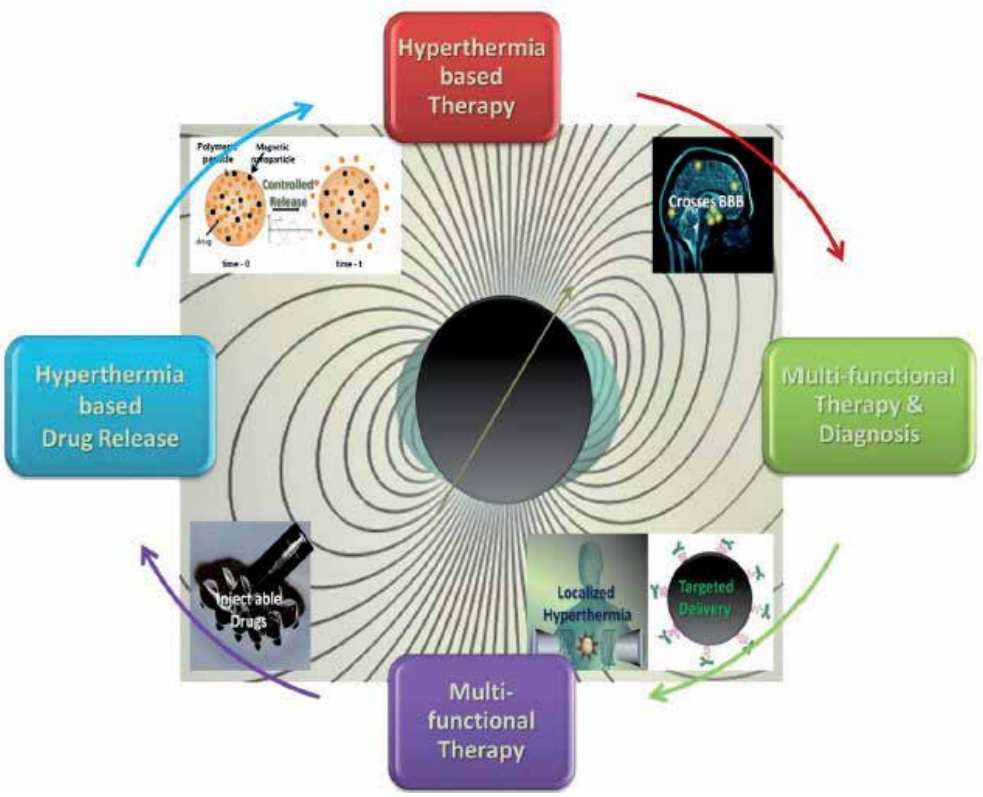

Figure 3. A schematic representation of some of the unique advantages of magnetic nanomaterials for hyperthermia-based therapy and controlled drug delivery (Kumar & Mohammad, 2011).

Magnetic losses in an alternating magnetic field to be utilized for heating arise due to different processes of magnetization reversal in the particle system: (1) hysteresis, (2) Néel or Brown relaxation, and (3) frictional losses in viscous suspensions (Hergt et al., 2006).

The magnetization of superparamagnetic nanoparticles can spontaneously change orientation under the influence of thermal energy. The magnetization oscillates between its two equilibrium positions. The typical time between two orientation changes is given by the Néel relaxation time $\tau_N = \tau_0 e^{\frac{KV}{k_B T}}$, where τ_0 is an attempt time with a value around 10^{-9}-10^{-10} seconds.

In the absence of a magnetic field, magnetic nanoparticles in solution move randomly, a movement called Brownian motion. When magnetic field is applied to magnetic nanoparticles in a fluid, magnetic nanoparticles rotate and progressively align with the magnetic field due to the torque generated by the interaction of the magnetic field with the magnetization. The time taken for a magnetic nanoparticle to align with a small external magnetic field is given by the Brown relaxation time: $\tau_B = \frac{3\eta V}{k_B T}$, where η is the solvent viscosity. The delay between the magnetic field rotation and the magnetization rotation

leads to a hysteresis. The area of this hysteresis loop is dissipated in the environment as thermal energy, which used in magnetic hyperthermia.

When an alternating magnetic field (AMF) is applied to a magnetic material, due to magnetic hysteresis, an energy is dissipated called the Specific Absorption Rate (SAR) and is expressed in W/g of nanoparticles.

The *SAR* of a given material is given by $SAR = Af$, where A is the area of the hysteresis loop and f the alternation frequency of the magnetic field. A is expressed in J/g and is also called the "specific losses" of the material, hence SAR may also be referred to as Specific Loss Power (SLP) in some studies.

The value of SAR estimated for the same material by several research groups may vary because it depends on several parameters like the physical and chemical properties of the carrier fluid, coating materials, frequency and amplitude of applied field, size and shape of Fe_3O_4 nanoparticles (Elsherbini et al., 2011).

An optimized SAR distribution in terms of A is developed by optimizing an algorithm to inversely determine the optimum heating patterns induced by multiple nanoparticle injections (Salloum et al., 2009). For hyperthermia applications, high SAR values are required. One way to achieve this is to increase the magnetic field strength but the average magnetic field strength should be kept below 30 mT to avoid the formation of eddy currents, which can induce toxicity (Alphandéry et al., 2012).

The study of SPA as a function of particle size shows that the average size and size distribution of the nanoparticles constituting a heating agent are central parameters for the design of efficient heating nanoparticles (Goya et al., 2008).

Unfortunately a direct comparison of particle composition and size is very difficult to make. In one study multidomain ferrite particles were prepared and SAR data is compared with small magnetite particles with and without dextran coating. Large ferrite particles (200-400 nm) had considerably lower power absorption per mass than smaller particles of the same composition although both particle size distributions were relatively broad (Jordan et al., 1993). However particles with large sizes are shown not reach inner cell (Martín-Saavedra et al., 2010).

By performing calorimetry measurements with Pluronic F127 coated Fe_3O_4 monodisperse particles it was shown that at a given frequency, heating rates of superparamagnetic particles are dependent on particle size, in agreement with earlier theoretical predictions. Results also indicate a broadening of SLP with sample polydispersity as predicted (Gonzales-Weimuller et al., 2009).

Similarly, a mean particle diameter in the single domain size range (20–70 nm) combined with a small size distribution width are shown to enhance SLP (Hergt et al., 2007).

Previous studies have shown a linear relationship between tissue iron concentration and heating rate in targeted magnetic hyperthermia treatment (Pardoe et al., 2003). A critical component of arterial embolization hyperthermia (AEH) is shown to be the concentration and distribution of ferromagnetic particles in the normal hepatic parenchyma (NHP), as well as in

the tumor tissue. If the distribution of particles in NHP is heterogeneous, with areas of high concentration, then unwanted areas of necrosis may result during AEH (Moroz et al., 2002).

In another study, several types of magnetic iron oxide nanoparticles representative for different preparation methods (wet chemical precipitation, grinding, bacterial synthesis, magnetic size fractionation) are used for a comparative study (Hergt et al., 2006). Commercially available very small superparamagnetic particles are claimed to be suboptimal for effective tumor heating. In contrast, superparamagnetic magnetite nanoparticles were shown to be appropriate for inducing hyperthermia with radiofrequency to Ehrlich tumors (Elsherbini et al., 2011).

No correlation was found between the magnetic moment of a single particle and SPA values for MNPs in the superparamagnetic regime. The optimum particle diameter is suggested to be near the critical size for the single- to multi-domain transition for Fe_3O_4 phase, although the relation between SPA mechanisms and incipient domain walls is still to be determined (Goya et al., 2008).

When using magnetic nanoparticles as a heating source for magnetic particle hyperthermia it is of particular interest to know if the particles are free to move in the interstitial fluid or are fixed to the tumor tissue. The immobilization state determines the relaxation behaviour of the administered particles and thus their specific heating power (Dutz et al., 2011). If the particles are not able to rotate and a temperature increase due to Brown relaxation can be neglected. An investigation showed that carboxymethyl dextran coated magnetic particles are fixed rather strongly to the tumor tissue after injection into a tumor (Dutz et al., 2011).

The effect of Néel relaxation on magnetic nanoparticles unable to move or rotate are studied and losses in linearly and circularly polarized fields are compared (De Châtel et al., 2009). In frequencies lower than the Larmor frequency, linear polarization is found to be the better source of heat power, at high frequencies (beyond the Larmor frequency) circular polarization is preferable. If Néel relaxation in isotropic sample is the dominant mechanism, the technical complications of generating a circularly polarized field in difficult geometry need not be considered.

In order to reach the required temperature with minimum particle concentration in tissue the specific heating power (SHP) of MNP should be as high as possible. The dependence of specific heating power of the size of superparamagnetic particles on the frequency and amplitude of the external alternating magnetic field is found to obey the predictions of relaxation theory. For small mean sizes (about 6 nm) the heating capability is negligibly small whereas larger particles deliver heating suitable for hyperthermia (Glöckl et al., 2006).

Data on SLP commonly reported in the literature show remarkable scattering of the orders of magnitude of 10–100W g^{-1} for a field amplitude of 10 kA m^{-1} and frequency of about 400 kHz (Hergt et al., 2006).

In summary:

1. The SLP of MNP must be considerably increased for achieving useful therapy temperatures in small tumors (at present smaller than 10mm diameter).

2. The main practical problem with MPH is an inadequate MNP supply to the tumor. For IT injection inhomogeneity of MNP distribution in tissue may lead to local temperature differences which do not allow for differentiation of hyperthermia and thermoablation. As a result of insufficient temperature enhancement in parts of the tumor there is a risk of proliferation of surviving tumor cells.
3. For systemic supply of MNP (e.g. antibody targeting) the target enrichment with MNP must be considerably enhanced for achieving therapy temperature. In particular, the therapy of small targets (metastases below presently diagnostic limit) seems to be a questionable hope (Hergt et al., 2007).

The specific loss power useful for hyperthermia is restricted by serious limitations of the alternating field amplitude and frequency. Large values of SLP of the order of some hundreds of W g^{-1} at 400 kHz and 10 kA m^{-1} are found for particles with mean size of about 18 nm provided that the size distribution is sufficiently narrow. A very large value of SLP of nearly 1 kW g^{-1} is found for bacterial magnetosomes having a mean diameter of the magnetite crystals of about 35 nm (Hergt et al., 2006).

MNPs modified with amino silane, which is commonly used in biomedicine, bacterial magnetosomes (BM) exhibit a better heating effect under AMF. Although both particles are found to enhance reduction in cell viability by hyperthermia using MNPs and magnetosomes of the same concentration, current of lower intensity is needed by BMs to produce a similar inhibitory effect in the tumor cell (Liu et al., 2012).

When chains of magnetosomes, which are bound to each other by a filament made of proteins, are incubated in the presence of cancer cells and exposed to an alternating magnetic field of frequency 198 kHz and average magnetic field strength of 20 or 30 mT, they produce efficient inhibition of cancer cell proliferation. This behavior is explained by a high cellular internalization, a good stability in solution and a homogenous distribution of the magnetosome chains, which enables efficient heating (Alphandéry et al., 2012).

When magnetosome chains are heated, the filament binding the magnetosomes together is denatured and individual magnetosomes are obtained which are prone to aggregation, are not stable in solution and do not produce efficient inhibition of cancer cell proliferation under application of an alternating magnetic field (Alphandéry et al., 2012).

Poly(ethylene glycol) methyl ether methacrylate and dimethacrylate with iron oxide as implantable biomaterials. It was demonstrated that the temperature of the hydrogels can be controlled by changing the AMF strength so that the gels either reached hyperthermic (42–45 °C) or thermoablative (60–63 °C) temperatures. The final temperature the hydrogel nanocomposites reach can be tailored to either one of these temperature ranges. The hydrogels were heated in an AMF, and the heating response was shown to be dependent on both iron oxide loading in the gels and the strength of the magnetic field (Meenach et al., 2010).

Cationic magnetoliposome containing both magnetic fluid and the photosensitizer-based complex (CB:ZnPc-ML) were prepared using the thin lipid film method. This result shows that the application of light and AC magnetic field together can be much more effective than the each of the two treatments applied separately (Bolfarini et al., 2012).

Combined effect of magnetic hyperthermia and chemotherapy was evaluated using drug loaded PCPG magnetoliposomes. Thermosensitive drug release took place under the influence of magnetic field and this combined therapy was shown to be more efficient than either treatment alone (Kulshrestha et al., 2012).

It was demonstrated that the temperature achieved with ferromagnetic MNPs was higher than that achieved with superparamagnetic MNPs, even with the same uptake amount into cells. This is due to heating efficiency differences between hysteresis loss and magnetic relaxation. Heat generation predominantly occurs by hysteresis loss rather than by magnetic relaxation. Heat produced by nanoparticles incorporated into cells and adsorbed on cell membranes should be critical for damaging cells, compared with heat produced from outside cells (Baba et al., 2012).

According to some, the well-known iron oxide ferro fluids become undesirable because their iron atoms are poorly distinguishable from those of hemoglobin. A suggested solution is to use mixed-ferrites (MFe_2O_4 where M¼Co, Mn, Ni, Zn) to have a range of magnetic properties. These ferrites have attracted special attention because they save time, and because of their low inherent toxicity, ease of synthesis, physical and chemical stabilities and suitable magnetic properties (Sharifi et al., 2012).

Giri et al. studied citrate coated ferrite particles below 100 nm sizes. Saturation magnetization is found to decrease for coated materials as magnetization is proportional to the amount of weight for the same magnetic material. The coercivity is found to be sufficient for hysteresis loss heating in hyperthermia. The magnetic hysteresis data indicate that these samples (coated) exhibit sufficient hysteresis losses to obtain the temperature required for the destruction of the tumorous cells.

Ferrite particles were prepared in a chitosan matrix at varying ratios (Park et al., 2005). The time period needed for reaching hyperthermia shortened upon increase of chitosan ratio while the saturation magnetization decreases. Optimization of ferrite-chitosan ratio may be promising for hyperthermia applications.

Co-Ti ferrite nanoparticles of 6-12 nm were shown to be suitable for hyperthermia applications (Ichiyanagia et al., 2012). Zn-Gd ferrite particles are suitable but if you cap them with poly (ethylene glycol) PEG, they are not useful (Yao et al., 2009). Co ferrite particles of 7.5 nm copolymerized with poly(methacrylate) and poly(2-hydroxyethylmethacrylate) were shown to be suitable for hyperthermia (Hayashi et al., 2012). $CoFe_2O_4$ ferrite particles (ferromagnetic) were shown to be suitable for hyperthermia (Skumiel, 2006).

Examining the heating produced by nanoparticles of various materials, barium-ferrite and cobalt-ferrite are unable to produce sufficient MFH heating, that from iron-cobalt occurs at a far too rapid rate to be safe, while fcc iron-platinum, magnetite, and maghemite are all capable of producing stable controlled heating. Iron-cobalt MNPs induce temperature changes that are too large, whereas barium-ferrite and cobalt-ferrite MNPs do not provide enough heat to treat a tumor. Simulations showed that magnetite, fcc iron-platinum, and maghemite MNPs are well suited for MFH, making it possible to heat tumors above 41 °C while keeping the surrounding healthy tissue temperatures below this value (Kappiyoor et al., 2010).

The thermoreversible hydrogels (poloxamer, chitosan), which accommodated 20% w/v of the magnetic microparticles, proved to be inadequate. Alginate hydrogels, however, incorporated 10% w/v of the magnetic microparticles, and the external gelation led to strong implants localizing to the tumor periphery, whereas internal gelation failed in situ. The organogel formulations, which consisted of precipitating polymers dissolved in single organic solvents, displayed various microstructures. A 8% poly(ethylene-vinyl alcohol) in DMSO (DMSO: Dimethyl sulfoxide) containing 40% w/v of magnetic microparticles formed the most suitable implants in terms of tumor casting and heat delivery (Le Renard et al., 2010).

Cell culture experiments showed that, by adjusting the amount of magnetic microspheres MMS and the time of exposure to AMF, heat treatments of mild to very high intensities could be achieved using maghemite nanoparticles embedded in mesoporous silica matrix (Martín-Saavedra et al., 2010). The heating effect of iron containing multi walled carbon nanotubes of 10-40 nm were studied and shown to be suitable (Krupskaya et al., 2009).

Several magnetic fluids are shown to be suitable for hyperthermia application. In a comparative study, 16 commercial magnetic fluids are investigated and most suitable ones are distinguished (Kallumadil et al., 2009). Magnetite microcapsules of 20-30 μm embedded in agar phantom exhibited heat generation under an alternating magnetic field (Miyazaki et al., 2012). Magnetite nanoparticles of 10 nm in an aerogel matrix are potential hyperthermia agents where the aerogel matrix can be used for drug loading for combined therapy (Lee et al., 2012).

As can be seen from the aforementioned studies, although there is not a clear definition for an ideal magnetic material for hyperthermia, there are several materials that can be employed, depending on the particular situation. Combined therapies of drug delivery and hyperthermia are promising future outlooks in this field.

Magnetic Drug Carriers

Being potential candidates for drug delivery due to their low toxicity and ability to be targeted, magnetic particles are often coated to stabilize them against precipitation, ensure their low cytotoxicity and to carry the drug in a matrix.

In *in vivo* applications SPION particles should be coated to prevent the drug molecule conjugations and to limit interactions with non-targeted cell sides, to prevent particle agglomeration and for enhanced drug loading and release. Different approaches in SPION coating resulting different assembly of polymers are summarized in Figure 4. In polysaccharide coating and coating with copolymers, the resulted particles are found as uniformly encapsulated cores. In another coating approach, polymer molecules anchored to the magnetic particle surface resulting in a brush like structure. Liposome and micelle forming molecules results a core shell structure with magnetic particles in the core. These structures can be used in drug encapsulation with retaining hydrophobic regions. (Veiseh et al., 2010).

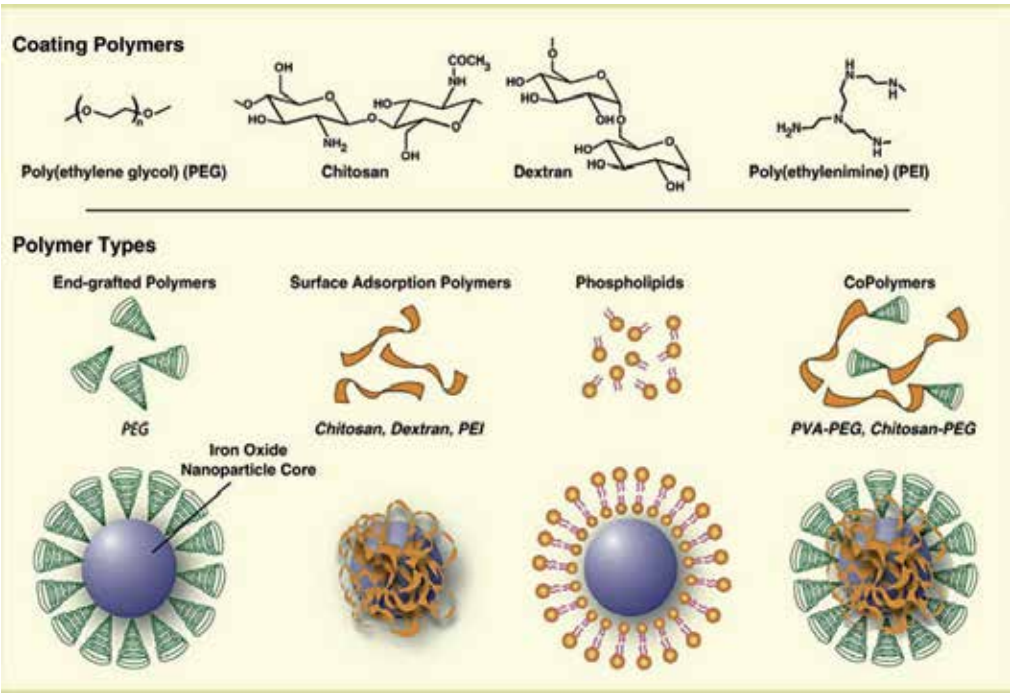

Figure 4. Illustration depicting the assembly of polymers onto the surface of magnetic nanoparticle cores (Veiseh et al., 2010).

Different particles are designed as drug delivery vehicles and a summary of these particles are given in Table 1.

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Core diameter of 10 - 15 nm Final diameter of 160 nm	chitosan/PAA multilayer	cefradine	Layer-by-Layer (LBL) The drug molecules were entrapped inside the hollow spheres through diffusion process	pH responsive	Zhang et al., 2006
Fe_3O_4	Final diameter of >1 μm	sodium carboxy methyl cellulose and chitosan	------	self-assembly shell composed of layers of carboxy methyl cellulose and chitosan around the magnetic core	----------	Cui et al., 2011
Fe_3O_4	Core diameter of 8 nm Final diameter of of 107 nm	chitosan	cefradine	cross-linking the particles with glutaraldehyde and the drug is embedded in the polymer matrix	pH responsive	Li et al., 2007
Fe_3O_4	Core diameter of 5 nm Final diameter of 1–1.5cm	Alginate / chitosan	insulin	insulin encapsulation in alginate/chitosan beads. The beads containing insulin were prepared in triplicate by extrusion method.	Magnetic field	Finotelli et al., 2010

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Final diameter of 200 nm	multiwalled carbon nanotubes (MWNTs)	doxorubicin	The MWNT-hybrid nanocomposites provided an efficient way for the extraction and enrichment of doxorubicin via π–π stacking of DOX molecules onto the polyaromatic surface of MWNTs.	pH responsive	Shen et al., 2011
Fe_3O_4	Core diameter of 3 nm	CNTs	---------	Magnetic nanoparticles adsorb on the CNT ends	------	Panczyk et al., 2010
Fe_3O_4	Core diameter of 5–10 nm. CNTs average diameter of about 30–50 nm and average length of about 100–500 nm	CdTe QDs and CNTs	-------	CNT-SPIO-CdTe nanohybrids via LBL assembly	----------	Chen et al., 2010
γ-Fe_2O_3	Core diameter of 10 nm	CNT	Diaminophenothiazine (methylene blue)	monodisperse, inherently open-ended, multi-wall CNTs loaded with magnetic iron-based nanoparticles that are encapsulated within the tube graphitic walls		Vermisoglou et al., 2011
Fe_3O_4	Core diameter of 8–12 nm mACs had a mean diameter of about 30 nm MWNTs= 40-60 nm	(mMWNTs) and magnetic-activated carbon particles (mACs)	gemcitabine (GEM)	Fe_3O_4 nanoparticles are on the outer surface of the PAA functionalized MWNTs and the drug is adsorbed on the surface .		Yang et al., 2011
$CoFe_2O_4$ nanoparticles	Core diameter of 6 nm MWCNTs with an outer diameter of 10–30 nm and an average length of 0.5–2 μm	MWCNT/cobalt ferrite ($CoFe_2O_4$) magnetic hybrids	doxorubicin	cobalt ferrite is on the outer surface of the MWCNT	pH responsive	Wu et al., 2011
γ-Fe_20_3	Core diameter of 5 nm Final diameter of 100 nm	DNA	fluorescein	Single-stranded DNA was immobilized onto the silica network, and the magnetic particles are loaded onto the network. The complementary DNA sequence was then attached to magnetic nanoparticles.	Temperature responsive	Ruiz-Hernandez et al., 2011

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Core diameter of of 8 nm Final diameter of 150 nm	PEG-functionalized porous silica shell	doxorubi-cin	DOZ conjugated magnetite particles are coated with silica to obtain core/shell nanoparticles and the whole composite is coated with PEG	the breaking of the bonding of the drug to the carrier or the swelling and degradation of the polymer.	Chen et al., 2010
α-Fe_2O_3	Core diameter of 13 nm micron-sized mesoporous molecular sieves (with 2.9-nm pores) MCM-41 and MCM-48 powders gave mean pore sizes of 3.7 and 3.5 nm, a size between 1 and 4 μm. and hollow silica microcapsules (pores of 2.7, average diameter being around 3 μm. and 15 nm. 250-nm wall thickness	hollow silica microcapsules	---------	Magnetic particles are encapsulated inside the hollow silica microcapsules	----------	Arruebo et al., 2006
Fe_3O_4	Core diameter of 10 nm Final diameter of 100 nm with 20 nm silica shell	SiO_2@ Fe_3O_4 core–shell NPs		Silica-magnetite nanocomposites are emulsified and self-assembly of magnetic-mesoporous heteronanorods at the interface of water-in-oil droplets takes place.		Zhang et al., 2011
Fe_3O_4	Particles between 150nm and 4.5 μm	silica,arabic acid and cross-linked polysaccharide	antibody	particles with starch derivative or polymeric arabic acid as matrix material functionalized with an antibody	---	Sieben et al., 2001
Fe_3O_4	Final diameter if 202 nm	β-cyclodextrin and pluronic polymer (F-127)	curcumin	multi-layer polymer coating around the magnetic particle and the drug is encapsulated via diffusion into polymer matrix	The initial burst of release was due to immediate dissociation of surface bound curcumin molecules that	Yallapu et al., 2011

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
					exist on the CD or F127 polymer matrix. The remaining sustained drug release was due to the slow release of the drug entrapped inside CD and/or F127 polymer layers.	
Fe_3O_4	Core diameter of 14.8 nm	2-hydroxypropyl-cyclodextrin (HCD) onto the gum arabic modified magnetic nanoparticles (GAMNP)	ketoprofen	polymers grafted onto magnetic particles(Multilayer polymer matrix)	drug molecules are rapidly released from HCDGAMNP, whereas some remains associated to degredation of HCD-GAMNP	Banerjee & Chen, 2009
Fe_3O_4	Final diameter of 13 nm.	(3-aminopropyl) triethoxysilane coated (APTES-MNPs) with b-cyclodextrin (β-CD).	-------	layer-by-layer	---------	Cao et al., 2009
Fe_3O_4	Core diameter of 9.2 nm	Oleic acid, sodium dodecyl benzene sulfonate SDBS, bovine serum albumin (BSA)	---	Oleic acid capped magnetic nanoparticles are embedded in the SDBS micelle and BSA adsorbs onto the micellar entity.	----	Yang et al., 2009
Fe_3O_4	Final diameter of 300 nm	poly (N-isopropylacrylamide) PNIPAAm and poly(D,L-lactide-co-glycolide) PLGA	Bovine serum albumin (BSA) and curcumin	(MLNPs) with a magnetic core and two shells made up of temperature-sensitive polymers (PNIPAAm) were encapsulated with PLGA. BSA was first loaded into PNIPAAm magnetic nanoparticles. Second, curcumin was loaded to PLGA to form the multilayer nanoparticles	Temperature responsive	Koppolu et al., 2010
Fe_3O_4	Final diameter of 150 nm	dextran	fluorescein (Fluo) or TEXAS RED® (Texas) fluorescent dye	By oxidizing Ferumoxides (FE) (suspension consisting of dextran- coated SPION) hydroxyl groups on the dextran coating are oxidized to aldehyde groups. Lysine fixable fluorescein (Fluo) or TEXAS RED® (Texas) fluorescent dye (supplied as lysine fixable dextran conjugates) was reacted with aldehyde FE and the fluorescent dye is conjugated to FE SPION (FL FE).	----	Lee et al., 2008

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Core diameter of 5 5 nm Final diameter of 4 μm,	Fe_3O_4/ PAH	fluorescein isothiocyanate (FITC)-Dextran	layer-by-layer (LbL) assembly FITC-dextran nanoparticle is coated with PSS polyelectrolyte which contains the magnetic particles forming a magnetic shell around the particle.	Magnetic field	Hu et al., 2008
Fe_3O_4	Core diameter of 12 nm	coated with starch, dextran, PEG or MPEG	----	Polymeric networks cover a large number of continuous magnetic monodomains.	----	Huong et al., 2009
magnetic fluids Carboxydextran coated DDM128 P6 (dextran–magnetite) Aminosilane coated (aminosilane–magnetite) MFL AS	DDM128 P6: core diameter of 3 nm MFL AS: core diameter of 15 nm.	dextran- or aminosilane-coated	----		-----	Jordan et al., 2006
Fe_3O_4	Core diameter of 7 nm	PVA and starch		PVA coated particles as large clusters where starch coated ones are be densely dispersed in the polymeric matrix		Voit et al., 2001
Fe_3O_4	Final diameter of 110±22 nm	starch	------	Core-shell particles	-----	Chertok et al., 2008
Fe_3O_4	coated with starch (G100) particles final diameter of 110 (±22) nm gumarabic polysaccharide Matrix (Gara) particles final diameter of 189nm Final diameter 225 nm after PEI addition	Polyethyleneimine (PEI)	--------	Surface modification of carboxyl-bearing Gara nano particles with PEI	--------	Chertok et al., 2010
Fe_3O_4	Final diameter of (140-190 nm)	Aminated, cross-linked starch and aminosilane coated Fe_3O_4 modified with PEG		To ensure that cross-linked starch particles was functionally similar to aminosilane coated particles, starch particles were covalently strengthened and aminated with concentrated ammonia to form aminated-precursor (DN). PEG is then linked to aminated precursors, DN and aminosilane particles with N-Hydroxysuccinimide (NHS) chemistry.		Cole et al., 2011

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Core diameter of 4-10 nm	PVA and PVA with partially exchanged carboxyl groups.	----		-----	Lee et al., 1996
Fe_3O_4	Core diameter of 10 nm	PVA matrix	---	the films of 200 mm depth and different concentrations of iron oxide particles in the PVA matrix.	----	Novakova et al., 2003
Fe_3O_4	Core diameter of 5–10 nm Final diameter of 108-155 nm	PVA	----	core-shell, all iron-oxide particles surrounded by a layer of PVA polymer.	----	Qui & Winnik, 2000
γ-Fe_20_3	Core diameter of 14, 19 and 43 nm Final particles are of diameter 43 nm	PNIPAM	doxorubicin	MNP cluster is coated with PNIPAM and the nanoparticl is dehydrated. Core shell morphology is achieved with dispersion free-radical polymerization	Thermoresponsive	Purushotham et al., 2009
Fe_3O_4	core diameter of 13 nm	PNIPAM	doxorubicin	Core shell morphology by dispersion polymerization where drug loaded PNIPAM shell contains magnetite clusters.	Thermoresponsive	Purushotham et al., 2010
Fe_3O_4	Core diameter of 11.21 nm Final particles are of diameter less than 250 μm	PMMA	fluorescein isothiocyanate (FITC)		Thermoresponsive	Urbina et al., 2008
γ-Fe_20_3	Core diameter of 20 nm Final particles are of diameter 400 nm	carbon	doxorubicin	Drug is released form the surface of on-coated or partially coated magnetic particles	released from the surface of our particles at a slow rate via desorption	Ibarra et al., 2007
Fe_3O_4	Final particles are of diameter ~10–20 nm	poly[aniline-co-sodium N-(1-onebutyric acid)] aniline (SPAnNa)	1,3-bis(2-chloroethyl)-1-nitrosoura	Microcapsule nanoparticles are encapsulated during the aggregation, forming the Fe_3O_4/SPAnH nanoparticles	Ultrasound and externally applied magnetic field.	Chen et al., 2010
Fe_3O_4	Core diameter of 8 nm Final particles of diameter 5.2 μm	PEs: poly(styrene sulfonate) (PSS, Mw~70000) and poly(allylamine hydrochloride) (PAH, Mw~50000).		Melamine formaldehyde microparticle is coated with polyelectrolytes (PE) in a layer-by-layer (LbL) assembly by solvent controlled precipitation of PE. The core is then dissolved and nanoparticles are infiltrated into the capsule core.		Gaponik et al., 2004
Fe_3O_4	Final particle diameter of 300–1300 nm	polystyrene		Similar technique to abovementioned method.		Madani et al., 2011

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4	Core diameter of 13 nm Final particle diameter of 3 μm	poly(sodium 4-styrenesulfonate) (PSS) and poly(allylamine hydrochloride) (PAH)	Dye	Similar technique to abovementioned method.	Magnetic heating	Katagiri et al., 2010
Fe_3O_4	Core diameter of 20 nm Final particle diameter of 2.82 μm	(PDDA/PSS)2/PDDA	Dye	Similar technique to abovementioned method.	Magnetic heating	Katagiri et al., 2011
Fe_3O_4 and γ-Fe_2O_3	Fe_3O_4 and γ-Fe_2O_3 core diameters of 9.5 and 4.3 nm, respectively	Ca alginate beads	-----	The nanoparticles were entrapped in Ca alginate beads, "egg-box like" structure of Ca alginate	-------	Finotelli et al., 2005
Fe_3O_4	Particle diameter of 58 nm	NP aggragates in humic acid (HA)	---	HA adsorbs onto magnetite particles	------	Hu et al., 2010
Fe_3O_4	Final particle diameter of 7.5 nm	amino silane(3-aminopropyl triethoxysilane)	---	nearly monolayer coating of amino silane on the magnetite particle surface	---	Ma et al., 2003
Fe_3O_4	Core particle diameter of 10–15 nm Final particle diameter of 400±80 nm	poly-L-lysine hydrochloride (PLL), poly-L-glutamic acid (PGA)	DNA	layer-by-layer (LbL) assembly on polycarbonate templates with subsequent removal of these templates. In the inner surface of polycarbonate templates, first poly-L-lysine hydrochloride (PLL) and poly-L-glutamic acid (PGA) are absorbed linking by electrostatic interactions as a polyelectrolyte layer. Then, multi polyelectrolyte layers are assembled on polycarbonate membrane and Fe_3O_4 nanoparticles are linked to PLL layer as Fe_3O_4/PLL bilayers.	----------	He et al., 2008
γ-Fe_2O_3	Core diameters of 12 nm. Final particle diameter of 35 nm (PEI) and 46 nm (PEI plus PEO-PGA)	Poly(ethylene imine) and Poly(ethylene oxide)-block-poly(glutamic acid)	---	MNPs stabilized with polymers in two layer-by-layer deposition steps.	----	Thunemann et al., 2006
Fe_3O_4	Core diameter of 12 nm	aminosilane coating	---	----	---	Maier-Hauff et al., 2011

Type of magnetic nanoparticle	Particle size	Coating agent	Drug	Design matrix	Drug release mechanism	Ref
Fe_3O_4 and γ-Fe_2O_3	Core diameters of 10 nm Final particle diameter of 96 ±15 nm	poly(ethylene glycol) (PEG)	doxorubicin	one-pot synthesis of colloids of SPION-DOX-PEG particles, PEG shell reduces the access of cellular enzymes to the drug-particle linkage and thus limits and/or delays the anticancer effect.	specific release mechanism for drug delivery is enzymatic cleavage, however the PEG shell seems to reduce the access of cellular enzymes to the drug-particle linkage and thus limits and/or delays the anticancer effect.	Shkilnyy et al., 2010

Table 1. Summary of magnetic nanomaterials used in drug delivery.

5. Conclusion

In this review, uses of magnetic nanoparticles in drug delivery are summarized. Magnetic nanoparticles gained a lot of interest due to their biocompatibility, low toxicity and their ability to be manipulated upon application of a magnetic field. These special properties allow them to be utilized as drug carrier vehicles, either by direct attachment of the drug onto the particle or often by using a natural or synthetic polymer to aid carry the drug and embedding the magnetic particles in the polymer matrix. Several types of drugs and coatings have been explored as drug carriers and a very limited selection is summarized in Table 1. The ease of surface modification of these particles opens the opportunity for targeting moieties to be attached onto particle surface, facilitating the targeting. Targeting with magnetic nanoparticles is predominantly carried out upon application of an external magnetic field, which act as an external force to localize the particles in the desired areas in the body. Applying an alternating magnetic field to magnetic particles once they are in the vicinity of a tumor, results in the temperature of the medium to rise up to 42 ^oC, which is the temperature required for hyperthermia, a complementary treatment along with chemotherapy and radiotherapy. We believe that these fascinating particles will find further potential applications along with more success in the present ones in the very near future.

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Chapter 8

Nanocarrier Systems for Transdermal Drug Delivery

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Additional information is available at the end of the chapter

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1. Introduction

The nanomedicine which is the application of technologies on the scale of 1 to 500 nm to diagnose and treat diseases, it has become a very relevant topic nowadays. During the last century, there has been a lot of new research and patents regarding nanomedicine in health sciences [1]. The objective of nanomedicine is to diagnose and preserve the health without side effects with noninvasive treatments. To reach these goals, nanomedicine offers a lot of new tools and capabilities. The manipulation that nanomedicine provides to the drugs and other materials in the nanometer scale can change the basic properties and bioactivity of materials. The solubility, increment in surface area, control release and site-targeted delivery are some characteristics that nanotechnology can manipulate on drug delivery systems.

Nanotechnology applied to health sciences contains new devices used in surgery, new chips for better diagnostics, new materials for substituting body structures and some structures capable to carry drugs through the body for treatment of a lot of diseases. These structures can be made of a lot of different materials and they are very different in structure and chemical nature. All these nanostructures are called nanocarriers and they can be administrated into the organisms by topical and transdermal routes [2]. Nanocarriers are a powerful weapon against a lot of illnesses since they are so small to be detected by immune system and they can deliver the drug in the target organ. For that reason, drug doses using nanocarriers and side effects decrease a lot.

The idea for using these tiny systems is not as new as we think but the use of nanocarriers in pharmaceutical products is not frequent, since the technology is expensive for certain types of nanoparticles and because nanocarriers need to be evaluated for demonstrating they do not have toxic effects. Nowadays the controversy of biological effects due to nanostructures

is an open discussion, in one hand, the nanotechnologist continue making new and more sophisticated nanocarriers and in the other hand, toxicologist continue evaluating possible damaging effects.

Whatever it happens, nanotechnology is the new era and nanomedicine cannot be taking off. New nanocarriers will be created and the entire scientist working in nanomedicine bet for it to be the cure of diseases that in this moment are difficult to deal with [3]

The application of preparations to the skin for medical purposes is as old as the history of medicine itself, with references to the use of ointments and salves found in the records of Babylonian and Egyptian medicine. The historical development of permeation research is well described by Hadgraft & Lane [4]. Over time, the skin has become an important route for drug delivery in which 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. In order to enhance drug transdermal absorption different methodologies have been investigated developed and patented [5,6]. 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 [3,7-10].

A number of excellent reviews that have been published contain detailed discussions concerning many aspects of transdermal nanocarriers [11-17]. The present chapter shows an updated overview of the use of submicron particles and other nanostructures in the pharmaceutical field, specifically in the area of topical and transdermal drugs. This focus is justified due to the magnitude of the experimental data available with the use of these nanocarriers. The development of submicron particles and other nanostructures in the pharmaceutical and cosmetic fields has been emerged in the last decades for designing best formulations for application through the skin [18-21].

2. The skin

The skin is the largest organ of the body [22-24], accounting for more than 10% of body mass, and the one that enables the body to interact more intimately with its environment. Essentially, the skin consists of four layers: The SC, that is the outer layer of the skin (non-viable epidermis), and forms the rate-controlling barrier for diffusion for almost all compounds. It is composed of dead flattened, keratin-rich cells, the corneocytes. These dense cells are surrounded by a complex mixture of intercellular lipids, namely, ceramides, free fatty acids, cholesterol, and cholesterol sulphate. Their most important feature is that they are structured as ordered bilayer arrays [25-28]. The other layers are: the remaining layers of the epidermis (viable epidermis), the dermis, and the subcutaneous tissue (**Figure 1**). There are also several associated appendages: hair follicles sweat ducts, glands and nails [29,30].

Many agents are applied to the skin either deliberately or accidentally, with either beneficial or deleterious outcomes. The main interest in dermal absorption assessment is related to: a) Local effects in dermatology (e.g., corticosteroids for dermatitis); b) transport through the skin seeking a systemic effect (e.g., nicotine patches, hormonal drug patches, etc.) [31]; c) surface effects (e.g., sunscreens, cosmetics, and anti-infectives) [32,33]; d) targeting of deeper tissues (e.g., nonsteroidal anti-inflammatory agents) [34-37]; and e) unwanted absorption (e.g., solvents in the workplace, pesticides or allergens) [38,39].

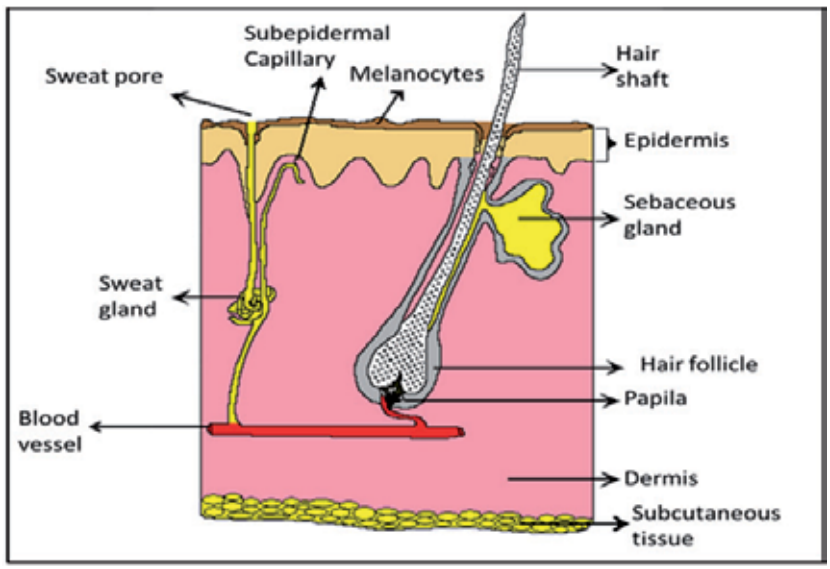

Figure 1. Schematic representation of skin layer.

2.1. Epidermis

2.1.1. Stratum corneum

The stratum corneum is the heterogeneous outermost layer of the epidermis and is approximately 10-20 μm thick. The stratum corneum consists of about 15 to 25 layers of flattened, stacked, hexagonal, and cornified cells embedded in an intercellular matrix of lipids. These lipid domains form a continuous structure so they are considered to play a crucial role in the maintenance of the skin barrier that helps avoid transepidermal water loss. Each cell is approximately 40 μm in diameter and 0.5 μm thick [40].

The stratum corneum barrier properties may be partly related to its very high density (1.4 g/cm^3 in the dry state) and its low hydration of 15–20 %, compared with the usual 70 % for the body. Each stratum corneum cell is composed mainly of insoluble bundled keratins (70 %) and lipid (20 %) encased in a cell envelope, accounting for about 5% of the stratum corneum weight. The permeability barrier is located within the lipid bilayers in the intercellular spaces of the stratum corneum [6-8] and consists of ceramides (40–50%), fatty acids (15–25%), cholesterol (20–25%) and cholesterol sulphate (5–10 %) [41-45].

The barrier function is further facilitated by the continuous desquamation of this horny layer with a total turnover of the stratum corneum occurring once every 2–3 weeks. The stratum corneum functions as a barrier are to prevent the loss of internal body components, particularly water, to the external environment. The cells of the stratum corneum originate in the viable epidermis and undergo many morphological changes before desquamation. Thus, the epidermis consists of several cell strata at varying levels of differentiation.

The origins of the cells of the epidermis lie in the basal lamina between the dermis and viable epidermis. In this layer there are melanocytes, Langerhans cells, Merkel cells, and two major keratinic cell types: the first functioning as stem cells having the capacity to divide and produce new cells; the second serving to anchor the epidermis to the basement membrane [46]. The basement membrane is 50–70 nm thick and consists of two layers, the lamina densa and lamina lucida, which comprise mainly proteins, such as type IV collagen, aminin, nidogen and fibronectin. Type IV collagen is responsible for the mechanical stability of the basement membrane, whereas laminin and fibronectin are involved with the attachment between the basement membrane and the basal keratinocytes. The cells of the basal lamina are attached to the basement membrane by hemidesmosomes, which are found on the ventral surface of basal keratinocytes [47]. Hemidesmosomes appear to comprise three distinct protein groups: two of which are bullous pemphigoid antigens (BPAG1 and BPAG2), and the other epithelial cellspecific integrins [48-50]. BPAG1 is associated with the organization of the cytoskeletal structure and forms a link between the hemidesmosome structure and the keratin intermediate filaments. The integrins are transmembrane receptors that mediate attachment between the cell and the extracellular matrix. Human epidermal basal cells contain integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$. Integrin $\alpha_6\beta_4$ and BPAG2 appear to be the major hemidesmosomal protein contributors to the anchoring of the keratinocyte, spanning from the keratin intermediate filament, through the lamina lucida, to the lamina densa of the basement membrane [51]. In the lamina densa, these membrane-spanning proteins interact with the protein laminin-5 which, in turn, is linked to collagen VII, the major constituent of the anchoring fibrils within the dermal matrix. It has also been suggested that both BPAG2 and integrin $\alpha_6\beta_4$ mediate in the signal transductions required for hemidesmosome formation and cell differentiation and proliferation. Integrin $\alpha_3\beta_1$ is associated with actin and may be linked with laminin-5. Epidermal wounding results in an up-regulation of these proteins that appears to be involved with cell motility and spreading. The importance of maintaining a secure link between the basal lamina cells and the basement membrane is obvious, and the absence of this connection results in chronic blistering diseases such as pemphigus and epidermolysis bullosa.

2.2. Dermis

The dermis is about 0.1–0.5 cm thick and consists of collagenous (70 %) and elastin fibres. In the dermis, glycosaminoglycans or acid mucopolysaccharides, are covalently linked to peptide chains to form proteoglycans, the ground substance that promotes the elasticity of the skin. The main cells present are the fibroblasts, which produce the connective tissue

components of collagen, laminin, fibronectin and vitronectin; mast cells, which are involved in the immune and inflammatory responses; and melanocytes involved in the production of the pigment melanin [51]. Nerves, blood vessels and lymphatic vessels are also present in the dermis.

Contained within the dermis is an extensive vascular network providing for the skin nutrition, repair, and immune responses for the rest of the body, heat exchange, immune response, and thermal regulation. Skin blood vessels derive from those in the subcutaneous tissues (hypodermis), with an arterial network supplying the papillary layer, the hair follicles, the sweat and apocrine glands, the subcutaneous area, as well as the dermis itself. These arteries feed into arterioles, capillaries, venules, and, thence, into veins. Of particular importance in this vascular network is the presence of arteriovenous anastomoses at all levels in the skin. These arteriovenous anastomoses, which allow a direct shunting of up to 60% of the skin blood flow between the arteries and veins, thereby avoiding the fine capillary network, are critical to the skin's functions of heat regulation and blood vessel control. Blood flow changes are most evident in the skin in relation to various physiological responses and include psychological effects, such as shock (''draining of color from the skin'') and embarrassment (''blushing''), temperature effects, and physiological responses to exercise, hemorrhage, and alcohol consumption.

The lymphatic system is an important component of the skin in regulating its interstitial pressure, mobilization of defense mechanisms, and in waste removal. It exists as a dense, flat meshwork in the papillary layers of the dermis and extends into the deeper regions of the dermis. Also present in the dermis are a number of different types of nerve fibers supplying the skin, including those for pressure, pain, and temperature [52]. Epidermal appendages such as hair follicles and sweat glands are embedded in the dermis [53].

2.3. Hypodermis

The deepest layer of the skin is the subcutaneous tissue or hypodermis. The hypodermis acts as a heat insulator, a shock absorber, and an energy storage region. This layer is a network of fat cells arranged in lobules and linked to the dermis by interconnecting collagen and elastin fibers. As well as fat cells (possibly 50% of the body's fat); the other main cells in the hypodermis are fibroblasts and macrophages. One of the major roles of the hypodermis is to carry the vascular and neural systems for the skin. It also anchors the skin to underlying muscle. Fibroblasts and adipocytes can be stimulated by the accumulation of interstitial and lymphatic fluid within the skin and subcutaneous tissue [54]. The total thickness of skin is about 2–3 mm, but the thickness of the stratum corneum is only about 10–15 μm.

2.4. Skin appendages

There are four skin appendages: the hair follicles with their associated sebaceous glands, eccrine and apocrine sweat glands, and the nails, but these occupy only about 0.1 % of the total human skin surface.

The pilosebaceous follicles have about 10 to 20 % of the resident flora and cannot be decontaminated by scrubbing. The hair follicles are distributed across the entire skin surface with the exception of the soles of the feet, the palms of the hand and the lips. A smooth muscle, the erector pilorum, attaches the follicle to the dermal tissue and enables hair to stand up in response to fear. Each follicle is associated with a sebaceous gland that varies in size from 200 to 2000 μm in diameter. The sebum secreted by this gland consisting of triglycerides, free fatty acids, and waxes, protects and lubricates the skin as well as maintaining a pH of about 5. Sebaceous glands are absent on the palms, soles and nail beds. Sweat glands or eccrine glands respond to temperature via parasympathetic nerves, except on palms, soles and axillae, where they respond to emotional stimuli via sympathetic nerves [51]. The eccrine glands are epidermal structures that are simple, coiled tubes arising from a coiled ball, of approximately 100 μm in diameter, located in the lower dermis. It secretes a dilute salt solution with a pH of about 5, this secretion being stimulated by temperature-controlling determinants, such as exercise and high environmental temperature, as well as emotional stress through the autonomic (sympathetic) nervous system. These glands have a total surface area of about 1/10,000 of the total body surface. The apocrine glands are limited to specific body regions and are also coiled tubes. These glands are about ten times the size of the eccrine ducts, extend as low as the subcutaneous tissues and are paired with hair follicles.

Nail function is considered as protection. Nail plate consists of layers of flattened keratinized cells fused into a dense but elastic mass. The cells of the nail plate originate in the nail matrix and grow distally at a rate of about 0.1 mm/day. In the keratinization process the cells undergo shape and other changes, similar to those experienced by the epidermal cells forming the stratum corneum. This is not surprising because the nail matrix basement membrane shows many biochemical similarities to the epidermal basement membrane [55,56]. Thus, the major components are highly folded keratin proteins with small amounts of lipid (0.1–1.0%). The principal plasticizer of the nail plate is water, which is normally present at a concentration of 7–12 %.

3. Skin functions

Many of the functions of the skin can be classified as essential to survival of the body bulk of mammals and humans in a relatively hostile environment. In a general context, these functions can be classified as a protective, maintaining homeostasis or sensing. The importance of the protective and homeostatic role allows the survival of humans in an environment of variable temperature; water content (humidity and bathing); and the presence of environmental dangers, such as chemicals, bacteria, allergens, fungi and radiation. In a second context, the skin is a major organ for maintaining the homeostasis of the body, especially in terms of its composition, heat regulation, blood pressure control, and excretory roles. It has been argued that the basal metabolic rate of animals differing in size should be scaled to the surface area of the body to maintain a constant temperature through the skin's thermoregulatory control [57]. Third, the skin is a major sensory organ in terms of

sensing environmental influences, such as heat, pressure, pain, allergen, and microorganism entry. Finally, the skin is an organ that is in a continual state of regeneration and repair. To fulfill each of these functions, the skin must be tough, robust, and flexible, with effective communication between each of its intrinsic components mentioned above.

The stratum corneum also functions as a barrier to prevent the loss of internal body components, particularly water, to the external environment. The epidermis plays a role in temperature, pressure, and pain regulation.

Appendage functions are following: hair follicle and sebaceous gland fulfill with protect (hair) and lubricate (sebum), eccrine and apocrine glands have the functions of cooling and vestigial secondary sex gland, respectively; and nails has the function of to protect.The hypodermis acts as a heat insulator, a shock absorber and an energy storage region. One of the major roles of the hypodermis is to carry the vascular and neural systems for the skin.

4. Routes of drug penetration through the skin

The determination of penetration pathways of topically applied substances into the skin is the subject of several investigations. The permeation of drugs through the skin includes the diffusion through the intact epidermis y through the skin appendages. These skin appendages are hair follicles and sweat glands which form shunt pathways through the intact epidermis, occupying only 0.1% of the total human skin [58]. It is known drug permeation through the skin is usually limited by the stratum corneum. Two pathways through the intact barrier may be identified, the intercellular and transcellular route (**Figure 2**):

a. The intercellular lipid route is between the corneocytes.

Interlamellar regions in the stratum corneum, including linker regions, contain less ordered lipids and more flexible hydrophobic chains. This is the reason of the non-planar 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 [59,60]. The hydrophilic molecules diffuse predominantly “laterally” along surfaces of the less abundant, water filled inter-lamellar 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 [61].

b. The transcellular route contemplates the crossing through the corneocytes and the intervening lipids [24].

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 [62]. The narrow aqueous transepidermal pathways have been observed using confocal laser scanning microscopy (CLSM). 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 better sealed and more transport resistant is the intra-cluster/inter-corneocyte pathway [63]. Hydrophilic conduits have openings between ≥5 μm (skin appendages) and ≤10 nm (narrow inter-corneocyte pores). So sweat ducts (≥50 μm), pilosebaceous units (5–70 μm), and sebaceous glands (5–15 μm) represent the largest width/lowest resistance end of the range. Junctions of corneocytes-clusters and cluster boundaries fall within the range [64]. It was determined that the maximally open hydrophilic conduits across skin are approximately 20–30 nm wide, including pore penetrant/opener thickness [63]. Another studies revealed the width of the negatively charged hydrophilic transepidermal pores expanded by electroosmosis to be around of 22–48 nm [65]. Lipophilic cutaneous barrier is governed by molecular weight and distribution coefficient rather than molecular size [66]. The relative height of cutaneous lipophilic barrier consequently decreases with lipophilicity of permeant, but molecules heavier than 400–500 Da are so large permeants to find sufficiently wide defects in the intercellular lipidic matrix to start diffusing through the lipidic parts of cutaneous barrier [64, 66,67].

The contribution to transdermal drug transport can increases with the pathways widening or multiplication, for example such that is caused by exposing the stratum corneum to a strong electrical (electroporation/iontophoresis), mechanical (sonoporation/sonophoresis), thermal stimulus, or suitable skin penetrants [59].

Recently, follicular penetration has become a major focus of interest due to the drug targeting to the hair follicle is of great interest in the treatment of skin diseases. However due to follicular orifices only occupying 0.1% of the total skin surface area, it was assumed as a non important route. But a variety of studies shown the hair follicles as could be a way to trough the skin [68- 73]. Such follicular pathway also has been proposed for topical administration of nanoparticles and microparticles and it has been investigated in porcine skin, because in recent studies the results have confirmed the *in vitro* penetration into the porcine hair follicles might be considered similar to those on humans *in vivo.* After topical application of dye sodium fluorescein onto porcine skin mounted in Franz diffusion cells with the acceptor compartment beneath the dermis, the fluorescence was detected on the surface, within the horny layer, and in most of the follicles confirming the similarity in the penetration between porcine and human skin [72]. So nanoparticles have been studied in porcine skin revealing in the surface images 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 [74]. In other investigations, it has been shown by differential stripping the influence of size microparticles in the skin penetration. It can act as efficient drug carriers or can be utilized as follicle blockers to stop the penetration of topically applied substances [73].

It has already been postulated that certain molecules can hydrogen bond to groups present on the surfaces of follicular pores [75]. However, more studies have to be made in order to identify all the molecular properties that influence drug penetration into hair follicles.

Nowadays, there are currently a number of methods available for quantifying drugs localized within the skin or various layers of the skin. To date, a direct, non-invasive quantification of the amount of topically applied substance penetrated into the follicles had not been possible. Therefore, stripping techniques, tape stripping and cyanoacrylate skin surface biopsy have been used to remove the part of the stratum corneum containing dye topically applied [76]. Thus, the "differential stripping" has been shown as a new method that can be used to study the penetration of topically applied substances into the follicular infundibula non-invasively and selectively [20,26,30,76,77].

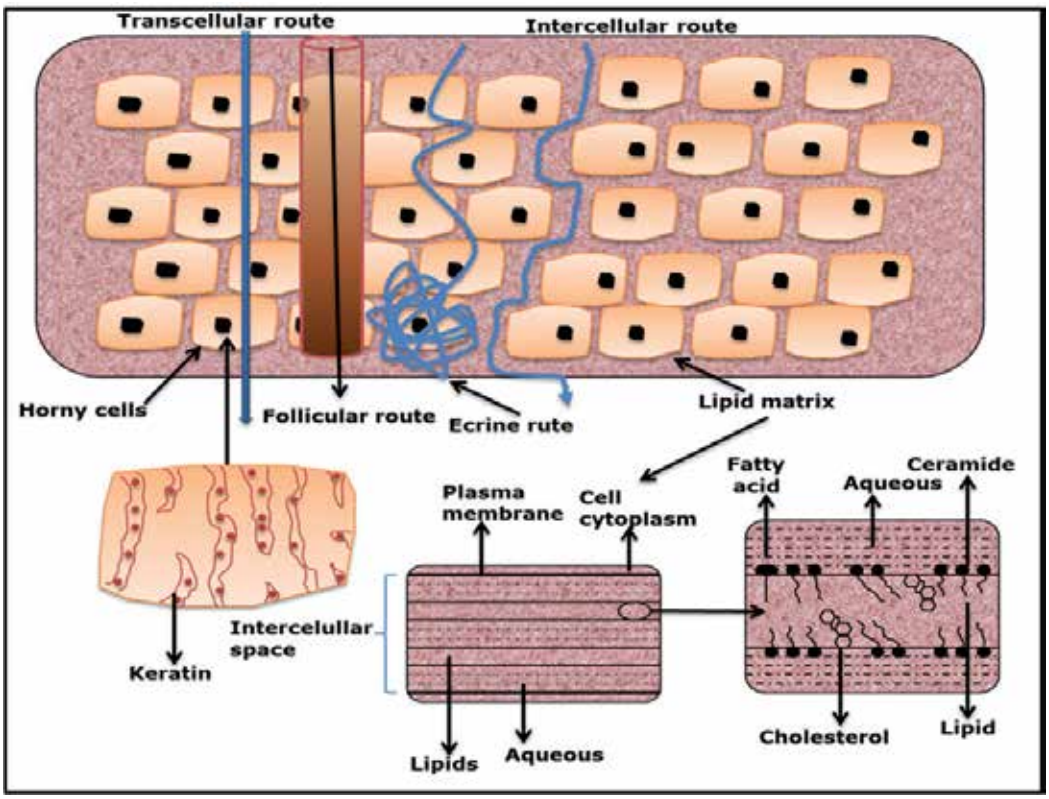

Figure 2. Schematic representation of penetration routes of drugs throughout the skin.

5. Advantages and disadvantages of transdermal drug delivery

Transdermal drug delivery systems offer several important advantages over more traditional approaches, in addition to the benefits of avoiding the hepatic first-pass effect, and higher patient compliance, the additional advantages and the disadvantages [78-80] that transdermal drug delivery offers can be summarized as follows in Table 1.

Advantages of transdermal drug delivery	Disadvantages of transdermal drug delivery
Longer duration of action	Possibility of local irritation at the site of application
Reduction in dosing frequency	Erythema, itching, and local edema can be caused by the drug, the adhesive, or other excipients in the patch formulation
More uniform plasma levels	The skin's low permeability limits the number of drugs that can be delivered in this manner
Useful for drugs that require relatively consistent plasma levels	
It is an alternative route of administration to accommodate patients who cannot tolerate oral dosage forms (specially for nauseated or unconscious patients)	
Improved bioavailability	
Reduction of side effects	
Flexibility of terminating the drug administration by simply removing the patch from the skin	

Table 1. Main advantages and disadvantages of transdermal drug delivery

6. Nanocarrier systems

Nanocarriers have demonstrated increased drug absorption, penetration, half-life, bioavailability, stability, etc. Nanocarriers are so small to be detected by immune system and they can deliver the drug in the target organ using lower drug doses in order to reduce side effects. Nanocarriers can be administrated into the organisms by all the routes; one of them is the dermal route. The nanocarriers most used and investigated for topical/transdermal drug delivery in the pharmaceutical field are shown in **Figure 3** and **Table 2**.

6.1. Nanoparticles

Nanoparticles are smaller than 1,000 nm. Nowadays, it is possible to insert many types of materials such as drugs, proteins, peptides, DNA, etc. into the nanoparticles. They are constructed from materials designed to resist pH, temperature, enzymatic attack, or other problems [81]. Nanoparticles can be classified as nanospheres or nanocapsules (See **Figure**

4). Nanospheres are solid-core structures and nanocapsules are hollow-core structures. Nanoparticles can be composed of polymers, lipids, polysaccharides and proteins [82,83]. Nanoparticles preparation techniques are based on their physicochemical properties. They are made by emulsification-diffusion by solvent displacement, emulsification-polymerization, in situ-polymerization, gelation, nanoprecipitation, solvent evaporation/extraction, inverse salting out, dispersion polymerization and other derived from these one.

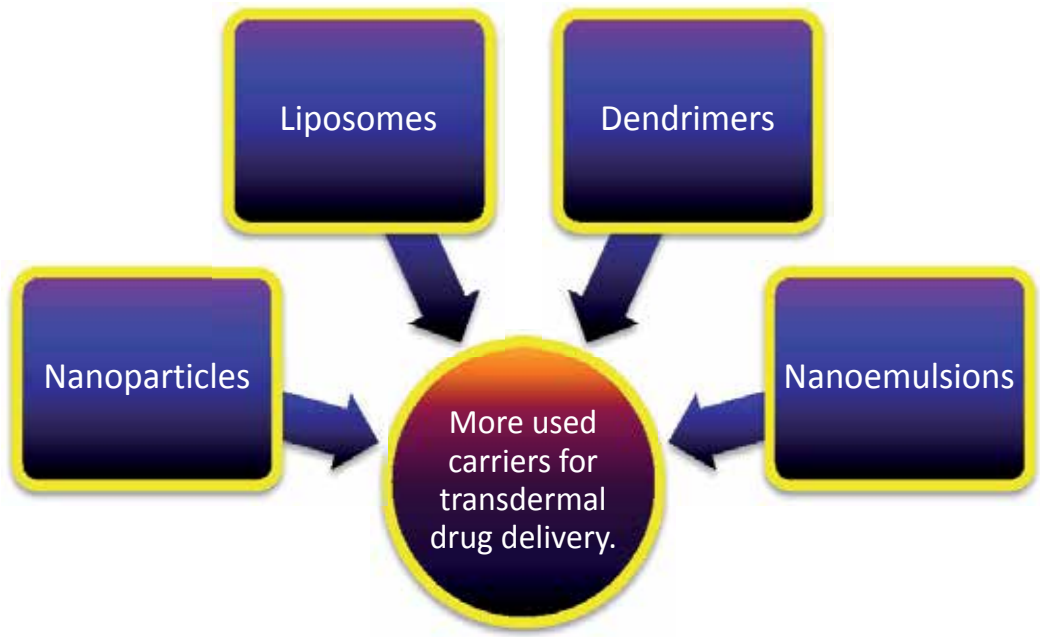

Figure 3. More used transdermal nanocarriers

6.2. Nanoemulsions

Nanoemulsions are isotropic dispersed systems of two non miscible liquids, normally consisting of an oily system dispersed in an aqueous system (o/w nanoemulsion), or an aqueous system dispersed in an oily system but forming droplets or other oily phases of nanometric sizes (100 nm). They can be stable (methastable) for long times due to the extremely small sizes and the use of adequate surfactants. Nanoemulsions can use hydrophobic and hydrophilic drugs because it is possible to make both w/o or o/w nanoemulsions [84]. They are non-toxic and non-irritant systems and they can be used for skin or mucous membranes, parenteral and non parenteral administration in general and they have been used 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 been reduced due to the stability problems inherent to this dosage form.

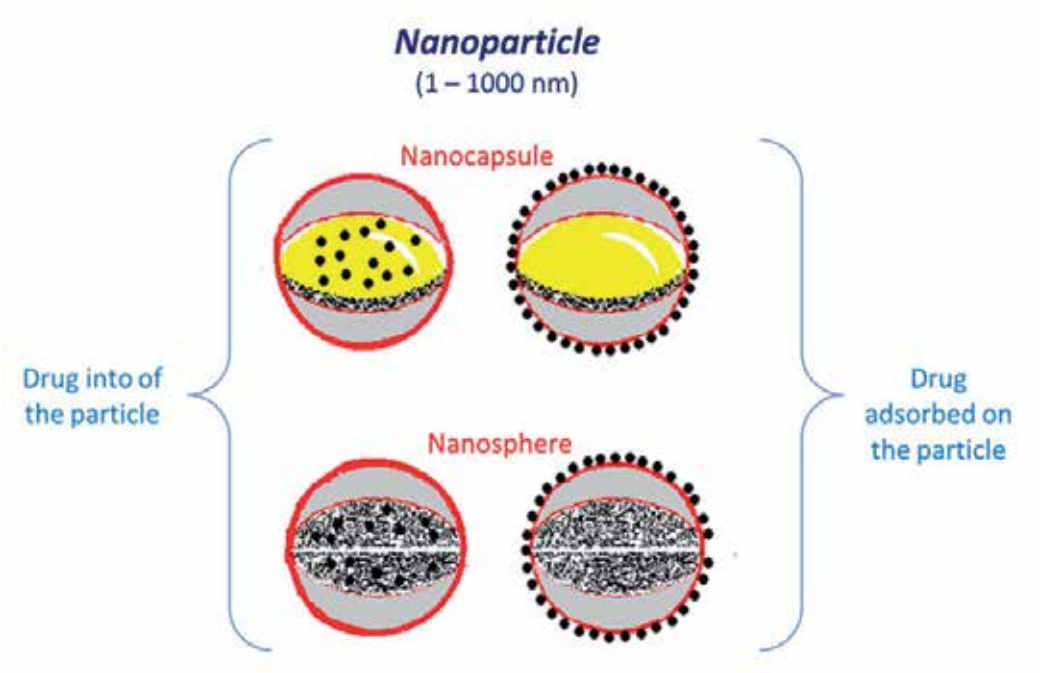

Figure 4. Nanospheres and nanocapsules are small vesicles used to transport drugs. Nanospheres are typically solid polymers with drugs embedded in the polymer matrix. Nanocapsules are a shell with an inner space loaded with the drug of interest. Both systems are useful for controlling the release of a drug and protecting it from the surrounding environment.

6.3. Liposomes

Liposomes are hollow lipid bilayer structures (**Figure 5**) that can transport hydrophilic drugs inside the core and hydrophobic drugs between the bilayer [85]. They are structures made of cholesterol and phospholipids. They can have different properties depending on the excipients included and the process of their elaboration. The nature of liposomes makes them one of the best alternatives for drug delivery because they are non-toxic and remain inside the bloodstream for a long time. Liposomes can be surface-charged as neutral, negative or positive, depending on the functional groups and pH medium. Liposomes can encapsulate both lipophilic and hydrophilic drugs in a stable manner, depending on the polymer added to the surface [86]. There are small unilamellar vesicles (25 nm to 100nm), medium-sized unilamellar vesicles (100 nm and 500nm), large unilamellar vesicles, giant unilamellar vesicles, oligolamellar vesicles, large multilamellar vesicles and multivesicular vesicles (500 nm to microns). The thickness of the membrane measures approximately 5 to 6 nm. These shapes and sizes depend of the preparation technique, the lipids used and process variables. Depending on these parameters, the behavior both in vivo and in vitro can change and opsonization processes, leakage profiles, disposition in the body and shelf life are different due to the type of liposome [86].

Liposomes preparation techniques follow three basic steps with particular features depending on safety, potential scale up and simplicity: 1) Lipid must be hydrated, 2) Liposomes have to be sized and 3) Nonencapsulated drug has to be removed. The degree of transdermal drug penetration is affected by the lamellarity, lipid composition, charge on the liposomal surface, mode of application and the total lipid concentrations [87].

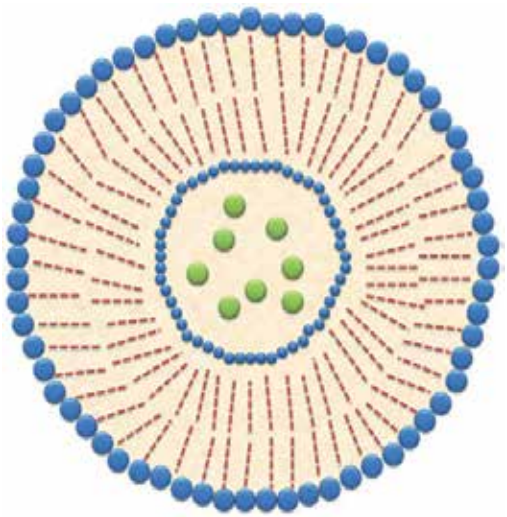

Figure 5. Liposomes are spherical vesicles that comprise one or more lipid bilayer structures enclosing an aqueous core. They protect encapsulated drugs from degradation. Liposomes can also be functionalized to improve cell targeting and solubility

6.4. Dendrimers

Dendrimers are monodisperse populations that are structurally and chemically uniform (**Figure 6**). They allow conjugation with numerous functional groups due to the nature of their branches. The amount of branches increases exponentially and dendrimers growth is typically about 1 nm per generation [88]. The dendrimers classification is based on the number of generations. After the creation of a core, the stepwise synthesis is called first generation; after that, every stepwise addition of monomers creates the next generation. This approach allows an iterative synthesis, providing the ability to control both molecular weight and architecture.

The kind of polymer chosen to construct the dendrimer by polimerization is very important with regard to the final architecture and features. In addition, the use of branched monomers has the peculiarity of providing tailored loci for site-specific molecular recognition and encapsulation. Notably, 3D and fractal architecture, as well as the peripheral functional groups, provide dendrimers with important characteristic physical and chemical properties. In comparison with linear polymers, dendritic structures have "dendritic voids" that give these molecules important and useful features. These spaces inside dendrimers can mimic the molecular recognition performed by natural proteins.

Furthermore, dendrimers have a high surface-charge density due to ionizable groups that help them to attach drugs by electrostatic forces, regardless of the stoichimetry. This dendrimer-drug association provides drugs with better solubility, increasing their transport through biological membranes and sometimes increasing drug stability. The number of molecules that can be incorporated into dendrimers is related to the number of surface functional groups; therefore, later-generation dendrimers are more easily incorporated into dendritic structure. However, not all the functional groups are available for interaction due to steric volume, molecule rotation or stereochemistry effects. Dendrimers can have positive and negative charges, which allows them to complex different types of drugs [89].

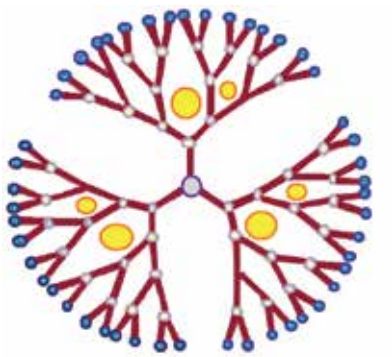

Figure 6. Dendrimers are highly branched polymers with a controlled three-dimensional structure around a central core. They can accommodate more than 100 terminal groups.

6.5. Advantages and limitations of using nanocarriers for transdermal drug delivery

As it is has been mentioned before, the search for new strategies able to enhance the topical and transdermal penetration of drugs has become essential [61]. Different carrier systems have been proposed in an attempt to favour the transport of drugs through the skin, enabling drug retention and in some cases allowing a controlled release. Skin penetration is essential to a number of current concerns, for example, contamination by microorganisms and chemicals, drug delivery to skin (dermatological treatments) and through skin (transdermal treatments), and skin care and protection (cosmetics) [103-106].

Follicular penetration has become a major focus of interest due to the drug targeting to the hair follicle is of great interest in the topical treatment of skin diseases. However due to follicular orifices only occupying ~0.1% of the total skin surface area, it was assumed as a non important route. But recently, a variety of studies have shown that the hair follicles represent a important way to trough the skin and some techniques have been used to test the penetration of drugs loaded nanoparticles/microsparticles [70,72,73,107-109]. Confocal laser scanning microscopy which permits optical sectioning of thick tissues and cells and their subsequent computerized three-dimensional reconstruction, has been used to study the entry of drugs through the skin [110]. It was visualized in the fresh human scalp skin

on-line the diffusion processes of a model fluorophore into the hair follicle at different depths [72]. Such follicular pathway also has been proposed for topical administration of nanoparticles and microparticles using porcine skin. Recent studies have confirmed that the *in vitro* penetration into the porcine hair follicles might be considered similar to those on humans *in vivo* [73]. Studies in porcine skin revealed in the surface images 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 [76]. In other investigations, it has been shown by differential stripping the influence of size microparticles in the skin penetration. It can act as efficient drug carriers or can be utilized as follicle blockers to stop the penetration of topically applied substances [75]. An alternative technique is multiphoton microscopy (MPM) especially two-photon excitation microscopy has been widely used in imaging biological specimens treated with nanoparticles [111-114]. The near-infrared light used in the two-photon microscope can penetrate deeper in highly scattering tissues such as *in vivo* human skin than confocal microscopes operated with ultraviolet excitation [115]. Futhermore, this technique provides both cellular and extracellular structural information, with subcellular resolution helpful for clinical dermatological diagnosis, both *ex vivo* and *in vivo*. In addition, it can be used to characterize stratum corneum structures, visualize and quantify transcutaneous drug delivery, detect skin cancers, explore collagen structural transitions, and watch laser–skin interactions [116,117]. A common method used for quantifying drugs localized within the skin or various layers of the skin are the tape stripping and cyanoacrylate skin surface biopsy techniques, which have been used to remove the part of the stratum corneum containing dye topically applied. Thus, the "differential stripping" has been shown as a new method that can be used to study the penetration of topically applied substances into the follicular infundibula non-invasively and selectively [118]. It has been reported in a previous *in vitro* permeation studies using tape stripping, that triclosan-loaded nanoparticles penetrated into the skin and their retention favoured a local effect. Moreover, polymeric nanoparticles are expected to be able to form a depot in the hair follicles, providing a targeted controlled drug delivery [33,119].

In general, the principal advantages of microparticles and nanoparticles over conventional formulations such as creams, solutions, ointments, lotions, gels, and foams, is that the second ones have different absorption characteristics and aesthetic properties, and they also have some major limitations, such as poor penetration and uncontrolled drug release. Furthermore, tolerability and safety end points, such as irritation, dryness, erythema, itching, stinging and burning will be key factors in determining its usefulness. It happens because using a traditional system, drug delivery is sometimes rapid and topical or plasmatic concentrations can result in toxic effects. However, for the case of nanoparticles a smaller amount of the drug is necessary and due to the targeted nature of delivery [120]. Topical or transdermal drug deliveries have many advantages over the other routes: fewer side effects, increased patient compliance, controlled release, and the lack of a hepatic first pass [121].

Nanocarrier	Size range	Preparation methods	Characteristics	References
Polymeric nanoparticles	10-1000 nm	-In situ polymerization -Emulsification-evaporation, -Emulsification-diffusion, -Emulsification-diffusion by solvent displacement.	Solid or hollow particles wich have entraped, binded or encapsulated drugs.	[33]
Solid lipid nanoparticles	50-1000 nm	-High-pressure homogenization.	Similar to polymeric nanoparticles but made of solid lipids.	[90]
Inorganic Nanoparticles	<50nm	-Sol-gel technique	Nanometric particles, made up of inorganic compounds such as silica, titania and alumina.	[91]
Liposomes	25 nm-100 μm	-Sonication -Extrusion, -Mozafari method	Vesicles composed of one or more concentric lipid bilayers, separated by water or aqueous buffer compartments.	[92]
Dendrimers	3–10 nm	-Polymerization	Macromolecular high branched structures.	[93]
Quantum dots	2-10nm	-Colloidal assembly, viral assembly, -Electrochemical assembly.	Made up of organic surfactants, precursors and solvents.	[94]
Lipid globules	1-100 nm	-Emulsification espontaneous systems.	Multicomponent fluid made of water, a hydrophobic liquid, and one or several surfactants resulting in a stable system.	[95]
Lipid microcylinders	<1 μm	-Self emulsification	Self organizing system in which surfactants crystallize into tightly packed bilayers that spontaneously form cylinders.	[96]

Ethosomes	<400 nm	-Cold method -Hot method	Non invasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation.	[97]
Aquasomes	60-300 nm	-Self-assembling of hydroxyapatite by coprecipitation method	The particle core is composed of noncrystalline calcium phosphate or ceramic diamond, and it is covered by a polyhydroxyl oligomeric film.	[98]
Pharmacosomes	<200 nm	-Hand-shaking method -Ether-injection method	Pure drug vesicles formed by amphiphilic drugs.	[99]
Colloidosomes	200nm – 1.5 μm	-Self-assembly of colloidal particles at the interface of emulsion droplets	Hollow capsules with elastic shells.	[100]
Niosomes	10-1000 nm	-Self-assembly of nonionic Surfactant	Bilayered structures made of non-ionic surfactant vesicles.	[101]
Nanoemulsions	20-200nm	-High-pressure homogenization. -Microfluidization. -Phase Inversion temperature	Submicron emulsions o/w or w/o.	[102]

Table 2. Examples of nanocarriers used for drug delivery.

It has been reported that nanoencapsulation of drugs (nano-medicines) increases their efficacy, specificity, tolerability and therapeutic index [122-124]. These nano-formulations are reported to be superior to traditional medicine with respect to controlled release, targeted delivery and therapeutic impact. The targeting capabilities of nanomedicines are influenced by particle size, surface charge, surface modification, and hydrophobicity. Of these, nanoparticle size distribution is an important factor in determining the interaction with the cell membrane and their penetration across physiological barriers, being dependent on the tissue, target site and circulation [125]. Example of this are the nanostructured lipid

carriers (NLC) by their structure (lipid nanoparticles with solid matrix) increase in loading capacity, physical and chemical long-term stability, triggered release and potentially supersaturated topical formulations with respect to solid lipid nanoparticles (SLN). Other advantages of NLC include improvement in stabilisation of incorporated compounds, controlled release, occlusivity, film formation on skin including in vivo effects on the skin. Lipid nanoparticles have been observed as a good option for transdermal delivery because they can be prepared in different sizes and it is possible to modify surface polarity in order to improve skin penetration [126,127]. From the upper skin, nanoparticles can reach deeper skin regions because they exhibit mechanical flexion [128].

Additionaly, transdermal nanocarriers are able to reach target organs because they can be attached to antibodies, antigens, vitamins and other molecules to be more specific. Nanoparticles can travel largely undetected by the immune system depending of the nanocarriers size of the antigen added as well as its composition. So, by hiding functional groups or protecting these groups with other molecules, drugs can be released specifically in the target organ. Consecuently, nanoparticles can even travel from the skin to lymph nodes, representing a promising tool for immunomodulation [129]. One of the first strategies for transdermal delivery were the liposomes. The nature of liposomes makes them one of the best alternatives for drug delivery because they are non-toxic and remain inside the bloodstream for a long time [130-133]. Nevertheless, some factors affect the degree of transdermal drug penetration such as the lamellarity, the lipid composition, the charge on the liposomal surface, the mode of application and the total lipid concentrations [89,134]. For that reason, flexible vesicles called transfersomes or transformable liposomes have been compared with those rigid vesicles to enhance penetration [135-142]. The lipids present in the liposome bilayer can interact with lipids present in the stratum corneum changing the structure of the upper skin. This change is beneficial for the penetration of lipophilic drugs into the stratum corneum [143]. Some liposomes may have a deformable structure and pass through the stratum or may accumulate in the channel-like regions in the stratum corneum, depending upon their composition [144,145]. In order to obtain transformable liposomes more flexible, they are prepared using surfactants or alcohol (ethosomes) in the lipid bilayer, to be able to deform them when a pressure is applied in the transdermal route.

Some limitations for nanocarriers are the important tests and regulations that should be carried out to ensure an adequate characterization, analytical evaluation, toxicological and pharmacological assessment, which is necessary to determine the efficacy of using these nanostructures in therapies and diagnosis because of their tiny size, their high surface energy, their composition, their architecture, their attached molecules, etc. Those things are frequently reviewed for the dendrimers. One of the main advantages is that they have multivalency and it is possible to get control of the functional groups on the surface [146,147]. Due to their form and size (1–10 nm), these molecules can carry drugs, imaging agents, and can interact with lipids present in membranes, because it was reported a better permeation in cell cultures and intestinal membranes. They also increased the permeation of lipophilic drugs instead of hydrophilic drugs. The main problems with this kind of

transdermal carrier are poor biodegradation and inherent cytotoxicity [148]. To obtain dendrimers less toxic, dendrimers have been linked to peptides. Dendrimers-peptides are formed from amino acids linked via peptide-amide bonds to the branches of dendrimers in the core or on the surface to get down the toxicity. Then, they are bio-transformed to produce amino-acid derivatives. Besides, the synthesis of these structures is less expensive and purification does not present any difficulty [149,150].

It is suggested in future research to elucidate the interactions between nanocarriers and other molecules as well as interactions between nanocarriers and biological entities. The toxicology of nanostructures is also a current concern. Materials behave very differently when they are diminished to nanosizes. Traditional laws do not work at this "meso-scale" in the same way as they function at the macro-scale. On the macro scale, bulk properties in a material predominate over surface properties. At the micro-scale, surface properties tend to dominate. At the meso-scale, both types of properties play significant roles [151,152]. Furthermore, the effects of metabolized/altered nanostructures on the biological system are difficult to predict. Regulatory agencies are taking action to assess new Nanotechnology-based products.

In addition, the fabrication of nanocarriers scaling up from the lab at the industrial production is difficult and the materials used to prepare nanocarriers are very expensive in the majority of the cases.

7. Applications of nanocarrier systems in topical/transdermal delivery

Nanocarriers as drug delivery systems were first intended for use in parenteral or oral routes of administration and as such still continue to be the focus of many studies [153]. However skin application of these nanocarriers, and especially for liposomes, polymeric and lipidic nanoparticles, also makes sense when considering surface effects (film formation and occlusive effects), local effects in the skin (drug delivery in the epidermis and dermis) and systemic effects (deeper drug permeation and transdermal delivery). In potential uses apart from those concerned with surface effects the nanocarrier has to overcome the SC barrier in order to deliver the drug more or less deeply into skin layers. Recent advances in the study of penetration mechanisms deal with the control of the intercellular penetration route by the crystalline state of lipids, and the penetration through skin appendages (the follicular pathway) that appears to contribute much more than was previously thought. Applications dependent on skin penetration that have received special attention include transdermal delivery of nano- and microparticles by hair follicles, especially for nanoparticles which penetrate hair follicles very efficiently targeting the skin immune system in order to develop new vaccination strategies, and problems relating to skin diseases [154,155].

Options for topical and transdermal delivery are the solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) [156]. Some drugs such as triptolide, triamcinolone acetonide acetate, cyclosporin A have been used to be entrapped in SLN [157-159]. SLN can be admixed to an already commercially available and established topical formulation, e.g. a

cosmetic day cream. Admixing the SLN leads to an increase in occlusivity while still maintaining the 'light character' of the day cream and avoiding the glossiness of more occlusive night creams. This phenomena is explained in **Figure 7**. However, having a highly occlusive night cream already, addition of SLN will have little or no effect [156].

Lipid nanoparticles are other options to load arthemeter and econazole nitrate [160,161]. Celecoxib [162]. It was compared the permeability of coenzima Q 10 incorporated in NLC and in an emulsion with the same lipid contain. The occlusion effect of the cream was also investigated. The result showed a higher permeability of the molecule and a higher occlusive effect for the NLC than for the emulsion as it could be observed in **Figures 8 and 9** [163].

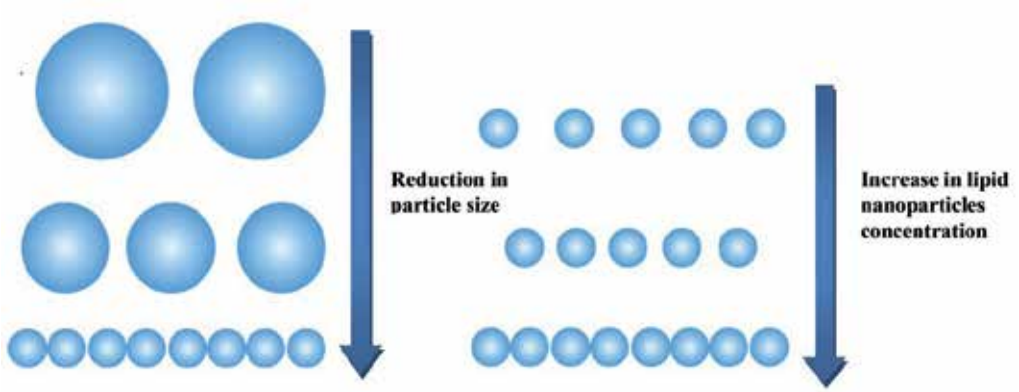

Figure 7. The occlusion factor of lipid nanoparticles depends on various factors: at identical lipid content, reducing the particle size leads to an increase in particle number, the film becomes denser (left) and therefore the occlusion factor increases. At a given particle size, increasing the lipid concentration increases particle number and density of the film (right) which also leads to a higher occlusion factor.

Different studies shown lipid nanoparticles were able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis. Enhancement of chemical stability after incorporation into lipid nanocarriers was proven for many cosmetic actives, e.g. coenzyme Q10 [164-166], ascorbyl palmitate [164,167], tocopherol (vitamin E) [165] and retinol (vitamin A) [168-170].

Three vitamin derivatives including vitamin C (ascorbyl tetraisoplamitate), vitamin E (tocopherol acetate) and vitamin A (retinyl palmitate) were also loaded in PLGA nanospheres, for skin whitening and anti-wrinkles/aging applications due vitamin C suppresses the blemishes because it limits the activity of tyrosinase, which promotes melanin production. Furthermore, it increases collagen formation to reduce wrinkles, and prevents cell oxidation by eliminating active oxygen. As to vitamin E and A, they also act as antioxidant and collagen promoter, respectively. They were able to reach the target areas in a stable form, sustain the pharmacological effect for a long time and be effective to reduce the wrinkles and produce a whitening effect [171]. In a sense, the idea of nanoparticle design for drug delivery systems in cosmetics applications is important.

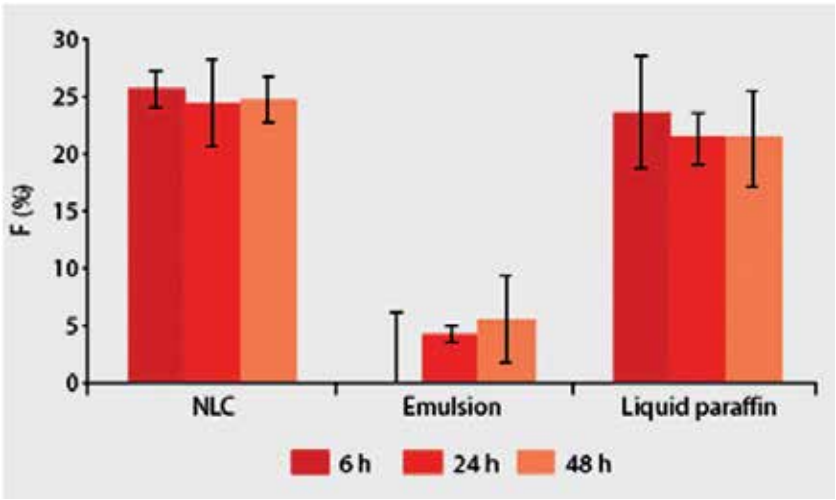

Figure 8. Occlusion factor (F) of NLC, emulsion and liquid paraffin (with permission from authors) [163].

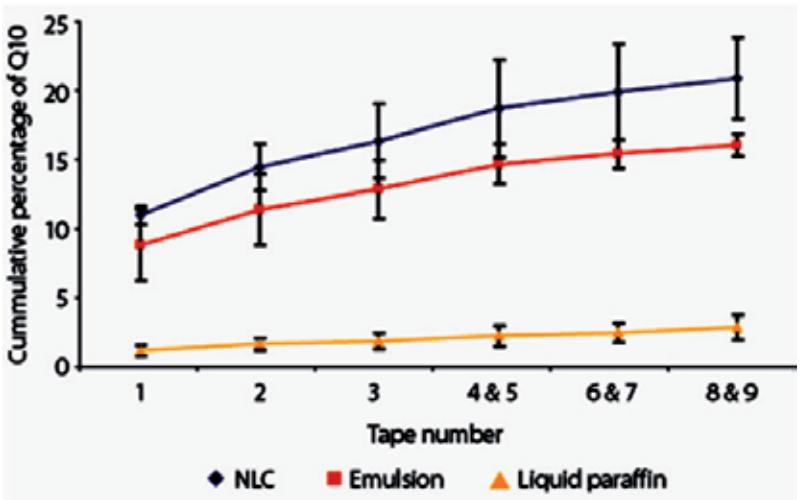

Figure 9. Tape stripping test: summation of coenzyme Q10 found in the tapes related to the applied amount (with permission from authors) [163].

As it was described before, the follicles are deep invaginations inside skin where the SC is thinner, the vascularisation is denser, and there are several targets of interest along a follicle structure from both cosmetic and pharmaceutical viewpoints [172]. Here, minoxidil which is an antihypertensive has been introduced in a block of copolymer poly (ε-caprolactone)-block-poly(ethyleneglycol) to treat the alopecia areata disorder , by widening blood vessels and opening potassium channels, it allows more oxygen, blood, and nutrients to the follicle. This disorder is an inflammatory condition, often reversible hair loss affecting mainly children and young adults. Clinically, round hairless patches appear on the scalp while hair follicles remain intact. This skin disorder is related with the distal part of the human hair follicle immune system, especially with the interacting intraepithelial T cells. The cause of

this condition is diverse and seems to involve T cell–mediated immunologic changes, neuropeptides, genetic disposition to autoimmunity, and distress [30,173,174]. As the infundibulum of the hair follicle is surrounded by an extensive capillary network and the permeability of its epithelium allows the transport of molecules or particles to the blood circulatory system. There is a high density of immune cells in and around the infundibulum epithelium which could be targets also for hair follicle immune system and topical vaccination.

The sebaceous glands associated with hair follicles provide another potential target for delivering drugs against acne, androgenetic alopecia and other sebaceous gland dysfunctions. Different nanoparticles formulations have been prepared in order to treat acne vulgaris, which is an inflammatory disease of the pilosebaceous units, most densely concentrated on the face and torso [175]. Pathogenesis is multifactorial, but *Propionibacterium acnes* a Gram-positive bacterium plays a central role in the promotion of inflammation in acne. The most commonly formulations are prepared with different topical antimicrobials, either alone or in combination with other drugs. It is expected that an agent able to inhibit *P. acnes* growth and to suppress the inflammatory response will provide significant benefits to patients with acne vulgaris [30,176,177]. For that reason triclosan has been used in several systems. It was reported the characterization of triclosan loaded polymeric nanoparticles. They showed a good encapsulation efficiency and also a good physical stability representing an alternative as a treatment of acne [33]. Triclosan loaded nanoparticles made of chitosan and cyclodextrins were prepared using a very simple ionic gelation technique. This new approach permits to enhance the entrapment of hydrophobic drugs by forming molecular inclusion complexes with cyclodextrins in aqueous media. Such a device could be of interest for conferring protection to some specific drug molecules through the complexation followed by entrapment in the polymer matrix [109]. Another drug used to treat this disorder is tretinoin (all-*trans*-retinoic acid) which is the active form of a metabolic product of Vitamin A, also called retinoic acid. Tretinoin-loaded nanocapsules improved tretinoin photostability, independently on the type of oily phase used (capric/caprylic triglycerides and sunflower seed oil) in this study, and represent a potential system to be incorporated in novel topical or systemic dosage forms containing tretinoin [178].

Formulations of nanoparticles are often used in combination with penetration chemical and physical enhancers to modify the physical state of the stratum corneum, affecting the degree of transdermal drug penetration. DNA has been entrapped in nanoparticle of polysaccharide such as chitosan/poly-γ-glutamic acid and in a multifunctional core-shell polymeric nanoparticle of PLGA core and a positively-charged glycol chitosan (GC) shell. Another drugs used in the preparation of nanoparticles made of propyl-starch derivatives are flufenamic acid, testosterone and caffeine [179,180]. Insulin is a protein which has also been introduced in chitosan nanoparticles [83]. Poly (lactide-co-glycolide) polymer has been used to prepare biodegradables nanoparticles containing dexamethasone phosphate and 5-Fluorouracil [181,182]. Chlorhexidine loaded polymeric nanoparticle are used to treat cutaneous infections [183].

Inflammatory skin diseases account for a large proportion of all skin disorders and constitute a major health problem worldwide. Psoriasis, atopic dermatitis, poison ivy, and eczema are another skin disorders. Contact dermatitis, atopic dermatitis, and psoriasis represent the most prevalent inflammatory skin disorders and share a common efferent T-lymphocyte mediated response. Oxidative stress and inflammation have recently been linked to cutaneous damage in T-lymphocyte mediated skin diseases, particularly in contact dermatitis [184]. Poison ivy and atopic dermatitis may also present with bullous and vesicular changes [185]. Lipid nanoparticles have been investigated to improve the treatments of skin diseases such as atopic eczema, psoriasis, skin mycosis and inflammations. Apart from the treatment of skin diseases by topical application, e.g. gastrointestinal side effects of non-steroidal anti-inflammatory drugs can be decreased by topical antirheumatic therapy. Drugs under investigations for dermal application using lipid nanoparticles at the present are for instance glucocorticoids, retinoids, non-steroidal anti-inflammatory drugs, COX-2 inhibitors and antimycotics. It was showed that it is possible to enhance the percutaneous absorption with lipid nanoparticles. These carriers may even allow drug targeting to the skin or even to its substructures. Thus they might have the potential to improve the benefit/risk ratio of topical drug therapy [186].

Perioral dermatitis is commonly seen in women aged 20–35 years. It presents as red papules that form superficial plaques around the perioral area, nasolabial folds and/or lower eyelids. It is minimally itchy [187,188]. Topical corticosteroids are the first-line therapy of acute exacerbations of atopic dermatitis and contact dermatitis. Prednicarbate is superior to the halogenated glucocorticoids because of an improved benefit/risk ratio. However, at the present the separation of desired anti-inflammatory effects and undesired antiproliferative effects is still not satisfying. Therefore, lipid nanoparticles were investigated as a delivery system for prednicarbate. The report show an improved extent of prednicarbate uptake by human skin *in vitro*, if applied as SLN dispersion or cream containing prednicarbate-loaded SLN. The authors found that a prednicarbate targeting to the epidermis [189]. This is particular relevant because prednicarbate in the dermis is responsible for the induction of irreversible skin atrophy while the inflammatory process is most pronounced within the epidermis [186]. Therefore, a better benefit/risk ratio is expected for the application of pretnicarbate in SLN containing topical formulations.

Tretinoin, a metabolite of vitamin A is used for topical treatment of various proliferateive and inflammatory skin diseases such as psoriasis, acne (as mentioned before), photo aging, epidermotropic T-cell lymphomas and epithelial skin cancer. One of the major disadvantages associated with the topical application of tretinoin is local skin irritation such as erythrema, peeling and burning as well as increased sensitivity to sunlight. To overcome these problems tretinoin was incorporated into SLN [190]. *In vitro* permeation studies through rat skin indicated that SLN-based tretinoin gel has a permeation profile comparable to that of the market tretinoin cream. Furthermore, Draize patch test showed that SLN-based tretinoin gel resulted in remarkably less erythremic episodes compared to the currently marketed tretinoin cream (**Figure 10**). Therefore, also for formulations containing tretinoin-loaded SLN a better benefit/risk ratio is expected.

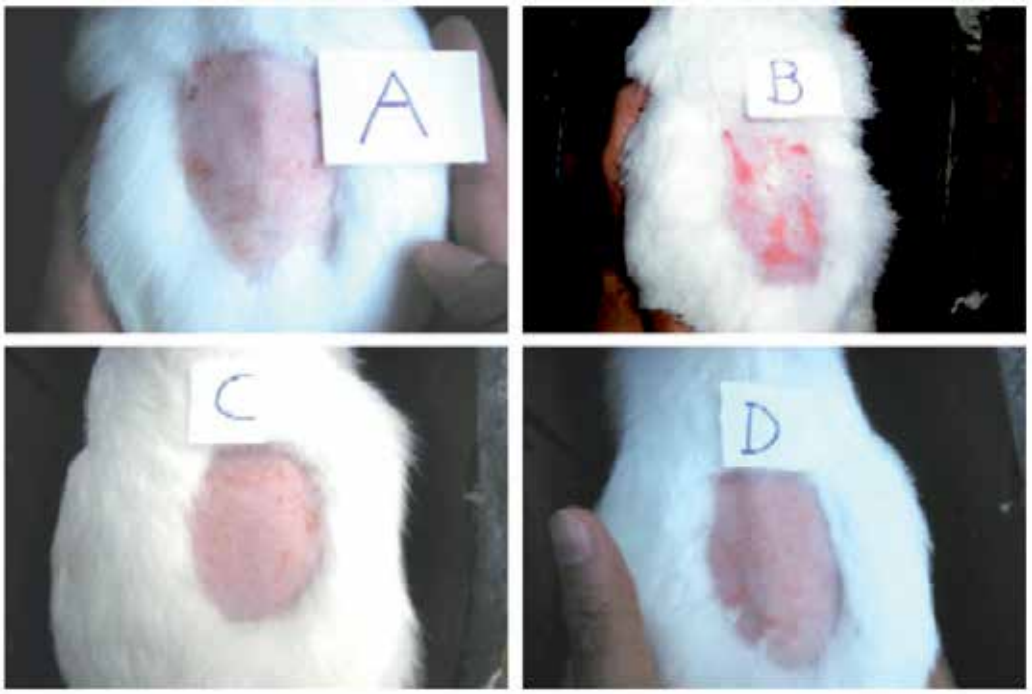

Figure 10. Pictures of Draize skin irritation studies carried out on New Zealand rabbits 24 h after application of (A) control (no application); (B) marketed formulation (Retino-A® cream); (C) SLN-based gel without tretinoin; (D) SLN-based gel containing tretinoin (0.05%, w/w). The Marketed tretinoin cream clearly shows erythemal lesions, which are not visible in SLN based tretinoin gel [190]. "Reprinted from International Journal of Pharmaceutics, 345/1-2, Kumar A. Shah, Abhijit A. Date,Medha D. Joshi,Vandana B. Patravale, Solid lipid nanoparticles (SLN) of tretinoin: Potential in topical delivery, 163-171., Copyright (2007), with permission from Elsevier"

Liposomes which were one of the first strategies for transdermal delivery are being successfully used in cancer therapy [139,141]. However to date, many liquid-type nanocosmetics 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 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. Some authors report the use of flexible vesicles called transfersomes or transformable liposomes in comparison with rigid vesicles to enhance penetration [148,191]. The application of transformable liposomes more flexible, which are prepared using surfactants or alcohol (ethosomes) in the lipid bilayer, to be able to deform them when a pressure is applied in the transdermal route has been increased.

In some researches, dendrimers are used for transdermal drug delivery. They show promising results in the delivery of drugs such as tamsulosin [192], indomethacin [193], ketoprofen and diflunisal [194] and 5-fluorouracil [195]. The main problems with this kind of transdermal carrier are poor biodegradation and inherent cytotoxicity [158]. In order to

get down the toxicity dendrimers have been linked to peptides (dendrimers-peptides) from amino acids linked via peptide-amide bonds to the branches of dendrimers in the core or on the surface [159,160].

For 5-fluorouracil (5FU) (log $P = -0.89$) which is one hydrophilic model drug used to treat skin diseases, has been reporte to have very poor penetration in skin [196-198]. Many strategies to increase skin permeation of this drug have been tested: prodrugs, terpenes, fatty acids, iontophoresis, sonophoresis, laser ablation and dendrimers which increased 5FU permeation across the skin by altering the skin structure [198-203].

Nowadays transdermal delivery using nanoemulsions it is not so used as nanoparticles or liposomes delivery due to the stability problems inherent to this dosage form. Nevertheless, Gamma Tocopherol, Caffeine, Plasmid DNA, Aspirin, Methyl Salicylate, Insulin, Nimesulide have been included in nanoemulsion. The use of these nanocarriers to deliver analgesics, corticosteroids, anti cancer agents, etc. is very important since these drugs are able to act immediately because they do not need to cross extra barriers. The drug is bioavailable easily and faster [204-210].

8. Conclusions

Nanocarriers have shown many advantages for topical and trasdermal delivery of drugs. It could be shown already for various drugs that topical/trandermal formulations containing nanoparticles can enhance the penetration into the skin increasing treatment efficiency, target the epidermis or follicles, reducing side effects. Furthermore, an increased activities as well as prolonged activities have been reported. These delivery systems can deliver both hydrophilic and lipophilic molecules. Advances with regard to materials, fabrication methods and techniques facilitate the development of new and better nanocarriers. Nonetheless, future researches must ensure the benefit and evaluate the risk ratio for many drugs included in nanocarriers.

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Chapter 9

Targeted Nanoparticles for Cancer Therapy

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Additional information is available at the end of the chapter

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1. Introduction

The World Health Organization (WHO) estimates that 84 million people will die of cancer between 2005-2015 [1]. According using the WHO mortality database it has been estimated the total number of cancer deaths in the European Union (EU) and in 2012 is predicted to be 1283101, of which 717398 men and 565703 women [2]. The most common types of cancer that will be diagnosed are lung (C33-C34), intestine (colon and rectum; C18-C21) and prostate (C61) for men, and breast (C50), intestine (C18-C21) and lung (C33-C34) for women.

Cancer is a group of diseases which cause an abnormal and uncontrolled cell division coupled with malignant behavior such as invasion and metastasis [3]. A tumor malignant is a neoplasm characterized by a failure in the regulation of tissue growth. The abnormal proliferation of tissues is caused by mutations of genes (oncogenes that promote cell growth and reproduction, and tumor suppressor genes that inhibit cell division and survival). Typically, changes in many genes are required to transform a normal cell into a cancer cell.

It is necessary to improve our knowledge of cancer physiopathology for effective cancer therapy, which will allow discover new anti-cancer drugs and develop novel biomedical technologies. The benefits of traditional chemotherapy are limited by the toxicity associated with anticancer drugs in healthy tissues. The common features of cancer and healthy cells make it difficult to achieve pharmacoselectivity of drugs at the target site.

The development of drug delivery systems that are able to modify the biodistribution, tissue uptake and pharmacokinetics of therapeutic agents is considered the great importance in biomedical research and the pharmaceutical industry. Controlled release in drug delivery can significance enhance the therapeutic effect of a drug. A constant concentration of a drug over an extended period of time keeping the drug concentration within the optimum range,

or a pulsatile drug release in response to an environmental change, can be achieved with controlled drug delivery systems [4]. In these type of systems, the drug is protected from degradation following administration, the delivery system can be administered close to the tumoral cells, the drug is released with a specific patron and the action of the drug on tumoral cells can be direct.

Nanotechnology, refers to the understanding and control of matter at dimensions between approximately 1 and 100 nanometers in at least one dimension. Nanomaterials have a large surface area to volume ratio and their biological and physicochemical properties, such as friction and interaction with other molecules, are distinct from equivalent materials at a larger scale. These new properties open opportunities in a wide variety of areas of technology, ranging from intelligent nanoscale materials to medicine and biology, where first nanotechnology applications have demonstrated an enormous potential [5]. Thus, the term nanomedicine has been taking shape and has been defined as the applications of nanotechnology for treatment, diagnosis, monitoring and control of biological systems by the National Institutes of Health [6]. Nanomedicine attempts to use sophisticated approaches to either kill specific cells or repair them one cell at a time, offering new possibilities towards the development of personalized medicine [7] focused on certain diseases which are currently being investigated, especially cancer.

One of the most important and hopeful tools employed in nanomedicine are nanoparticles (NPs), which are solid, colloidal particles consisting of macromolecular substances that are being developed to: improve drug bioavailability, abrogate treatment-induced drug resistance, and reduce nonspecific toxicity in the field of medicine. Depending on the method of preparation NPs can be constructed to possess different properties and release characteristics for the best delivery or encapsulation of the therapeutic agent [8]. In all these types, drugs can be absorbed onto the surface, entrapped inside, or dissolved within the matrix of the NPs [9]. One advantage of NPs is their ability to overcome various biological barriers and to localize into the target tissue. The first generation of NPs comprises passive delivery systems that, in case of cancer, reach the tumor through the fenestrations in the adjacent neovasculature [10]. The unique mechanism of driving systems to the tumor site is the nanometer size of particles, not specific recognition of the tumor or neovascular targets.

In order to optimize the therapeutic index of antitumor drugs, decreasing their toxicity to normal tissues, a second generation of nanosystems includes additional functionalities that allow for molecular recognition of the target tissue or for active or triggered release of the payload at the disease site. Thus, the presence of reactive pendant groups in NPs make easy their vectorization forward specific cell motif by binding of ligands. These include various ligands [11-13] that bind to specific target cell surface markers or surfacemarkers expressed in the disease microenvironment. Responsive systems, such as pH-sensitive polymers, are also included in this category. Hence, over the past

years, efforts have been focused on the development of nanomedicines such as NPs, liposomes, micelles or dendrimers for the specific delivery of anticancer drugs to tumor tissues [14].

2. Physiological characteristics of solid tumors

Tumors are characterized by poorly differentiated, highly chaotic arrangement of vessel which have endothelial cell-cell junctions and discontinuous basement membrane. Angiogenesis is not only a prerequisite for the transformation from a small, often dormant cluster of cancer cells to a solid tumor, but is also required for the spread of tumor. Microvascular network is absolutely essential for the development of solid tumors. Once a tumor cell cluster, whether in its initial stage as a primary tumor or in later stages when forming metastases, induces an angiogenic switch, its vasculature and microenvironment changes dramatically, and abnormal cellular organization, vessel structure, and physiology function develops (Figure 1). Angiogenesis is defined as the formation of new blood vessels from existing ones. For solid tumors of 1-2 mm^3, oxygen and nutrients can reach the center of the tumor by simple diffusion. Because of their non-functional or non-existent vasculature, non-angiogenic tumors are highly dependent on their microenvironment of oxygen and the supply nutrients. When tumor reaches 2 mm^3, a state of cellular hypoxia begins, initiating angiogenesis.

Angiogenesis is regulated by a fine balance of activators and inhibitors [15]. The vascular endothelial grown factor (VEGF), also called vascular permeability factor (VPF), plays an important role in regulating the process of tumor angiogenesis. VEGF has been shown to stimulate the proliferation, migration and invasion of endothelial by interacting with a family of tyrosine kinase receptor expressed on vascular endothelium. VEGF is also known to have the ability to enhance the permeability of microvessels, favoring the rapid and reversible increases in extravasation of plasma protein in tissue [16]. In the angiogenesis process, different phases can be distinguished: Dilation of existing vessels, endothelial cell activation, migration and proliferation, hyperpermeability of postcapillary venules and vessel destabilization, basement membrane degradation by proteases such as matrix metalloproteases, cathepsines, urokinase and plasmin, endothelial cell migration, vessel formation and angiogenic remodeling [17].

The new tumor vessels formed during angiogenesis differ markedly from those of normal tissues and the neovasculatures is characterized by an irregular shape, high density, and heterogeneity, and also have different oxygenation, perfusion, pH and metabolic states. The abnormal vascular architecture plays a mayor role for an EPR (Enhanced Permeability and Retention) effect [18]. Extensive angiogenesis and hypervasculature, lack of smooth-muscle layer, pericytes, defective vascular architecture: fenestrations, no constant blood flow and direction, inefficient lymphatic drainage that leads to enhanced retention in the interstitium of tumor and slow venous return that leads to accumulation from the interstitium of tumor.

Physiological changes in blood flow within the tumors and in transport properties of tumor vessels are consequences of these vascular abnormalities. The osmotic pressure in tumors is high [19]. The interstitial compartment of tumors is significantly different to that of normal tissues. Primarily, as a result of vessel leakiness and hyperpermeability with a concomitant bulk flow of free fluid into the interstitial space that cannot be removed effectively due to a lack of functional lymphatics, due to cancer cells compress lymphatic vessels causing their collapsed. The lymphatic network transports interstitial fluid and immune cells out of normal tissue and is essential for immune function and maintenance of fluid balance in tissue interstitium. In tumor cells the vessels are compressed by solid stresses. The function of lymphatic vessels depends on their localization, when they are at the periphery of the tumor or the periphery tumor interface possesses functionality, while those within the tumor are functionality defective. VEGF factors (VEGF-C and VEGF-D) and their corresponding receptors have been identified as specific lymphangiogenesis factors in several tumors, and have been implicated in increased lymphatic metastases in numerous tumors [20].

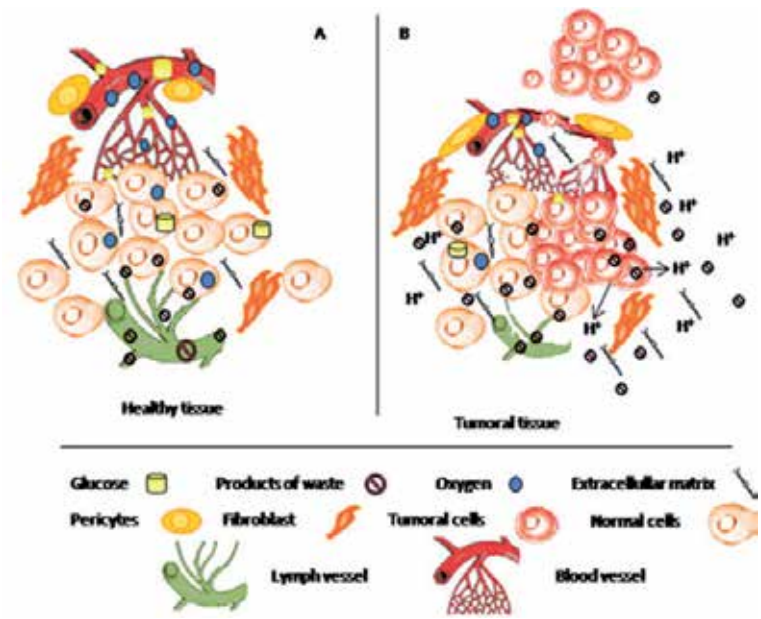

Figure 1. Differences between healthy and tumor tissues. A) Healthy tissue is characterized by a good flow in blood vessel. These vessels are supported by pericytes with a good physiological organization and structure. They provide adequate amounts of glucose and oxygen to normal cells. Collagen fibres, fibroblasts and macrophages are present in the extracellular matrix. Lymph vessels are present and allow the elimination of waste products. B) Tumoral tissue is characterized by vascular disorganization, with fenestrations and discontinuous basement membrane, that promotes the metastasis of abnormal cells to other tissues, inadequate supply of nutrients and poor lymphatic network that does not drain properly increasing the amount of waste products in these tissues and also increasing the protons concentration which decrease the physiological pH. Components of extracellular matrix (collagen fibres, fibroblasts and macrophages) in this type of tumor tissue are also increased

Leaky tumor vasculature and dysfunctional lymphatics in tumor interstitium result in undesirable accumulation of vascular contents in the tumor leading to interstitial hypertension [19]. In normal tissues the interstitial fluid pressure (IFP) is approximately 0 mm Hg, and the pressure in the capillary is around 1-3 mmHg, this gradient facilitates the transport of macromolecules. In tumor tissues the pressure gradient is contrary, consequently, interstitial hypertension results in reduce convection across the walls of tumor blood vessels. IFP tends to be higher at the center of solid tumors, diminishing toward the periphery, creating a mass flow movement of fluid away from the central region of tumor. The microvasculature pressure in tumors is also one to two orders of magnitude higher than in normal tissues.

Abnormal tumor vasculature reduces blood flow and limit delivery of oxygen throughout the tumor resulting in regions of hypoxia. There are different types of hypoxia: inadequate perfusion (ischemia), increased diffusion distance (chronic hypoxia), anemia and hypoxemia [20]. The hypoxic condition initiates signaling events that trigger the upregulation of multiple pro-angiogenic factors in the tumor lesion, another consequence, the lack of oxygen promotes an anaerobic metabolism of tumor cells and an extracellular acidosis in tumor tissues, primarily due to excessive production of lactic acid and CO_2 [20].

So, while the intracellular pH of cells within healthy tissues and tumors is similar, tumors exhibit a lower extracellular pH than normal tissues. Accordingly, although tumor pH may vary according to the tumor area, average extracellular tumor pH is between 6.0 and 7.0, whereas in normal tissues and blood the extracellular pH is around 7.4 [21-22]. Low pH and low pO_2 are intimately linked and a variety of insights now support their roles in the progression of tumor from in situ to invasive cancer [23]. The low extracellular tumor pH mostly arises from the high glycolysis rate in hypoxic cancer cells. However, ATP hydrolysis, glutaminolysis, and ketogenesis also contribute to this extracellular acidic pH.

Therefore, due to the cancer cell presents differences compared to normal cell including vascular abnormalities, interstitial pressure, oxygenation, pH, metabolic states, and abnormal lymphatics, a preferential accumulation of encapsulated drug at desired sites can be obtained either by passive or active targeting.

3. Targeted drug delivery nanoparticles

Targeted NP therapeutics have shown great potential for cancer therapy, as they provide enhanced efficacy and reduced side effects [24]. NP drug delivery can be either an active or passive process. Passive delivery refers to NP transport through leaky tumor capillary fenestrations into the tumor interstitium and cells by passive diffusion or convection [25]. Selective accumulation of NP and drug then occurs by the already mentioned characteristics of the tumor microenvironment (Figure 2).

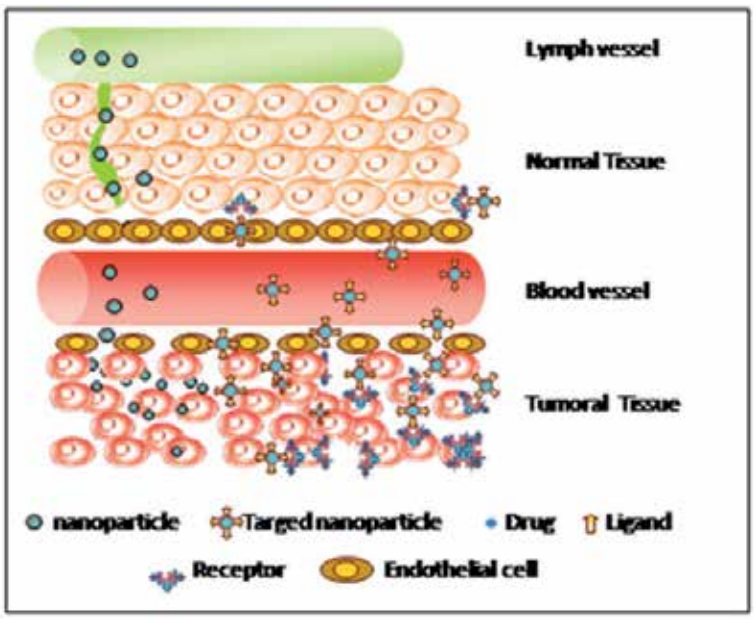

Figure 2. NPs are more able to reach tumor cells through passive targeting due to the characteristics of tumor tissue (vascular disorganization, fenestrations, discontinuous basement membrane, etc.). In normal tissues the lower amount of nanoparticles that able to reach it are removed by lymph vessels while, in tumor tissues, lymphatic network is too damaged to perform its function promoting the accumulation of nanoparticles in the tissue. The functionalized nanoparticles are internalized not only by passive targeting but also by active targeting. This active targeting is more effective in the tumor tissue due to tumor cells overexpress some receptors that allow them a better uptake of functionalized nanoparticles.

Active targeting involves drug delivery to a specific site based on molecular recognition. One such approach is to couple a ligand, such monoclonal antibodies, lectins, aptamers, folate, and peptides, to a NP so that the ligand can interact with its receptor at the target cell site (Figure 2). Depending on the type of ligand-receptor interaction, the rate of cellular internalization would differ. This is an important factor as rates of internalization could affect the accumulation of NP in tumor sites. The use of a targeting moiety also facilitates cellular uptake of the drug by receptor mediated endocytosis, which is an active process requiring a significantly lower concentration gradient across the plasma membrane than simple endocytosis. Thus there is plenty of room to improvise these systems to address the above-mentioned issues and different groups are working to improve the targeting properties of NPs and for the development of targeted therapeutics [26]. In table 1 are shown various nanocarriers evaluated to deliver therapeutic agents into cancer cells.

Ligand	Nanosystem	Drug	Cell/tumor model	Reference
AS1411 DNA apt	nucleolin /liposomes	cisplatin	MCF-7	[27]
	PEG-PLGA NPs	PTX	C6 glioma cells	[28]
Sgc8c apt	SWNTs	Dau	acute lymphoblastic leukemia T-cells (Molt-4).	[29]
Thrombin apt	Mesoporous silica NP	Dtxl	HeLA cells	[30]
EGF	SWNTs	Cisplatin	HNSCC	[31]
antiHER-2 ab: Trastuzamab	PLGA/MMT NPs	PTX	breast cancer	[32]
Tf	PLA-PEG NPs	PTX	BT4C rat glioma model	[33]
	PLGA–NPs	PTX	C6 glioma cells PC3 Prostate cancer cell	[34]
	G4 PAMAM dendrimers	Dox,	C6 glioma cells	[35]
FA	G5 PAMAM dendrimers	MTX	human KB tumor xenografts	[36]
	PEG liposomes	Dox	KB cells	[37]
	NIPA-NPAM-2AAECM	5-FU, TMX	T47D cells, HeLa cells	[38]
cRGD	PEG-PTMC micellar NPs	PTX	U87 MG cells	[39]
Antiαv integrin ab	HSA NPs	Dox	Melanoma cells	[40]

Table 1. Examples of nanocarriers used for active targeted drug delivery

3.1. Aptamers

Originally discovered in 1990, aptamers are short nucleic-acid-based single stranded ligands (DNA, RNA, oligonucleotide), whose size could vary from 20 to 80 nucleotides [27,41], that, through intramolecular interactions, fold into unique tertiary conformations capable of binding to target proteins with high affinity (K_D=10 pmol/l to 10 μmol/l) and specificity. This property makes them an attractive class of targeting molecules as they are also nonimmunogenic and exhibit remarkable stability. Aptamers can tolerate a moderate change in temperature, pH (4-9), and ionic strength, and can be processed with organic solvents without a lost of activity [13]. Aptamers are chemically synthesized and they possess additional advantages over natural antibodies [42] including a smaller size, and single-chain variable fragment antibodies what allows for more efficient penetration into biological compartments [43] and due to which they accumulate quickly within the tumor tissue. It is possible to chemically modify aptamers to facilitate covalent conjugation to nanomaterials, for example, with 50 or 30 amino or thiol groups. These properties in aptamers enable them to withstand the common production conditions encountered during NP preparation. However, due to this small size, aptamers can be cleared quickly by the

kidneys. To delay their clearance, polyethylene glycol (PEG) or cholesterol can be added to aptamer NPs [44-45]. Aptamers that are internalized by cells can be used to study internalization pathways or used as drug targeting agents [24].

Traditionally, a number of compounds were assayed to isolate a ligand for the production of aptamers. However, development of a technique called 'in vitro selection' or Systematic Evolution of Ligands by Exponential Enrichment (SELEX) has allowed the rapid and selective production of aptamers. Briefly, the SELEX method starts with a random library of 1013–1016 single-stranded DNA or RNA and uses an iterative process that specifically amplifies sequences that have high binding affinity to the target molecules [46-47]. Although many complex forms of SELEX exist, there are two basic forms of SELEX (Cell-SELEX and Automated SELEX) [41].

Aptamers can be designed as targeting ligands, and can differentiate diseased cells from healthy cells, thus enabling the selective delivery of therapeutic compounds to target cells [41,47]. A large number of aptamers have been raised against cancer-associated antigens such us AS1411 aptamer for targeting nucleolin protein, which is highly expressed in the membrane of cancer cells [48-49], aptamers CPG 7909 and IMO 2055, that target Toll-like receptor 9 (TLR9), which is expressed by certain immune cells, TD05 aptamer, which was selected for the Burkitt's lymphoma Ramos cell line [47], Sgc8c aptamer which targets leukemia biomarker protein tyrosine kinase-7 (PTK7) [46,50] and can recognize target leukemia cells, DNA aptamers to leukemic lymphoid (CEM) cells [46], and fruoropyrimidine RNA aptamers which target Prostate-specific membrane antigen (PSMA) [51] for targeting prostate cancer. And also another aptamers against antigens such as pigpen [52] for targeting the tumor microvasculature, or mucin 1 (MUC1) [53] for targeting various epithelial neoplasms that upregulate MUC1, whose expression has been associated with carcinomas.

Aptamer-functionalized NPs have also been widely used for cancer cell specific drug delivery. Aptamers that were conjugated to NPs resulted in increased targeting and more efficient therapeutics, as well as more selective diagnostics. For instance, it has been synthesized NPs of poly(D,L-lactide-coglycolyde) [PLGA] and PEG triblock copolymer using aptamers as a targeting ligand for PSMA and Docetaxel (Dtxl)-encapsulated demonstrated that they bind and are taken up by LNCaP prostate ephitelial cells resulting in a significantly enhanced in vitro cellular toxicity as compared with nontargeted NP [24]. In the same way A10 aptamer is being explored for the targeted delivery of several anticancer agents, by including paclitaxel (PTX) and cisplatin in NPs [51,54]. Guo and coworkers conjugated DNA aptamers to a PEG-PLGA NP as a novel drug delivery system capable of targeting cancer cells and endothelia cells in angiogenic blood vessels [28]. In the tested C6 glioma cells, aptamer-nucleolin specific binding resulted in the cellular association of NPs and thereby enhanced the cytotoxicity of the PTX delivery. They suggested the potential of utilizing Ap-PTX-NP as therapeutic drug delivery platform for gliomas treatment [28].

Besides organic NPs, inorganic systems of Au-Ag nanorods (NRs) were synthesized to serve as a platform for binding several aptamer molecules. Thus, Au-Ag NRs have been

conjugated with multiple anti-PTK7 aptamers, such as scg8 aptamer, for targeted cancer photothermal therapy [48,55]. By using Au-Ag NRs that can be conjugated around 80 aptamers, 26 times higher binding affinity was obtained compared to individual aptamer strands [48]. By functionalizing the surface of Au NPs with an RNA aptamer that binds to PSMA, NP–aptamer conjugates were used for targeted molecular computed tomography imaging and treatment of prostate cancer [56].Yin et al. reported a one-step method for the synthesis of DNA-aptamer templated fluorescent silver nanoclusters (AgNCs) [57]. The Sgc8c aptamer strands were immobilized onto AgNCs through cytosine-rich sequence, and the resulting Sgc8c-modified AgNCs showed specific targeting to CCRF-CEM cancer cell over control cells.

In addition to their ability to recognize a target molecule with high specificity, certain aptamers can also modulate the activities of proteins implicated in pathological conditions, making aptamers potentially useful as pharmaceutical agents. For instance, one of the most important success of aptamers so far has been the development of aptamers that are able to bind VEGF [58] such as Pegaptanib sodium aptamer (Macugen, Pfizer, and Eyetech). However, as aptamers are expensive to produce, it is more economical to use aptamers as targeting agents rather than as therapeutic agents. Another example is AS1411, that binds specifically to nucleolin, a bcl-2 mRNA binding protein involved in cell proliferation, which is found on the surface of many cancer cells. Once bound, the AS1411 aptamer is taken into the cancer cell, where it causes death by apoptosis [47,59].

Furthermore, antidotes for anticancer agent toxicities are of interest to regulate drug activity. Thus, aptamers can also be prepared as antidotes for anticancer drugs to modulate anticancer effects. In this way, cDNA aptamer was recently designed for inhibiting cisplatin activity. The multifunctional carrier system consisted of cisplatin as the anticancer agent, which was encapsulated within a liposomal system and conjugated to AS1411-derived aptamer. In the absence of cDNA, the targeted NP showed cell-specific targeting and an improved cytotoxicity. When de cDNA aptamer was administered, it inhibited the cytotoxic activity of cisplatin. However, the interval between the administration of cDNA and NP seemed to be critical [11,27].

3.2. Human epidermal receptor

The Human epidermal receptor (HER)-family tyrosine kinases play a central role in the proliferation, differentiation, and development of cells as they are known to mediate a cell signaling pathway for growth and proliferation in response to the binding of the growth factor ligand [60]. The family consists of four members: epidermal growth factor receptor (EGFR or HER1), HER2 (also known as ERBB2 or HER-2/neu), HER3 and HER4. Each of these receptors has an extracellular region, a single transmembrane region, and a cytoplasmic sequence containing a tyrosine kinase domain and a C-terminal tail [61].

EGFR has six known endogenous ligands: EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), and epiregulin [60]. Using any of these ligands as targeting moieties offers a method for targeting the EGFR; especially

TGF-α and EGF as are the most commonly detected in humans. Ligand binding to EGFR results in activation of intracellular signaling cascades in cancer cell proliferation, apoptosis, migration, sensitivity to chemoradiation therapy, and tumor angiogenesis, and the complex is internalized for destruction and recycling [12, 62-63]. Over one-third of all solid tumors have been shown to express EGFR, and in many of these tumors, EGFR expression characterizes a more advanced disease stage [60]. The presence of EGFR corresponds directly to the metastatic capabilities in various types of cancer, such as colorectal [12]. Among the wide range of tumors that overexpress EGFR are breast, lung, colorectal, pancreatic cancers [63], glioblastomas [64], and brain cancers [65].

Hence EGF target delivery systems have been used in cancer molecular imaging diagnosis and therapy [63]. Thus, cisplatin and EGF were attached to single-wall carbon nanotubes (SWNTs) to target squamous cancer cells HNSCC which overexpress EGFR. Through Qdot luminescence and confocal microscopy, it was shown that SWNT–Qdot–EGF bioconjugates was rapidly internalized into the cancer cells, and HNSCC cells were selectively killed in vitro, while tumor growth was regressed in vivo [31]. A current cancer treatment that targets EGFR is the monoclonal antibody Cetuximab, which targets the extracellular domain of EGFR and small-molecule inhibitors of tyrosine kinase activity [66]. One study showed that boronated immunoliposomes with conjugated Fab' fragments of Cetuximab mAb delivered ~8 times more boron to EGFRpositive cells (F98EGFR) than non-targeted IgG immunoliposomes [67].

With regard to HER2, among tumor biomarkers the HER2 membrane receptor is one of the most promising targets for immunotherapy. The surface accessibility, the high level of expression in certain primary and metastatic tumors and the internalization of these antigens via receptor-mediated endocytosis [68] promote preferential intracellular accumulation of drug nanocarriers [69]. The gene encoding HER2 protein is present in normal cells as a single copy and is expressed at low levels in many normal epithelial cells. Amplified HER2 gene and its over-expressed protein product are found in many types of cancers, including breast, ovary, lung, pancreas [63,70], stomach and renal. The overexpression of HER2antigens (*c-erb*B-2, neu) in 20–30% of breast and ovarian cancers is correlated with a high occurrence of metastasis and angiogenesis processes, as well as with a poor prognosis [71]. The ligand binding to the extracellular domain of HERB2 causes the dimerization of the receptor and in this way the activation of many intracellular signaling proteins and physiological pathways, such as the mitogen-activated protein kinases (MAPK) pathway, phospahtidylinositol 3-kinase/AKT/mTOR pathway, and Src tyrosine kinase [61].

Thus, antibodies and antibody fragments, consisting of only the Fab binding regions, against the HER2 receptor are common examples of receptor targets. These antibodies generally exhibit strong interactions with corresponding receptors, with dissociation constants in the nanomolar range. The advantages of the antibody fragments is that they are smaller, and do not contain the Fc region of the antibody which can induce immunogenicity and antigenicity [72]. Antibody-labeled NP is one of the most coveted modes of active targeting of NPs. Blocking the activity of the upregulated receptor by binding it with a ligand, such as

monoclonal antibody (mAb) represented on the nanovector, would ensure arrest of the signalling pathway(s).

Anti-Her2 mAbs (trastuzumab; Herceptin®), a humanized mAb designed to specifically antagonize HER2 function, was approved in 1998 for metastatic breast cancer overexpressing HER2 antigens [70]. Hence Herceptin® is used as a targeting moiety for various NP systems. For instance, incorporation of anti-HER2 antibodies onto the surfaces of PEGylated liposomes has indeed shown greater efficiency for drug delivery compared to non-targeted PEG-liposomes [73] and significantly higher intracellular accumulation was observed with targeted liposomes in xenografts of the HER2 overexpressing BT-474 tumors compared to MCF-7 tumors [69]. It has also been used PTX-loaded anti-HER2 immunonanoparticles (NPs-PTX-HER) which were prepared by the covalent coupling of humanized monoclonal anti-HER2 antibodies (trastuzumab, Herceptin®) to PTX-loaded poly (DL-lactic acid) NPs (NPs-PTX) for the active targeting of tumor cells that overexpress HER2 receptors [71]. NPs-PTX were thiolated and conjugated to activated anti-HER2 mAbs to obtain immunonanoparticles. The immunoreactivity and the *in vitro* efficacy of NPs-PTX-HER were tested on SKOV-3 ovarian cancer cells that overexpress HER2 antigens and it was demonstrated the greater cytotoxic effect of NPs-PTX-HER compared to other PTX formulations. Lyu and coworkers [74] used a single-chain Fv antibody (scFv23) targeting HER-2/neu to deliver tumor necrosis factor (TNF) to TNF-resistant pancreatic cancer cells and compared the cell responses to TNF alone, scFv23/TNF, herceptin, and combinations of scFv23/TNF with various chemotherapeutic agents including 5-Fluorouracil (5-FU), cisplatin, doxorubicin (Dox), gemcitabine, and etoposide. Their results indicated that delivery of TNF to HER2/neu-expressing pancreatic cancer cells using HER2/neu as a targeting molecule may be an effective therapy for pancreatic cancer especially when utilized in combination with 5-FU.

3.3. Transferrin receptor

Transferrin (Tf) (M_w=80 kDa) is the fourth most abundant serum nonheme iron-binding glycoprotein. It is synthesized by the liver and secreted to plasma, where it binds to endogeneous iron, forming the iron-transferrin chelate which is an important physiological source of iron for cells in the body. It helps to transport iron to proliferating cells [75], which is required as a cofactor for DNA synthesis [76], and it also plays a pivotal role in the transportation of iron for the synthesis of hemoglobin. Based on these facts, Tf can be potentially utilized as a cell marker for tumor detection.

Normally at a cell, Tf offloads the iron onto a transferrin receptor (TfR). The natural ligand for TfR, Tf, binds to its receptor with a dissociation constant of around 40nM. TfR, also known as CD71, is a dimeric transmembrane glycoprotein (180 kDa) [77]. The receptor for Tf, referred to as TfR1, is ubiquitously expressed at low levels in most normal human tissues. A second member of the TfR family is TfR2, a protein that is homologous to TfR1 but whose expression is largely restricted to hepatocytes [76]. This receptor is an attractive molecule for the targeted therapy of cancer since it is upregulated on the surface of many

cancer types and is efficiently internalized. Serving as the main port of entry for iron bound Tf into cells, TfR1 is a type-II receptor that resides on the cell membrane and cycles into acidic endosomes into the cell in a clathrin/dynamin dependent manner [78]. The low pH environment triggers dissociation of the iron and the iron-poor Tf is released out of the cell for recycling. As cancer cells rapidly proliferate, the TfR is overexpressed in the surface of malignant cells due to the increased requirement of iron [12]. In this sense, many studies have indicated that the expression level of TfR on tumor cells is much higher than that on normal cells [79], such as the surface of cerebral endothelium and brain tumor cells [80], breast cancer, prostate cancer, and squamous cell carcinomas [63]. This enhanced TfR expression, at levels correlating with the grade of malignancy [81], can be exploited for actively delivering anticancer agents specifically to tumor tissues. This receptor can be targeted in two ways: 1) for the delivery of therapeutic molecules into malignant cells or 2) to block the natural function of the receptor leading directly to cancer cell death [78].

A wide variety of therapeutic agents have been used for TfR-targeted cancer therapy. They include chemotherapeutic drugs, bacterial toxins, plant toxins, DNA, oligonucleotides, short inhibitory RNA (siRNA), and enzymes. Vast types of anti-cancer drugs that have been delivered into cancer cells employing a variety of receptor binding molecules including the use of its natural ligand Tf, anti-TfR antibodies, or TfR-binding peptides alone or in combination with carrier molecules including NPs and viruses [78].

With regard to NPs, Tf has a number of properties that allow it to be successfully incorporated as a targeting ligand in NP systems, such as its stability over a wide pH range (3.5-11) and that has shown to be unaffected by repeated freeze-thaw cycles; hence, it can be subjected to processing conditions commonly encountered during NP preparation [11]. Furthermore, Tf is available in recombinant version (Optiferrin) [82] and, as a human protein, has low immunogenicity [83]. Normally, Tf can be conjugated to NPs less than 100 nm in size to obtain an enhanced cytotoxic activity. If the NPs are greater than 100 nm, it may lead to poor accumulation of these NPs in the tumor cells, which results in moderate anticancer activity. To overcome this issue, the actively targeted system can be directly administered into the tumor tissue by intratumoral injection [84].

Tf-conjugated NPs have been explored in a number of studies for the delivery of anticancer agents. Thus, gold NPs were conjugated with Tf molecules for targeting, imaging and therapy of breast cancer cells (Hs578T, ATCC), showing that, the Tf–TfR-mediated cellular uptake of gold NPs is six times of that in the absence of this interaction [85].

It has also been [86] prepared PTX loaded NPs with shells formed of the biodegradable polymer, PLGA, conjugated to Tf via epoxy linkages. The Tf-conjugated NPs demonstrated greater cellular uptake and reduced exocytosis, yielding greater antiproliferative activity and more sustained effects compared to the free drug or unconjugated NPs. In a similar way, particulate nanodrugs consisting of PLGA loaded with PTX were conjugated to Tf (PTX–NPs–Tf) using an epoxy compound (Denacol-EX-521) [84]. These PTX–NPs–Tf showed a 70% in vitro inhibition of proliferation in human prostate cancer PC3 cells, while at the same concentration the NPs without ligand exhibited 25% inhibition, and PTX in

solution resulted in a 35% [84]. Tf-conjugated lipid-coated PLGA NPs carrying the aromatase inhibitor, 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD), were synthesized and evaluated for aromatase inhibition efficiency in SKBR-3 breast cancer cells. PLGA NPs loaded with the 7α-APTADD were significantly more effective preventing proliferation of the human breast cancer cell line SK-BR-3 than non-targeted NPs. These results suggested that the aromatase inhibition activity of the Tf-NPs was enhanced relative to that of the non-targeted NPs, which was attributable to TfR mediated uptake [87]

Gan and coworkers observed that Tf-conjugated poly(lactide)-D-α-tocopheryl polyethylene glycol succinate diblock copolymer NPs loaded with Dtxl could be more efficient eliciting cytotoxicity against C6 glioma cells than other nontargeted formulations [88].

Transferrin–PEG–adamantane (Tf-PEG-AD) conjugates synthesized for NP modification have been used to target malignant tumors including Ewing's sarcoma [89-90]. Thus, several Tf-NPs have been successfully entered into clinical trials. CALA-01 [91] is one of the first clinically successful transferrin-conjugated nanoparticulate system. This system consists of a duplex of synthetic nonchemically modified siRNA, which self-assembles to a cationic copolymer containing cyclodextrin, AD-PEG as a stabilizing agent, and AD-PEG-Tf as the targeting moiety. After administration, the nanocomplex provides siRNA protection from nucleases in the serum, minimizes erythrocyte aggregation, and reduces complement fixation. At the tumor site, the Tf binds to the tumor cell TfR, which leads to preferential uptake of the complex within the tumor cell. In the cell, the polymer unpacks from the small interfering RNA allowing it to interfere with RNA resulting in reduced tumor growth [92].

Hydroxycamptothecin (HCPT)-loaded stealth niosomes(NS) modified with transferrin (Tf-PEG-NS) were prepared with poly(methoxy-polyethylene glycol cyanoacrylate-co-hexadecyl cyanoacrylate) (MePEG-PHDCA) as surface modification material [93]. Tf-PEG-NS demonstrated the strongest cytotoxicity to three carcinomatous cell lines (KB, K562 and S180 cells), the greatest intracellular uptake especially in nuclei, the highest drug concentration and largest area under the intratumoral HCPT concentration curve, as well as the most powerful anti-tumor activity compared with other niosomes. More reciently Tf modified stealth NPs (Tf-PEG-NP) encapsulating PEG-HCPT conjugate were prepared and was studied the possibility of combination of the functions of passive and active targeting by Tf-PEG-NP, as well as sustained drug release in tumor by PEGylated drug for most efficient tumor targeting and anti-tumor effects enhancement. The advantages of such system included prolonging drug residence time in circulation and increasing EPR effect by the sterically stabilising action of PEG-PHDCA NPs, active targeting function of transferrin by transferring receptor-mediated endocytosis, and sustained releasing drug in tumor by PEGylation of the drug. The prepared Tf-PEG-NP showed more sustained in vitro release profile. The pharmacokinetic and biodistribution studies found that Tf-PEG-NP demonstrated the longest retention time in blood, the highest tumor accumulation, as well as the most powerful anti-tumor activity with the inhibition rate up to 93% against S180 tumor in mice [94].

A pH-sensitive dual-targeting drug carrier (G4-Dox-PEG-Tf-TMX) was synthesized with Tf conjugated on the exterior and Tamoxifen (TMX) in the interior of the fourth generation (G4) Poly(amido amine))(PAMAM) dendrimers for enhancing the blood brain barrier (BBB) [35].

The pH-triggered Dox release was 32% at pH 4.5 and 6% at pH 7.4, indicating a comparatively fast drug release at weak acidic condition and stable state of the carrier at physiological environment. MDR proteins, such as P-glycoprotein (P-gp), MRP4 (ABCC4), and breast cancer resistance protein (BCRP), are over expressed on the BBB and glioma cells, thus causing the block of overcoming the BBB and low uptake of drugs by the tumor cells [95]. The in vitro assay of the drug transport across the BBB model showed that G4-Dox-PEG-Tf-TMX exhibited higher BBB transportation ability. The carrier was internalized into C6 glioma cells upon crossing the BBB model by the coactions of TfR-mediated endocytosis and the inhibition effect of TMX to the drug efflux transports. Moreover, it also displayed the in vitro accumulation of DOX in the avascular C6 glioma spheroids made the tumor volume effectively reduced.

But, besides of its natural ligand, Tf, it have also been used other ligands conjugated to NPs. Thus, antibodies and antibody fragments against the TfR are common examples of receptor targets [72]. Among these are the mAbs A24 [96], Rat anti-murine TfR RI7 217 and YE1/9.9, Murine anti-human TfR Antibody HB21(also known as 5E9), Antibody 454A12, Antibody B3/25, Antibody OKT9, R17217 and OX26 mAb [95,97-99].

For instance the R17217, a rat IgG2a antibody against the mouse TfR which binds to this receptor on mouse cells [99], and the OX26, a murine Ab to the rat TfR, which is used for the delivery of peptides across the BBB [100], have been used in NP systems. Hence, it has been developed human serum albumin (HSA) NPs to which Tf was coupled, and was evaluated the potential of these NPs to deliver drugs across the BBB and, in addition, the possibility of achieving similar results by the coupling of the above-mentioned mAbs against the TfR receptor to the NPs was investigated. The analgesic Loperamide was chosen as the model drug since it does not cross the BBB [101]. HSA NPs coupled to Tf or TfR-mAb are enabling a significant loperamide transport across the BBB into the brain. The loperamide-loaded, Tf- or TfR-mAb-coupled HSA NPs achieved strong antinociceptive effects, whereas IgG2a-modified HSA NPs were not able to transport this drug across the BBB [80]. Therefore, these novel NPs with attached Tf or TfR-mAb represent very useful carriers for the transport of drugs into the brain. It have also been used fluorescein labeled Chitosan (CS) nanospheres conjugated with PEG obtained with the PRINT (Particle Replication In Non-wetting Templates) technology that were bioconjugated either with the OKT9 murine anti-human TfR antibody (NPs–OKT9) or with human Tf (NPs–hTf) [102]. In both cases greater than 80% uptake was observed in several human tumor cell lines (HeLa, Ramos, H460, SKOV-3, HepG2, and LNCaP) compared to bovine Tf conjugated NPs (NPs–bTf) or control IgG1 (NPs–IgG1). The targeting efficiency was dependent on nanocarrier concentration, ligand density, dosing time, and level of cell surface receptor expression. For these cells a strong correlation was found between the viability and the amount of ligand (OKT-9 or hTf) that can be conjugated to the surface of the NPs, with lower cell viability associated with higher percentage of ligand conjugate, suggesting that the polyvalency of the moiety targeting TfR plays a role in the toxicity in some malignancies [102].

3.4. Folate receptor

Folic acid (FA) or folate, a member of the B complex group of vitamins with small-molecular weight (441 Da), is required by eukaryotic cells as is an important co-factor in one-carbon

transfer reactions for biosynthesis of nucleotide bases (purines and pyrimidines) and plays a key role in DNA and RNA synthesis, epigenetic processes, cellular proliferation, and survival [103-104]. Since folic acid is required for essential cell function, the cargo attached the ligand is retained within an endocytic vesicle or released into the cytoplasm. FA conjugates have the ability to deliver a variety of drugs or imaging agents to pathological cells without causing harm to normal tissues. Furthermore FA targeting is an interesting approach for cancer therapy because it offers several advantages over the use of monoclonal antibodies. Thus, FA is known to be stable, inexpensive, non-toxic, non-immunogenic, easy to conjugate to carriers [105], and FA-conjugated drugs or NPs are rapidly internalized via receptor-mediated endocytosis.

Distinct transporters mediate cellular FA uptake. Among them, the FA transporter named as the folate receptor (FR) [9]. Three FR isoforms (FR-α, FR-β and FR-γ) have been identified in human tissues and tumors. FA can be internalized in cells by a low-affinity (K_D of approximately 1-5 μmol/l) membrane-spanning protein, which transports reduced FAs directly into the cytosol or it can be endocytosed by a high-affinity glycoprotein (K_D of approximately 100 pmol/l). FR, often referred to as the high affinity folate-binding protein, is a 38 kDa cell surface glycosyl-phophatidylinositol (GPI)-anchored glycopeptides that characteristically binds folic acid and transports it by a nonclassical endocytic mechanism [106]. The receptor-mediated uptake of FA conjugates proceeds through a series of distinct steps [107]. The process begins with the conjugate binding to FRs on the cell surface. The plasma membrane then invaginates and eventually forms a distinct intracellular compartment. The endocytic vesicles become acidified, and then lysozymes are activated allowing the FR to release the FA conjugates. The membrane-bound FRs recycle back to the cell surface, allowing them to mediate the delivery of additional FA conjugates. Concurrently, the FA conjugates released from FRs escape the endosome, resulting in drug deposition in the cytoplasm. Functional FRs are largely localized to the apical surfaces of polarized epithelia [105]. Normal tissues express insignificant level of FR-α and low level of FR-β (such as liver), and FR-γ is only found in haematopoietic cells. However, FR-α and FR-β are vastly overexpressed in many human tumors such as uterus, colon, lung, prostate, ovaries, mammary glands, nose, throat and brain [11,107-108] which makes it a rational target for drug delivery to tumor tissues. At the tumor site, FA has a very high affinity for tumor cell surface FR and the complex is rapidly internalized into tumor cells ($3x10^5$ FA molecules/h) [109]. Studies have shown a significant correlation between FR-α expression and the grade and differentiation status of the tumor, thus poorly differentiated and aggressive tumors express high levels of FR-α [110]. However, immunochemistry studies have shown the overexpression of FA receptors in normal tissues like placenta and kidneys [13].

A wide range of chemical conjugates of FA, antifolate drugs, and immunological agents have been used for developing therapeutic and imaging agents for various diseases. Thus, it is not surprising that FA targeted NPs have shown to be effective in a number of tumors. A range of polymers with an improved biocompatibility have been used for the development of FR-targeted NPs [11]. In a typical FR-targeted NP, the anticancer agent is encapsulated in a stabilizing polymer and the FA is conjugated on the surface of the polymer. PEG is often used as a polymer in a FR-targeted nanoparticulate system to enhance its circulation time

and also to improve the association of the targeted NP with the tumor cells [111]. The surface density and length of PEG chains should be optimal to maintain the system targeting and stealth properties [72]. The mole fraction of FA added to a NP system is also thought to affect the cytotoxic capability of the system. It is presumed that higher ligand content would give an enhanced targeting ability. However, when excessive FA molecules are present on the surface of the NPs, they can self-assemble to form dimers, trimers or tubular quartets, which cannot interact with FR (only one molecule of FA can bind to FR) [112]. FA-PEG-liposome loaded with Dox showed a 45-fold higher uptake in FR-rich KB cells compared to nontargeted liposomal-doxorubicin and 86-fold greater cytotoxicity. In mice bearing KB cell tumor xenografts, treatment with FA-targeted liposomal Dox produced a 31% inhibition of tumor growth [37].

Similar to PEG, PLGA NPs can be coated with FA to target the FR to further enhance accumulation of these NPs into tumor cells [113]. Copolymeric nanohydrogels based on N-isopropylacrylamide (NIPA), N-(pyridin-4-ylmethyl)acrylamide (NPAM) and tert-butyl-2-acrylamidoethyl carbamate (2AAECM), as well as FA-conjugate copolymeric nanogels, were synthesized and evaluated for antitumor therapy by loading them with TMX and 5-FU. Nanohydrogels were assayed as TMX and 5-FU delivery systems in vitro. Cell culture experiments were performed using MCF7, T47D and HeLa cells which have different degrees of FR expression. FA-targeted nanohydrogels showed a larger uptake into T47D and HeLa cells due to the fact that these cells are FR-positive. Furthermore, TMX-loaded and 5-FU-loaded nanohydrogels showed effective elimination of carcinoma cells [38]. Loaded with the same drugs, it have also been synthesized FA-conjugate poly[(*p*-nitrophenyl acrylate)-co-(N-isopropylacrylamide)] systems. TMX and 5-FU-loaded folate-systems present effective elimination of both MCF7 and HeLa cellular lines, and the presence of folate in the particles enhances their internalization, especially in HeLa cells [114-115].

A natural polymer (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHBHHX) was used as a base matrix for the production of a novel nanotherapeutic including antineoplastic agent, etoposide and attached FA as a ligand on the NPs. In the cytotoxicity tests, etoposide loaded and folic acid attached PHBHHX NPs were observed as more effective on HeLa cells than etoposide loaded PHBHHX NPs without attached folic acid. Furthermore the cytotoxicity of folic acid conjugated PHBHHX NPs to cancer cells was found to be much higher than that of normal fibroblast cells, demonstrating that the FA conjugated NPs has the ability to selectively target to cancer cells [116].

CS NPs have also been conjugated to FA to target contrast dye to tumor tissues. The mucoadhesive property of CS provides sustained interaction with the target cells and the FR-mediated uptake leads to an enhanced imaging effect [117]. The cytotoxic activity of CS NP conjugated to FA has also been explored to show a higher cellular cytotoxicity due to enhanced uptake by receptor-mediated endocytosis complemented with a depot effect, which leads to sustained drug release providing grater apoptosis and enhanced cell cycle arrest [118]. An alginate-complexed FA CS NP has been reported for photodynamic early detection of colorectal carcinoma. These NPs are readily engulfed by the cancer cells through FR-mediated endocytosis, furthermore an improved release in the cellular lysosome was observed when they are loaded with 5-aminolevulinic acid (5-ALA) [119].

In other system, FA was coupled with HSA NPs through carbodiimide reaction resulting in the formation of HSA-NPs spheres. The cellular binding and uptake was studied in normal foreskin fibroblasts (HFF), human neuroblastoma cells UKF-NB3, and in rat glioblastoma cell lines. An inceased NP uptake was observed in cancer cells, but not in normal HFFs [9].

3.5. Integrin

Integrins are heterodimeric cell-surface receptors that consist of α- and β-subunits, such as integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and which are barely detectable or entirely absent from normal blood vessels but are abundantly expressed on tumor-associated endothelial cells [120-121]. Furthermore the $\alpha_v\beta_3$ integrin is important in the calcium-dependent signaling pathway leading to endothelial cell migration [122]. Endothelial cells undergoing angiogenesis experience at least three cellular alterations, including an increase in proliferation, increase in locomotion, and endothelial cell interaction with the ECM. These alterations are directly related to the adhesion processes of the $\alpha_v\beta_3$ integrin [122]. Thus, integrins represent potential pharmacological targets for antiangiogenic therapy. Several antibodies and peptides capable of functionally blocking the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been demonstrated to inhibit neovascularization in tumor-bearing mice. The targeting scheme for the $\alpha_v\beta_3$ integrin has centered upon the three amino acid sequence RGD. An important characteristic of the $\alpha_v\beta_3$ integrin is that it is intrinsically associated with VEGFR-2 signaling. Upon $\alpha_v\beta_3$ integrin binding to the components that harbor the RGD sequence, there is an upregulation of VEGF signaling in cell cultures. By blocking $\alpha_v\beta_3$ integrin binding, there would be a reduction in VEGF signaling, proving the use of $\alpha_v\beta_3$ blocking agents for anti-angiogenesis [120]. Targeting the $\alpha_v\beta_3$ integrin with an active targeting NP system increases the effectiveness of anti-angiogenic treatments by the downregulation of VEGF.

Park and coworkers [123] reported the development of self-assembled hydrogel NPs capable of imbibing a peptide sequence that specifically binds to $\alpha_v\beta_3$ integrin. The authors observed that NPs made of hydrophobically modified CS could release the peptide in a sustained manner, and showed that they might be useful for monitoring or destroying angiogenic vessels. Peptides that contain RGD domains can preferentially bind cells in tumor microvasculature that express the $\alpha_v\beta_3$ integrin [13]. However, RGD sequences also act as adhesive molecules and can non-specifically bind tissues that also express its integrin complement. Integrin receptors are also expressed on the cell membrane of macrophages [124] and it is shown that RGD bioconjugates aggregate in spleen and liver tissues due to macrophage clearance [125].

Using an RGD-targeted stealth system, NPs carrying Dox were found to accumulate faster and in higher concentrations in the liver and the spleen [126]. The ligands are incorporated as RGD-PEG-lipid conjugates, which indicates their extension from the NP surface as a consequence of the brush-like state. A report showed that short peptide-targeted NPs exhibited lower cell-bindings abilities when higher mol% of PEG_{2000} was included into the formulation [127-128]. As a sufficient PEG coating is essential for avoiding recognition by the RES, ligands should be extended away from NP surfaces, to avoid shielding by the

polymer chains. Another study reported the targeting and imaging of MDA-MB-231 human breast cancer cells using RGD peptide-labeled Fluorescent silica NPs (FSiNPs). The FSiNPs exhibited high target binding to $\alpha_v\beta_3$ integrin receptor (ABIR)-positive MDA-MB-231 breast cancer cells in vitro [129].

Peptide-labeled NPs may also be used for targeted gene silencing. A study shows that RGD-CS-NP is a highly selective delivery system for siRNA with the potential for broad applications in human disease [130]. Binding of RGD-CS-NP with $\alpha_v\beta_3$ integrin and antitumor efficacy were examined, resulted in significant inhibition of tumor growth compared with controls. The targeted RGD non-peptide mimetic coupled to NPs were coupled to cDNA encoding ATPμ-Raf tagged with the FLAG epitope [131] and were proven to cause tumor regression in M21-L melanomas. Peptides harboring RGD sequences have also shown high efficiencies in targeting SLK tumor endothelial cells derived from Kaposi's sarcoma. A cyclic RGD pentapeptide was conjugated to the surface of Dox-loaded micelles at different densities. A higher density of RGD sequences led to a higher level of cellular internalization of the micelles over the range of RGD densities. A 30-fold enhancement in micelle internalization was achieved with 76% RGD-functionalized Dox-loaded micelles as compared to the non-targeted micelles [132].

There are studies with other ligands. Thus, integrin-targeted C16Y peptide-modified liposomes (C16Y-L) were prepared to enhance the intracellular uptake of drugs and genes specifically into tumor tissues [133]. The C16Y peptide is a 12-amino acid modified C16 synthetic peptide (DFKLFAVYIKYR-GGC), which is derived from the laminin γ1 chain, and binds to integrins $\alpha_v\beta_3$ and $\alpha_5\beta1$ [134]. The cellular uptake of C16Y-L by both endothelial cells and cancer cells was higher than uptake of the un-labeled and scramble peptide-modified liposomes. Moreover, to evaluate whether the uptake depended on an integrin–ligand interaction, they examined the inhibition of C16Y-L uptake using recombinant integrin $\alpha_v\beta_3$ and found that the cellular uptake of C16Y-L treated with $\alpha_v\beta_3$ integrin decreased. This result suggests that C16Y-L can selectively target cells that highly express integrin $\alpha_v\beta_3$.

Finally, a new strategy is to use a multi-targeting NP systems. For instance, PTX-loaded NPs based on Herein, an hyperbranched amphiphilic poly[(amine-ester)-co-(D,L-lactide)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer (HPAE-co-PLA/DPPE), which was modified with two targeting ligands, RGD and Tf were synthesized [135]. Thus, these dual-targeting NPs may achieve more accumulation and improved lethality of the PTX-loaded NPs in tumors. Active tumors targeting can be achieved in two steps: the ligand RGD enhances the targeting migration and accumulation of NPs to the $\alpha_v\beta_3$ integrin-expressing tumor vasculature and Tf then improves the cellular uptake of NPs by TfR-expressing tumor cells. In addition, a heterobifunctional cross-linker, p-maleimidophenyl isocyanate (PMPI), used for hydroxyl to sulfhydryl coupling was introduced to the HPAE-co-PLA/DPPE copolymer for the successful modification of targeting ligands [136-137]. Results showed the cytotoxicity and cellular uptake of PTX-loaded NPs against human cervical carcinoma (HeLa) cells for their tumor-targeting effects [135].

4. Tumor cell targeting

Cancer cells express different targets on their surface, some of them specific of each type of cancer. Active targeting of nanosystems for cancer treatment has been usually associated with a type of cancer and so with a specific target.

While chemotherapy has been the standard of care for patients with different types of cancer, efforts have shifted toward evaluating novel targeted agents in an attempt to improve outcome. These targeted agents are directed towards key components in several signaling pathways. The potential of targeted therapies has stimulated the study of targeted nanocarriers that can allow synergistically act by binding and inhibiting cancer pathways while delivering therapeutic payloads. Tumor cell targeting involves many targets associated with the uncontrolled cell proliferation and the angiogenesis and others specifics for the different types of cancer (Table 2)

CANCER TYPE	TARGET	AGENT	REFERENCE
Lung cancer (NSCLC)	VEGFR	Axitinib (Pfizer Inc., USA)	[138]
		Cediranib (Recentin®, AstraZeneca plc, UK)	[139]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[140]
		Erlotinib (Tarceva®, Genentech/Roche, Switzerland)	[141]
	IGF-1R	Figitumumab (CP-751871, Pfizer, USA)	[142]
Colorectal cancer	VEGF-A	Bevacizumab (Avastin®, Roche-Pharma AG, Germany)	[143-145]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[146]
		Panitumumab (Amgen Inc; Thousand Oaks, USA)	[147]
Breast cancer	HER2	Transtuzumab (Herceptin®, Genentech)	[148]
		Transtuzumab-DM1 (T-DM1; Genetech Inc/Roche)	[149]
		Pertuzumab (Omnitarg®; Genentech/Roche)	[150]
	PARP	Olaparib (AZD2281; AstraZeneca)	[151]
Prostate cancer	17-α-hydroxylase	Abiraterone acetate (Zytiga, Cougar Biotechnology)	[152]
	AR	MDV3100	[153]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[154]
	HER2	Transtuzumab (Herceptin®, Genentech)	[155]
	HER3	MM-121 (humanized antibody)	[156]
	PSMA	J591 (monoclonal antibody)	[157]

Table 2. Examples of targets for different types of cancer

4.1. Lung cancer

Non-small cell lung cancer (NSCLC) involves signaling pathways that influence angiogenesis, tumorigenesis and tumor growth, and different targeted agents have been used towards vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), EGFR and insulin-like growth factor 1 receptor (IGF-1R) [158-159]. Furthermore, there is an increasing interest in using combinations of targeted agents to inhibit more than one pathway.

Among agents that target VEGFR in the treatment of advanced NSCLC, axitinib [138] has resulted to be a potent selective inhibitor of these types of receptors; also cediranib [139] has been assayed in combination with carboplatin and PTX in the treatment of this kind of cancer.

Over-expression of EGFR has been associated with angiogenesis and poor prognosis in NSCLC [160]. Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA) is a chimeric mAb that targets the EGFR pathway by binding to the extracellular domain of the receptor and in this way inhibiting the receptor-associated tyrosine kinase (TK) activity [140]. Furthermore, inhibitors of TK activity of EGFR have been used for targeting the receptor pathway. Small-molecule, such as erlotinib (Tarceva®, Genentech/Roche, Switzerland) and gefitinib (Iressa®, AstraZeneca plc, UK), compete reversibly with ATP to bind to the intracellular catalytic domain of EGFR TK and, thus, inhibit EGFR autophosphorylation and downstream signaling [161].

IGF-1R is a key signaling pathway that leads to the growth and survival of tumor cells [162] and is commonly overexpressed in lung cancer cells. Figitumumab (CP-751,871, Pfizer, USA) is a fully human monoclonal antibody that is a specific and potent inhibitor of IGF-1R. In combination with carboplatin/PTX, figitumumab has shown to be a promising antitumor agent as first line treatment of NSCLC [142]. Several other anti-IGF-1R mAbs are being investigated in the treatment of advanced NSCLC, among them IMC-A12 (cixutumumab; fully human IgG1 monoclonal antibody), MK0646 (dalotizumab; a humanized IgG1 monoclonal antibody) and R1507 (fully humanized monoclonal antibody) [163].

Lung cancer is a heterogeneous disease with multiple mutations, and it is unlikely that any single signaling pathway drives the oncogenic behaviour of all tumors. In fact, multilevel cross-stimulation among the targets of the new biological agents can contribute to the relative failure of some target therapies. In this way, combining targeted therapies is a promising research approach to the treatment of NSCL, and an exhaustive review has been recently published by Custodio and coworkers [163]

4.2. Colorectal cancer

The systemic treatment of metastatic colorectal cancer (mCRC) involves the use of active cytotoxic drugs and biological agents either in combination or as single agents.

Initial chemotherapy of mCRC is based on using several cytotoxic regimens [164]. These clinical trials are based on the results of key phase III studies conducted over the past

decade. The IFL regimen [irinotecan (I), 5-FU and leucovorin (LV)] has been extensively used [143]. Furthermore, combination of oxaliplatin and 5-FU/LV (FOLFOX4) [165] has improved the overall survival of mCRC patients.

A significant percentage of patients with CRC receive a biological agent targeting the vascular endothelial growth factor A (VEGF-A) or EGFR over their treatment course. The currently available anti-VEGF-A agent is bevacizumab (Avastin®, Roche-Pharma AG, Germany), a humanized mAb. Different key clinical trials incorporating bevacizumab have been carried out. In the AVF2107 trial [144] the combination of IFL and bevacizumab improved the progression-free survival and the overall survival. The trial of bevacizumab plus oxaliplatin-based chemotherapy (FOLFOX4) or plus capecitabine/oxaliplation (XELOX) [143,145] showed a significantly increase in the progression-free survival, mainly with XELOX.

The anti-EGFR mAbs indicated for mCRC treatment are cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA; a chimeric monoclonal antibody) and panitumumab (Amgen Inc; Thousand Oaks, USA; a fully human monoclonal antibody). Both of them are efficacy in the treatment of patients whose mCRC tumors express wild-type KRAS. Different clinical trials combining anti-EGFR agents and chemotherapy have been carried out. Thus, mCRC therapy (wild-type KRAS patients) based on cetuximab and FOLFIRY (CRYSTAL trial) [146] showed a significantly improved progression-free survival and overall survival. In a similar way, the combination of panitumumab and FOLFOX4 (PRIME trial) had a very positive impact on survival parameters in wild-type KRAS patients [147].

4.3. Breast cancer

Breast cancer is the most common cancer affecting females and one of the main causes of mortality of women. This disease shows a high heterogeneous nature in terms of genetic features, molecular profiles and clinical behaviour. The high mortality caused by breast cancer can be attributed to the development of metastatic breast cancer [166]. The discovery of "genetic signatures" in breast cancers can provide key insights into the mechanisms underlying tumorigenesis and can be proven useful for the design of targeted therapeutic approaches [167-168].

The HER2 is over-expressed 15-30% of invasive breast carcinomas [167]. Extracellular domain of HER2 has been the target of several monoclonal antibodies created in order to inhibit the proliferation of human cancer cells. Transtuzumab, a recombinant humanized anti-HER2 monoclonal antibody was approved by the FDA for immunotherapy of women with metastatic HER2 over-expressing breast carcinoma. This antibody provokes cell cycle arrest during G1 phase [148]. Transtuzumab has been extensively used to target different drug-loaded nanocarriers to breast cancer cells [169-170].

Many hormone receptor positive breast cancers are resistant to hormone therapies. Thus, clinical trials have been developed combining therapies with biological and targeted agents (anti EGFR and HER2) for the treatment of estrogen receptor (ER) positive breast cancer.

Combination of geftinib, an EGFR TK inhibitor, with anastrozole [171] or TMX [172] has conducted to a light prolongation of progression-free survival of patients. Clinical trials based on combination of transtuzumab and letrozole (Femara), an oral non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer, in patients with ER+/HER2+ metastatic breast cancer have demonstrated clinical benefit [173]. A large proportion of HER2+ cancers have developed resistance to HER2-targeted therapeutics, including resistance of tumor cells to trastuzumab. Several agents have been developed to overcome resistance to this monoclonal antibody. The conjugation of maytansinoid DM1 with transtuzumab has generated transtuzumab-DM1 (T-DM1; Genetech Inc/Roche), that is active on HER2 overexpressing breast cancer and also transtuzumab-refractory tumors [149]. Another innovative targeted agent, which belongs, to the class of HER2-dimerization inhibitors, is pertuzumab (Omnitarg; Genentech/Roche), a recombinant humanized monoclonal antibody. Pertuzumab is directed against the highly conserved dimerization domain of HER2 [150]. The efficacy of adding pertuzumab to trastuzumab plus Dtxl for the first-line treatment of HER2-positive metastatic breast cancer was demonstrated in the randomized, double-blind, multinational, phase III CLEOPATRA trial [174].

Basal like breast cancers are the result of specific mutations. DNA lesions such as single-stand breaks (SSBs) and double-strand breaks (DSBs) are common in the normal cellular metabolism, and can be repaired by specific DNA repair mechanisms. In one of these DNA repair mechanisms, poly-(adenosine diphosphate ribose) polymerase 1 (PARP1) is an important key of the pathway. PARP1-inhibitors (PARP1-I) have been developed for the treatment of advanced breast cancer. Olaparib (AZD2281; AstraZeneca)), a PARP1-I has been evaluated in BRCA (a tumor suppressor protein) mutated patients [151]. Also iniparib (BSI 201; 4-iodo-3-nitrobenzamide; Sanofi-Aventis), an irreversible PARP1-I, is under study in patients with metastatic triple-negative breast cancer [175]. However, studies carried our by Liu and coworkers [176] shown that Iniparib nonselectively modifies cysteine-containing proteins in tumor cells, and the primary mechanism of action for iniparib is likely not via inhibition of PARP activity.

4.4. Prostate cancer

The current standard treatment of localized prostate cancer consists of prostatectomy and radiation therapy, sometimes supplemented with hormonal therapies to prevent testosterone production, which include anti-androgens and luteinizing hormone-releasing hormone (LH-RH) agonists. In locally advanced or widespread prostate cancer, the disease gradually transforms to a metastatic hormone-refractory state. Despite castrate levels of testosterone, the tumor will finally become independent of androgens resulting in death within a few years from diagnosis. In fact, the mortality rate of metastatic prostate cancer is extremely high. Thus, novel therapies [177-178] are on demand for the treatment of the malignant forms of prostate cancer that recur after initial therapies, including hormone refractory (HRPC) and castration resistant prostate cancer (CRCP).

Different molecules have been assayed as androgen and androgen receptor inhibitors. In this way, abiraterone acetate (Zytiga, Cougar Biotechnology) in combination with

prednisone has been recently approved by FDA for the treatment of CRCP in men who have received prior Dtxl chemotherapy [152]. Arbiraterone inhibits 17-α-hydroxylase, an enzyme of the testosterone biosynthesis pathway, decreasing circulating levels of the hormone. This enzyme is expressed in testicular, adrenal and prostate tumors. Regarding of androgen receptor inhibitors (AR-I), MDV3100 is an oral androgen receptor antagonist [153], which irreversibly binds to this intracellular receptor and causes no transcription of the gen.

The EGFR family (EGFR/HER) receptors have long been implicated in prostate cancer initiation and progression. EGFR is overexpressed in 18-37% prostate cancers [179], and a direct correlation of HER2 overexpression with the risk of death and recurrence in prostate cancer has been reported [180]. Thus, monoclonal antibodies have been studied as treatment options for prostate cancer. The efficacy of combining cetuximab with mitoxantrone plus prednisolone have been analyzed in a phase II clinical trial in men with CRCP after receiving Dtxl, but the time to progression and overall survival did not improve with the addition of cetuximab [154]. In order to evaluate whether dual inhibition of EGFR and HER2 would prolong the effectiveness of androgen withdrawal therapy (AWT) treatment in prostate cancer, studies using EGFR inhibitors (erlotinib and AG1478) and HER2 inhibitors (trastuzumab and AG879) were realized [155]. Results indicate that dual EGFR/HER2 inhibition, administered together with AWT, sensitize prostate cancer cells to apoptosis during AWT. In general, studies using inhibitors of EGFR/HER1 and HER2 show that these molecules fared poorly in prostate cancer clinical trials.

Recent research suggests that another family member HER3 (ErbB3) abets emergence of the castration resistant phenotype. The prostate cancer, in comparison to the normal tissue, overexpresses HER3 protein, which indicate poor prognosis. Antibody-based therapy that prevents ligand binding to ErbB3 appears promising and fully-humanized antibodies that inhibit ligand-induced phosphorylation of HER3 (ErbB3) are currently in early development [181]. HER3's signaling functions depend upon ligand binding to its extracellular domain and inhibitors are generated to disrupt this interaction. A recently-characterized, HER3-specific humanized antibody MM-121 blocked ligand-dependent HER3 activation induced by the HER1, HER2 or MET receptors [156].

The IGF-R signaling pathway plays a role in prostate cancer. In fact, an increase risk of prostate cancer has been directly correlated with the circulating IGF-1 (one ligand of the IGF-R) levels [182]. An inhibitor de the IGF pathway is the anti-IGF-R mAb cixutumumab (IMC-A12; ImClone Systems), which was effective in both androgen-dependent and androgen-independent human prostate cancer in animal models [183].

PSMA has been identified as an ideal antigenic target in prostate cancer. PSMA is the most well-established, highly restricted prostate cancer cell surface antigen. It is expressed at high density on the cell membrane of all prostate cancers, and after antibody binding, the PSMA-antibody complex is rapidly internalized along with any payload carried by the antibody. J591 is the first IgG mAb developed to target the extracellular domain of PSMA, and it has been deimmunized (humanized) to allow repeated dosing in patients. Three phase I studies have been carried out, two using the β-emitting radiometals yttrium 90 and lutetium 177

(177Lu), and a third using a cytotoxin (DM1) linked to J591 [157]. A phase II clinical trial (NCT00859781) to test the effectiveness of the radiolabel monoclonal antibody, 177Lu-J591 in combination with ketoconazole and hydrocortisone against prostate cancer is in progress.

5. Conclusion

The development of drug delivery systems that are able to modify the biodistribution, tissue uptake and pharmacokinetics of therapeutic agents is considered of great importance in biomedical research. Controlled release in drug delivery can significantly enhance the therapeutic effect of a drug. Among drug delivery systems, nanocarriers are the smallest devices for transport of drugs, and they comprises a variety of the type of nanoparticles developed for cancer, including liposomes, nanoshells, nanocapsules, dendrimers, polymer-drug conjugates, polymeric nanogels and micelles, and polynucleotide nanoparticles. The attractive properties of nanomedicines include their ability of controlled release of drugs, the targeting of specific tissues and the biocompatibility. Because of their size, nanocarriers can be taken up, in many cases, very efficiently by cells, internalized and stored into cytoplasm or different organelles. Nanocarrier uptake into a cell depends on the cell-type, since some cells are more susceptible to include nonfunctionalized systems via their design. The unique attributes of tumors support extravasation of polymeric nanomedicines through large pores on the endotheliallayer and via the disordered neoplastic tissue architecture. Thus, nanoparticles target the tumor passively via the EPR effect if their size is smaller than 100nm. Therefore, current research involves novel strategies to attach targeting ligands with high affinity for receptors overexpressed on tumors or ways to utilize the tumor´s own microenvironment as a stimulus for drug release. An active targeting strategy can improve the efficacy of the therapy and diminish side effects associated with drugs, since not all nanocarriers can overcome the cell membrane barrier without a targeting motif. Nanoparticle systems are able to target various portions of the tumor using specific targeting moieties and evade the problems associated with multi-drug resistance. Thus, to increase the delivery of a given drug to a specific target site, targeting ligands are conjugated to carriers. The presence of reactive pendant groups in nanogels make easy their vectorization forward specific cell motif by binding of ligands. Furthermore, it is an important fact that targeting ligands lead to macrophage recognition and faster clearance compared to the non-targeted nanoparticles. Various molecules, that include folates, transferrin, antibody and antibody fragments, peptides, aptamers, small molecules, and carbohydrates, have been used to target nanocarriers to specific receptors on tumoral cell surfaces. In many cases, ligand-targeted nanoparticles demonstrate better internalization by cancer cells and more effective intracellular drug delivery than other preparatios. The search for more molecular targets will advance the ability to improve delivery at the tumor level while decreasing toxicity to normal tissue. As a result, moieties-targeted drug-loaded nanoparticles, searching for new tumor targets, novel ligands, new strategies for targeting, and particle stabilization, are generally considered as promising candidates for cancer chemotherapy and we can expect their extensive clinical evaluation in the near future.

Nomenclature

2AAECM: *tert*-butyl-2-acrylamidoethyl carbamate
5-ALA: 5-aminolevulinic acid
5-FU: 5-Fluorouracil
7α-APTADD: 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione)
ABIR: $\alpha_v\beta_3$ integrin receptor
AD: adamantane
Apt: aptamer
AR-I: androgen receptor inhibitor
AWT: androgen withdrawal therapy
BBB: blood brain barrier
BCRP: breast cancer resistance protein
CEM: leukemic lymphoid cells
CRCP: castration resistant prostate cancer
CS: Chitosan
Dox: doxorubicin
DSBs: double-strand breaks
Dtxl: Docetaxel
ECM: extracellular matrix
EGF: epidermal growth factor
EGFR (or HER1): epidermal growth factor receptor 1
EPR: Enhanced Permeability and Retention
ER: estrogen receptor
EU: European Union
FA: Folic acid or folate
FDA: Food and Drug Administration
FR: folate receptor
FSiNPs: Fluorescent silica nanoparticles
GPI: glycosyl-phophatidylinositol
HB-EGF: heparin-binding epidermal growth factor
HCPT: Hydroxycamptothecin
HER: Human epidermal receptor
HER2: epidermal growth factor receptor 2
HER3: epidermal growth factor receptor 3
HFF: Human foreskin fibroblasts
HNSCC: Head and neck squamous cell carcinoma
HPAE-co-PLA/DPPE: hyperbranched amphiphilic poly[(amine-ester)-co-(D,L-lactide)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer
HRPC: hormone refractory prostate cancer
HSA: human serum albumin
I: irinotecan
IFL regimen: irinotecan + 5-FU + leucovorin
IFP: interstitial fluid pressure
IGF: insulin-like growth factor

IGF-1R: insulin-like growth factor 1 receptor
LH-RH: luteinizing hormone-releasing hormone
LV: leucovorin
mAb: monoclonal antibody
MAPK: mitogen-activated protein kinases
mCRC: metastatic colorectal cancer
MePEG-PHDCA: poly(methoxy-polyethylene glycol cyanoacrylate-co-hexadecyl cyanoacrylate)
MTX: Methotrexate
MUC1: mucin 1
NCs: nanoclusters
NIPA: *N*-isopropylacrylamide
NPAM: *N*-(pyridin-4-ylmethyl)acrylamide
NPs: nanoparticles
NRs: nanorods
NS: niosomes
NSCLC: Non-small cell lung cancer
PAMAM: Poly(amido amine)
PARP1: poly-(adenosine diphosphate ribose) polymerase 1
PARP1-I: poly-(adenosine diphosphate ribose) polymerase 1 -inhibitors
PDGFR: platelet-derived growth factor receptor
PEG: polyethylene glycol
P-gp: P-glycoprotein
PHBHHX: poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PLGA: poly(D,L-lactide-coglycolyde)
PMPI: p-maleimidophenyl isocyanate
PRINT technology: Particle Replication In Non-wetting Templates technology
PSMA: Prostate-specific membrane antigen
PTK7: protein tyrosine kinase-7
PTX: Paclitaxel
RES: reticuloendothelial system
RGD: tripeptide arginine–glycine–aspartic acid
SELEX: Systematic Evolution of Ligands by Exponential Enrichment
siRNA: short inhibitory RNA
SSBs: single-stand breaks
SWNTs: single-wall carbon nanotubes
Tf: Transferrin
TfR: Transferrin receptor
TGF-α: transforming growth factor-α
TK: tyrosine kinase
TLR9: Toll-like receptor 9
TMX: Tamoxifen
TNF: tumor necrosis factor
VEGF: vascular endothelial grown factor
VEGFR: vascular endothelial grown factor receptor

VPF: vascular permeability factor
WHO: World Health Organization

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Miscellaneous

Chapter 10

Oral Delivery of Insulin: Novel Approaches

Amani M. Elsayed

Additional information is available at the end of the chapter

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1. Introduction

1.1. Insulin: physicochemical properties and function

Insulin is a hormone that is synthesized in the β-cells of the pancreas as a proinsulin precursor and is converted to insulin by enzymatic cleavage. The resulting insulin molecule is composed of 51 amino acids arranged into two polypeptide chains - the A and B chains - which are connected by two interchain disulphide bridges. In the A chain, there is an additional intrachain disulphide linkage [1]. The primary structure of human insulin is shown in **Figure 1 a**. In the secondary structure, chain A consists of two antiparallel α-helices (A_2 to A_8 and A_{13} to A_{20}), while chain B forms a single α-helix "B_9 to B_{19}" followed by a turn and a β strand "B_{21} and B_{30}" [2]. The folding of insulin into a tertiary structure is essential for its biological activity (Figure 1b). Insulin has an isoelectric point (pI) of 5.3 and a charge of -2 to -6 in the pH range 7-11. Another intrinsic property of insulin is its ability to readily associate into dimmers, hexamers and higher-order aggregates. At the low concentrations found in the blood stream ($< 10^{-3}$ μM), insulin exists as a monomer, which is its biologically active form. Following biosynthesis, insulin is stored as crystalline zinc-bound hexamers in vesicles within the pancreatic β-cells from which secretion occurs in response to elevated blood glucose levels [3]. The biological actions of insulin are initiated when insulin binds to its cell surface receptor. Insulin is an anabolic hormone and when binding to its receptor begins, many protein activation cascades occur. These include: the translocation of the glucose transporter to the plasma membrane and the influx of glucose, glycogen synthesis, glycolysis and fatty acid synthesis. Insulin has been observed as promoting the transport of some amino acids and potassium ions. Insulin also inhibits the liberation of free fatty acids and glycerol from the adipose tissue [3].

Insulin is used for the treatment of diabetes, a disease which results from a defect in the secretion or action of insulin.

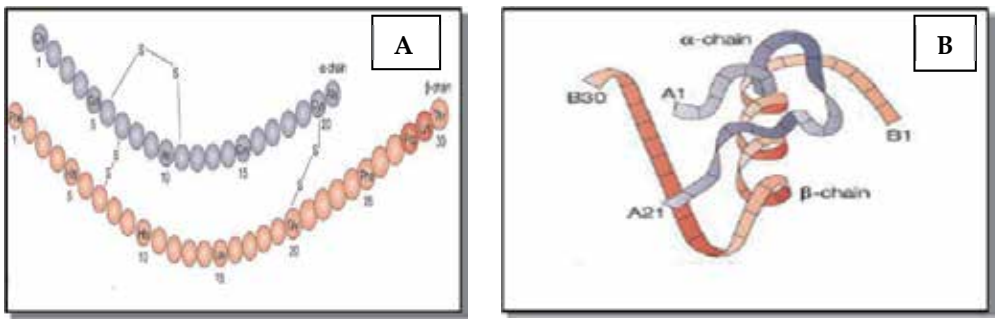


Figure 1. Insulin: a) Primary Structure b) Tertiary Structure

1.2. Oral delivery of insulin: Why?

Insulin is introduced by the parenteral route and two or three injections are needed for the better control of diabetes and in order to reduce the long term complications of hyperglycaemia (retinopathy, neuropathy and nephropathy). Patient non-compliance is common with the parenteral route. In addition, repeated injections will cause lipoatrophy or lipohypertrophy. Moreover, insulin injected into the subcutaneous tissues goes directly into general circulation and leads to peripheral hyperinsulinemia, which is associated with peripheral hypertension, the development of atherosclerosis, cancer, hypoglycaemia and other adverse metabolic effects [4]. Thus, the conventional subcutaneous injection of insulin is unphysiological because it deprives patients of the benefits of portal insulin since the liver is the major metabolic modulator of the glucose metabolism.

Oral insulin is a dream of patients and a challenge for scientists. For patients, not only are the pain and stress of injections relieved but it may also protect beta cells, avoid the weight gain associated with insulin injections and correct the blunting of the first-phase release of insulin [5]. All these effects are due to the fact that oral route provide insulin directly to the liver through portal circulation, resembling that which occurs in the non-diabetic individual [6]. The achievement of an adequate level of insulin in portal circulation has been associated with more a rapid and significant lowering of plasma glucose and haemoglobin A1c levels, the normalization of the plasma levels of three carbon precursors - such as lactate, pyruvate and alanine - and the hormones cortisol, growth hormone and glucagon [7].

Another advantage of oral insulin is that the gastrointestinal tract is immune tolerant compared to other routes of drug administration since immunogenicity has become a major issue for most biotechnology products. Immunogenicity decreases in the following order: (inhalation > subcutaneous > intramuscular > intravenous > oral).

For scientists, greater effort is needed to develop a nontoxic, stable, bioactive oral insulin delivery system. To develop such systems, many barriers must be explored.

1.3. Obstacles to oral delivery

The major barrier is that of absorption through the gastrointestinal membrane. Generally, the absorption of molecules can occur through the paracellular or the transcellular route.

The former is the preferred route for small hydrophilic molecules with a molecular weight below 500 Da [8]. Of course, molecules with a high molecular weight - such as insulin (about 6 KDa) - would not penetrate through this route. This large molecular size, its charge and its hydrophilicity all preclude insulin absorption by transcellular diffusion.

Another obstacle is those enzymes that are located throughout the GIT. In the stomach there is a family of aspartic proteases called pepsins. In the small intestine, pancreatic proteases consisting of the serine endopeptidase (trypsin, α-chymotrypsin, elastase and exopeptidases, carboxypeptidases A and B) are responsible for the degradation of proteins [9]. Other enzymes are located at the brush–border membrane (various peptidases) or within the enterocytes of the intestinal tract. It was demonstrated by Aoki et al. [10] that the enzymatic degradation activity for insulin in the mucous/glycocalyx layers tends to increase towards the upper small intestine in the following order: duodenum > jejunum > ileum.

Another challenge for the formulator is the stability of insulin. Insulin has a delicate structure, and both formulation and processing parameters could influence its stability [11]. The most common degradation reactions are deamidation and polymerization. Extensive deamidation at the residue Asn^{A21} of insulin occurs in acid solutions, while in neutral formulations deamidation takes place at Asn^{B3} at a substantially reduced rate [12]. High temperatures accelerate the formation of covalent insulin dimer and covalent insulin polymer [13].

The formulator could handle the enzyme and stability barriers. A well-formulated nanocarrier would protect insulin from enzymes. Also, and with the proper choice of excipients and a properly designed method of production, the stability of insulin could be preserved. Many investigated systems have overcome these two barriers. However, no system exhibits a reproducible and pronounced absorption.

1.4. Approaches explored to overcome the obstacles

To improve the bioavailability of insulin, different approaches have been explored, including chemical modification [14], co-administration with absorption enhancers and/or enzyme inhibitors [15] and incorporation into carriers, such as liposomes]16], mixed micelles, lipid-based systems, microspheres and nanoparticles [17-20].

1.4.1. Nanocarriers

These are carriers with a particle size of less than 1000 nm. Nanocarriers have received more attention recently due to their submicron size and their large specific surface area, both of which favour their absorption compared to larger carriers.

1.4.1.1. Types

Nanocarriers are categorized into: polymeric nanoparticles, nanovesicles and solid lipid nanoparticles **(Figure 2).** There are two types of polymeric nanoparticles: the matrix particles termed 'nanospheres' and the reservoir-type named 'nanocapsules'. Vesicles have a hydrophilic core and hydrophobic bilayers. Conventionally, liposomal vesicles were

developed by the self-assembly of phospholipid molecules in an aqueous environment. Recently, polymeric vesicles were prepared from amphiphilic polymers which form aggregates in aqueous solutions [21]. Solid lipid nanoparticles (SLN) are submicron colloidal carriers prepared from solid lipids (lipids being solid at room and body temperatures), such as triacylglycerols, complex acylglycerol mixtures or waxes, and dispersed either in water or in an aqueous surfactant solution [22]. The important features of different nanocarriers are illustrated in Table 1.

1.4.1.2. Applications

1.4.1.2.1 Liposomes

Insulin-loaded liposomes containing different kinds of bile salts (glycocholate, sodium taurocholate or sodium deoxycholate) were prepared by a reversed-phase evaporation method and their hypoglycaemic activity was assessed after oral administration to male Wistar rats. Liposomes containing sodium glycocholate elicited higher bioavailability - of approximately 8.5% and 11.0% - in non-diabetic and diabetic rats, respectively [23]. A hepatic-directed vesicle insulin system (HDV-I) was developed by Diasome Pharmaceuticals, Inc. The vesicles contain a specific proprietary hepatocyte-targeting molecule - biotin-phosphatidylethanolamine - in their phospholipid bilayer. Clinical trials in adult patients with type 1 diabetes mellitus demonstrated that the postprandial glycaemic control produced by 0.1 and 0.2 U/kg oral HDV-I was similar to that produced by 0.07 U/kg SC Humulin R [7].

1.4.1.2.2. Polymeric nanovesicles

Poly(lactic acid)-b-Pluronic-b-poly(lactic acid) block copolymers were synthesized [21]. This amphiphilic block copolymer aggregates in an aqueous solution to form vesicular nanoparticles. The oral administration of insulin-loaded vesicles to diabetic mice resulted in the reduction of blood glucose levels - 25% of the initial glucose level - which was maintained at this level for an additional 18.5 h [21].

1.4.1.2.3. Solid Lipid Nanoparticles (SLN)

Sarmento et al. [24] prepared insulin-loaded cetyl palmitate solid lipid nanoparticles and demonstrated their potential to deliver insulin orally. The drug loading capacity in solid lipid nanoparticles was improved by enhancing insulin liposolubility. Insulin was solubilized into mixed reverse micelles of sodium cholate and soybean phosphatidylcholine and transformed into SLN using a novel reverse micelle-double emulsion technique. Stearic acid and palmatic acid were used as a biocompatible lipid matrix [25]. The surface of the nanoparticles was modified by chitosan to enhance their penetration through GIT. In addition, chitosan was able to provide stealth properties to SLN, resulting in the absence of phagocytosis. Pharmacological availability values of 5.1–8.3% for SLN and 17.7% for chitosan-coated SLN were reported [26]. Lectins are proteins that bind sugar reversibly and are involved in many cell recognition and adhesion processes. They have been extensively adopted to target both absorptive enterocytes and M cells [27]. Wheat germ agglutinin binds (WGA) specifically to cell membranes and is taken up into cells by receptor-mediated endocytosis [28]. Zhang et al. [29] utilized the advantages of WGA and formulated SLN

Nanocarrier	Advantages	Shortcomings
Polymeric nanocarriers	Various polymeric materials (hydrophobic and hydrophilic) can be used to modulate the physicochemical properties of NPs (e.g., surface charge and mucoadhesivity), encapsulation efficiency, drug release profile and biological behaviour	Denaturation can occur during encapsulation in synthetic polymers, due largely to exposure to organic solvents, elevated temperatures and aqueous organic interfaces. Cytotoxicity of polymers after internalization into cells
Liposomes	Good permeation property since their bilayer structure is similar to that of the cell membrane	Poor entrapment efficiency Stability in biological fluids was relatively weak
Polymeric nanovesicles	More stable than liposomes The properties of polymeric vesicles, such as the size and the thickness of the bilayer, can be varied by changing the molecular weight and block composition of the polymer	Cytotoxicity of polymers after internalization into cells
Solid lipid nanoparticles	Tolerability, Biodegradability Composed of physiological lipids, which minimizes the risk of acute and chronic toxicity. Possibility of production on a large industrial scale	Poor encapsulation efficiency of water-soluble proteins and peptides into the lipid core The high temperatures required to melt the lipid may affect the stability of insulin Slow degradation

Table 1. Nanocarriers: general advantages and shortcomings

modified with WGA to enhance the oral delivery of insulin. Insulin-loaded SLNs or WGA-modified SLNs were administered orally to rats and elicited relative pharmacological bioavailability values of 4.46% and 6.08% and relative bioavailability values of 4.99% and 7.11%, respectively, in comparison with the subcutaneous injection of insulin.

Polymeric nanoparticles developed from biocompatible and biodegradable polymers are good candidates for insulin delivery

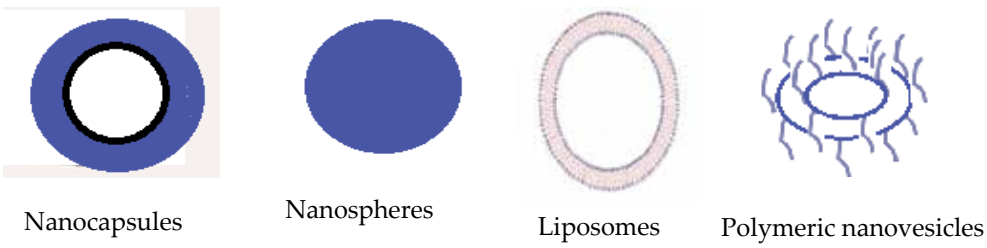


Figure 2. Types of nanocarriers

1.4.1.3. Polymers used for the fabrication of polymeric nanoparticles

Both synthetic and natural polymers were investigated for the production of nanosystems. These polymers may be used alone or in combination to develop nanoparticles. Several fabrication techniques have been developed and can generally be subdivided into two categories, according to whether a preformed polymer is used or else whether nanoparticles are formed during the polymerization reaction. Methods from the first category include: emulsification/solvent evaporation, solvent displacement and interfacial deposition, emulsification/solvent diffusion, salting out with synthetic polymers, ionotropic gelation, coacervation and polyelectrolyte complexation. Meanwhile, the methods of the second category are: emulsion polymerization, interfacial polymerization and interfacial polycondensation. These methods were thoroughly discussed by Reis et al. [30].

1.4.1.3.1. Synthetic polymers

Usually, these are well-defined structures that can be modified to yield reasonable degradability and functionality. Synthetic biodegradable polymers such as poly ε-caprolactone (PCL) poly (lactic-co-glycolic acid) (PLGA) and polylactides (PLA) are widely used in drug delivery due to their good biocompatibility, biodegradability and novel drug release behaviour. The chemical structures of synthetic polymers were depicted in Table 2 and an example of insulin nanoparticles fabricated from these polymers is illustrated in Table 3.

1.4.1.3.1.1. PLGA

Poly (lactic-co-glycolic acid) (PLGA) is an aliphatic polyester synthetic biodegradable biopolymer which is successfully used for the development of nanomedicines. It was also investigated for the delivery of insulin. In the work of Yang et al. [31], insulin was encapsulated in PLGA nanoparticles. The administration of insulin-loaded PLGA nanoparticles for diabetes mellitus induced a rapid decrease in blood glucose levels for up to 24 h and increased insulin levels. The loading capacity was 78.35%. To facilitate loading efficiency, the lipophilicity of the insulin was increased by complexation with sodium lauryl sulphate or sodium oleate. Insulin encapsulation efficiency reached up to 90%. [32, 33]. Mucoadhesive PLGA nanoparticles were prepared to enhance the oral bioavailability of the negatively charged PLGA nanoparticles. The PLGA nanoparticles were coated with chitosan or Eudragit® RS (RS). The pharmacological availability of two kinds of nanoparticles - PLGA nanoparticles and chitosan-coated PLGA nanoparticles - relative to SC injection was calculated and found to be 7.6% and 10.5%, respectively, at an insulin dose of 15 IU/kg [34]. Meanwhile, the pharmacological availability of the 50 IU/kg Eudragit® RS (RS) coated PLGA nanoparticle was 9.2% [35]. The main shortcomings of PLGA are that the degradation products arising from degradation of PLGA (lactic and glycolic acid) result in the generation of acidic species which can provoke problems for long-term stability when encapsulating bioactive molecules. Antacid-insulin co-encapsulated PLGA were investigated with a view to increasing the microclimate pH and preventing structural losses and aggregation [36]. The antacids assessed were magnesium hydroxide and zinc carbonate. However, short-term stability was not reported.

1.4.1.3.1.2. PLA

Polylactides (PLAs) have similar properties to PLGAs but they are more hydrophobic than PLGAs and they degrade more slowly due to their crystallinity [37]. Cui et al. [38] reported enhanced insulin entrapment efficiency (up to 90%) in PLA and PLGA nanoparticles, where insulin was complexed with phosphatidylcholine (SPC) to improve its liposolubility. An oral bioavailability of 7.7% relative to subcutaneous injection was obtained.

1.4.1.3.1.3. PCL

Another interesting biodegradable polyester polymer is poly-ε caprolactone (PCL). Compared with PLGA and PLA, PCL is semi-crystalline, has superior viscoelastic properties and possesses easy formability. PCL has the advantage of generating a less acidic environment during degradation as compared with PLGA-based polymers [37]. Nevertheless, the hydrophobic nature of PCL affects the encapsulation of hydrophilic substances, such as peptides, enzymes and other proteins. Damgé et al. [39] prepared nanoparticles from a blend of a biodegradable polyester poly (ε-caprolactone) and a polycationic non-biodegradable acrylic polymer (Eudragit® RS). These nanoparticles were investigated as a carrier for the oral administration of insulin and demonstrated prolonged hypoglycaemic effect of insulin in both diabetic and normal rats. The same author loaded nanoparticles with regular insulin ((Actrapid, Novo Nordisk) or insulin-Aspart ((Novorapid). Regular insulin-loaded nanoparticles reduced glycaemia in a dose dependent manner with a maximal effect observed with 100 IU/kg. In contrast, insulin analogue did not elicit a dose-dependent hypoglycaemic effect. The maximal effect was observed with 50 IU/kg insulin while lower (25 IU/kg) and higher doses (100 IU/kg) did not show any significant reduction in glycaemia. The authors attributed these discrepancies to the saturation of the receptors when the dose of aspart-insulin increases to 100 IU/kg [40].

1.4.1.3.1.4. PACA

Poly (alkyl cyanoacrylate) is a biocompatible and biodegradable polymer. It is degraded by esterases in biological fluids and produces certain toxic products that will stimulate or damage the central nervous system. Thus, this polymer is not authorized for application in humans [41]. However, PACA polymers are used to encapsulate insulin using emulsion or interfacial polymerization. Damge et al. [42] prepared an insulin-loaded poly (alkyl cyanoacrylate) nanocapsule. The oral administration of nanocapsules dispersed in Miglyol 812 to diabetic rats resulted in a 50% reduction of initial glucose levels from the second hour for up to 10-13 days. This effect was shorter (2 days) or absent when the nanocapsules were dispersed in water, whether with surface active agents or not. Insulin-loaded poly (ethyl cyanoacrylate) nanoparticles were prepared from microemulsions with a different microstructure and were administered orally to diabetic rats. A consistent and significant hypoglycaemic effect over controls was found for up to 36 h depending upon the type of monomer (ethylcyanoacrylate or butyl cyanoacrylate). However, no significant serum insulin levels were detectable [43].

1.4.1.3.1.5. Poly (Acrylic acid)

These are non-degradable polymers with mucoadhesive properties based on acrylic or methacrylic acid. Anionic polymers, such as methyl acrylic acid (Eudragit L-100) and methyl methacrylate (S-100), have been used to formulate pH sensitive nanocarriers. Polymethacrylic acid–chitosan–polyethylene glycol nanoparticles were developed by Pawar et al. for the oral delivery of insulin. These nanoparticles displayed excellent binding efficiency on mucin from porcine stomach and elicited pH dependent release profiles in vitro [44]. The nanoparticles were formed by a complex coacervation method using EudragitL100-55 and chitosan of various molecular weights. Insulin release from these nanoparticles was pH-dependent [45]. The distribution, transition and bioadhesion of insulin-loaded pH-sensitive nanoparticles prepared from EudragitL100-55 and chitosan were investigated. The addition of the hydroxypropylmethylcellulose reduced the stomach- and intestine-emptying rates and enhanced the adhesion of the nanoparticles to the intestinal mucosa [46].

1.4.1.3.2. Natural polymers

The naturally-occurring polymers of particular interest in the oral delivery of insulin are either polysaccharides or else proteins. Polysaccharides include chitosan, hyaluronan, dextran, cellulose, pullulan, chondroitin sulphate and alginate. Meanwhile, the protencious polymers are casein and gelatin. They are nontoxic, biocompatible, biodegradable and hydrophilic. The structure of these natural polymers is illustrated in **Table 4**. Examples of the natural polymers used to prepare insulin-loaded nanoparticles and the methods used for their fabrication are shown in **Table 5.**

1.4.1.3.2.1. Dextran

Dextran sulphate is an exocellular bacterial polysaccharide consisting of linear 1,6-linked D-glucopyranose units and branches beginning from α-1,3-linkages with approximately 2.3 sulphate groups per glucosyl residue. It is a nontoxic, highly water-soluble, biodegradable and biocompatible branched negatively charged polyion. A nanoparticle insulin delivery system was prepared by the polyelectrolyte complexation of oppositely charged natural polymers - dextran sulphate and chitosan in an aqueous solution. These pH sensitive nanoparticles released insulin in the intestinal medium [47]. The natural uptake processes of the intestine were utilized for the oral delivery of peptides and proteins. Vitamin B12 is an example of such carriers and was investigated for delivering different peptides [48]. Due to the susceptibility of vitamin B12/peptide conjugate to gastrointestinal degradation, dextran nanoparticles were coated with vitamin B12 and used as a carrier for the oral delivery of insulin [49]. These nanoparticles were found to be targeted at the systemic circulation through vitamin B12-intrinsic factor receptor ligand-mediated endocytosis via ileocytes of the intestine [49]. The % pharmacological availability of nanoparticle conjugates containing 2, 3 and 4% w/w insulin was 1.1, 1.9 and 2.6 times higher, respectively, compared with nanoparticles without VB12.

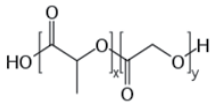
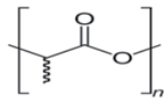
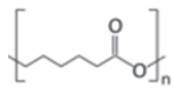
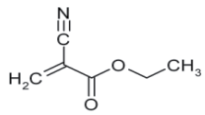
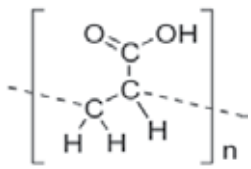
Polymer	Chemical Structure
PLGA	 x = number of units of lactic acid y = number of units of glycolic acid
PLA	
Polycapolactone	
Ethyl cyanoacrylate monomer	
Poly (acrylic acid)	

Table 2. Chemical Structure of Synthetic Polymers

1.4.1.3.2.2. Alginate

Alginate is a naturally occurring polysaccharide obtained from marine brown algae. It is a linear copolymer composed of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid residues that gel in the presence of divalent cations. It is a nontoxic and biodegradable polyanion that forms polyelectrolyte complexes with polycations, such as chitosan. Insulin-loaded nanoparticles were prepared by the ionotropic pre-gelation of alginate with calcium chloride followed by complexation between alginate and chitosan [50]. The pharmacological effect of insulin-loaded nanoparticles was evaluated in diabetic rats. The pharmacological availability was 6.8% and 3.4% for the 50 and 100 IU/kg doses, respectively [51]. Alginate/chitosan nanoparticles form complexes with cationic β-cyclodextrin polymers. The nanoparticles protect insulin against degradation in simulated gastric fluid [52]. Reis et al. [53] evaluated nanoparticle systems composed of alginate/chitosan cores coated with chitosan-polyethylene glycol-albumin. Albumin was added to prevent protease attacks on the insulin and chitosan for its mucoadhesive properties, while PEG served as a nanosphere stabilizer to improve the half-live of the insulin and increase the residence time along the intestine. Chitosan-PEG-albumin coated nanospheres demonstrated a more than 70% blood glucose reduction, increased insulinemia by a factor of seven and significantly improved the

response to the glucose oral tolerance test following oral administration to diabetic rats. In contrast, nanospheres lacking albumin and PEG in the coating material were ineffective. Multilayer nanoparticles consisting of calcium cross-linked alginate, dextran sulphate, poloxamer 188, chitosan and an outermost coating of albumin were developed. A 3-factor 3-level Box–Behnken statistical design was used to optimize the nanoparticle formulation. Solutions of 0.20% calcium chloride, 0.04% chitosan and 0.47% albumin constituted the optimum formulation of nanoparticles for orally-dosed insulin [54]. The relative pharmacological availability and bioavailability were calculated after oral administration of 50 IU/kg of insulin-loaded multilayered nanoparticles to diabetic rats and were found to be 11% and 13%, respectively [55].

Polymer	Method of preparation	Particle size (nm)	Pharmacological availability (PA)/ or relative bioavailability (RBA)	Ref.
PGLA	Double-emulsion/solvent evaporation technique	208	**Not calculated**	31
PLGA and chitosan	Water-in oil-in-water solvent evaporation technique	150	**PA: 10.5%**	34
PLGA, Eudragit® RS (RS) and Hydroxypropyl methylcellulose phthalate	Multiple emulsions solvent evaporation technique.	285	PA: 9.2%	35
PCL and Eudragit® RS	Double emulsion method	331	RBA: 13%	39

Table 3. Synthetic polymers used for the preparation of nanoparticles and the methods used for their fabrication

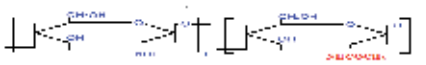
Polymer	Chemical Structure
Dextran	
Alginate	
Chitosan	

Table 4. Chemical Structure of Natural Polymers

As illustrated in Table 5, the most widely explored polymer is chitosan. This is because of its favourable biological properties, safety, low cost and easy modification. Compared to synthetic polymers, the degradation products of chitosan are amino sugars, which are easily metabolized by the body. Therefore, there is no concern of an acidic microclimate being generated by chitosan particles [56].

Polymer	Method of preparation	Particle size (nm)	Pharmacological Availability (PA)/ or Relative bioavailability (RBA)	Ref.
Chitosan and Poly (γ-glutamic acid)	Ionic-gelation method: the nanoparticles were freeze-dried and placed in enteric-coated capsules	232.9	RBA: approximately 20%.	70
Chitosan and Poly (γ-glutamic acid)	A penetration enhancer (DTPA) was covalently conjugated on poly (g-glutamicacid) followed by ionic-gelation with chitosan	246.6	RBA: approximately 20%	71
Chitosan, Alginate, Dextran, Poloxamer, and BSA	Ionotropic pre-gelation followed by polyelectrolyte complexation	396	RBA: 13.2±2.9	52
Dextran	Cross-linking of emulsion to prepare dextran nanoparticles. The surface was modified with succinic anhydride and conjugated with amino VB12 derivatives of the carbamate linkage.	160-250	PA: 26.5	49

Table 5. Natural polymers used for the preparation of nanoparticles and the methods used for their fabrication

What is Chitosan?

Chitosan is a linear copolymer consisting of ß (1-4)-linked 2-amino-2-deoxy–D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units **(Table 4)**. It is obtained by the alkaline N-deacetylation of chitin, which is the primary structural component of the outer skeletons of crustaceans. Chitosan is a weak poly base due to the large quantities of amino groups on its chain [57]. Both high molecular weight (HMWC) and low molecular weight chitosans (LMWC) are available. The latter were obtained by the depolymerization of HMWC. This can be carried out by enzymatic [58,59], physical [60, 61] or chemical methods [62, 63]. Another important property of chitosan is the degree of deacetylation (DDA), defined in terms of the percentage of primary amino groups in the

polymer backbone. The properties of chitosan and its biological role are dependent on the DDA and M.wt [64]. Chitosan dissolves easily at low pH due to the protonation of the amino groups, while it is insoluble at higher pH ranges since the amino groups become deprotonated, as the pH approach the pKa of chitosan (6-6.5). The solubility of chitosan depends upon the molecular weight and DDA.

Chitosan nanoparticles have been prepared using ionotropic gelation with tripolyphosphate or even simply polyelectrolyte complexation between insulin and chitosan. The interaction of chitosan and polyanions leads to the spontaneous formation of nanoparticles in an aqueous environment without the need for heating or the use of organic solvents [65]. In addition, to ease of preparation under mild conditions, a high level of drug entrapment can be achieved so that the protein secondary structure and biological activity is preserved [66]. Insulin-loaded chitosan nanoparticles were prepared by the ionotropic gelation of chitosan with tripolyphosphate anions [67]. These nanoparticles were effective at lowering the serum glucose level of streptozotocin-induced diabetic rats when administered orally at insulin doses of 50 U/kg and/or 100 U/kg. However, they dissociate easily in acidic gastric conditions. To protect insulin from harsh GIT conditions, chitosan nanoparticles were formulated with an enteric coating polymer - hydroxypropyl methylcellulose phthalate (HPMCP) - and evaluated for the oral delivery of insulin. HPMCP-chitosan nanoparticles showed a 2.8-fold increase in their hypoglycaemic effect when compared with chitosan nanoparticles without HPMCP [68]. Self-assembled nanoparticles were developed by mixing the anionic poly-γ-glutamic acid (γ-PGA) solution with the cationic chitosan solution in the presence of $MgSO_4$ and sodium tripolyphosphate (TPP). TPP and sulphate salts were physically added to crosslink chitosan by ionic gelation, while physical gelation may occur between Mg^+ and the carboxylate ions on γ-PGA via an electrostatic interaction. Chitosan-γ-PGA nanoparticles remained intact within the pH range of 2.0–7.2; however, at lower pH values they disintegrated. The pharmacodynamics and pharmacokinetics of insulin were evaluated in a diabetic rat model and the relative bioavailability was 15% [69]. For the further enhancement of bioavailability, two approaches were investigated: in the first, chitosan- γ-PGA nanoparticles were freeze-dried and placed in an enteric-coated capsule, while in the second, a penetration enhancer - diethylene triamine pentaacetic acid (DTPA) - was added. In both cases the bioavailability was approximately 20% [70,71].

The problem of the low solubility of chitosan in the neutral environment of the intestine was solved by synthesis of a partially quaternized derivative of chitosan - Trimethyl chitosan (TMC). TMC has good solubility and a permeation enhancing effect [72]. The targeting of trimethyl chitosan chloride to goblet cells was achieved through modification with a CSKSSDYQC (CSK) targeting peptide. The significant internalization of insulin via clathrin- and caveolae-mediated endocytosis on goblet cell-like HT29-MTX cells results in a better hypoglycaemic effect with a 1.5-fold higher relative bioavailability compared with unmodified TMC nanoparticles [73]. Trimethyl chitosan-cysteine conjugate and N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan were synthesized and demonstrated high mucoadhesion capability compared with TMC/insulin nanoparticles or native chitosan [74, 75]. Quaternized derivatives of chitosan have a high positive charge, which can easily

interact with negatively-charged blood corpuscles, resulting in haemolysis and toxicity [76]. To overcome these problems, chitosan derivatives were modified with polyethylene glycol to reduce the interaction between the cationic polymers and cell membranes [77]. Chitosan was also modified with hydrophobic fatty acids, such as anacardic acid. Anacardoylated chitosan spontaneously formed nanoparticles in an aqueous insulin solution that sustained the release of insulin in the intestinal environment [78].

1.4.1.4. Mechanisms of the absorption of nanoparticles

The absorption of the nanoparticles was thoroughly reviewed by des Rieux [8]. A particle can traverse the intestinal epithelium by the paracellular (between cells) or transcellular route (through the cells). The transcellular route is the most common. With the transcellular transport of nanoparticles, the particles are taken up by cells through the endocytic process - which takes place at the cell apical membrane - transported through the cells and released at the basolateral pole. Two types of intestinal cells are important in nanoparticle transcytosis: the enterocytes lining the gastrointestinal tract and the M cells mainly located in Peyer's patches. The uptake of nanoparticles takes place by one of three endocytotic mechanisms: pinocytosis, macropinocytosis or clathrin-mediated endocytosis. Clathrin vesicles are for particles smaller than 150 nm while phagocytosis is for particulate matters of up to several μm. The uptake of particles, microorganisms and macromolecules by M cells occurs by fluid phase endocytosis, adsorptive endocytosis and phagocytosis [8].

1.4.2. Lipid-based systems

Lipid-based delivery systems (LDS) range from simple oil solutions to complex mixtures of oils, surfactants, cosurfactants and cosolvents [79]. The bioavailability of several peptides was improved when incorporated into LDS - e.g., cyclosporine (Neoral®). The enhancement in absorption was attributed to an increase in membrane permeability, the inhibition of efflux transporters, a reduction in cytochrome P450 enzymes, an increase in chylomicron production and lymphatic transport [80].

1.4.2.1. Types

The LDS investigated for the delivery of insulin are: multiple emulsions, microemulsions and solid in-oil-in water systems.

1.4.2.1.1. Water-in-oil-in-water

A water-in-oil-in-water (W/O/W) emulsion has been proposed to protect peptides against proteolysis and enhance their absorption. Multiple emulsions containing unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) have been reported to enhance the ileal and colonic absorption of insulin without tissue damage [81]. The transport enhancement of the W/O/W emulsion prepared with octanoic acid triacylglycerol was found to be affected by the size of oil droplets. When the oil-droplet median was 2.3 μm, an earlier hypoglycaemic response was observed compared with a multiple emulsion, having a diameter of 3.8 μm. In contrast, the emulsion with a diameter of 0.7 μm exhibited no effect [82].

1.4.2.1.2. Microemulsions

Microemulsions are clear, stable, isotropic mixtures of oil, water and surfactant, frequently in combination with a cosurfactant [83]. The average particle size of microemulsions falls in the range of 5-100 nm; they are polydispersed in nature and the polydispersity decreases with decreasing particle size [84]. Insulin-loaded microemulsions were developed using didodecyldimethylammonium bromide as the surfactant, propylene glycol as the co-surfactant, triacetin as the oil phase and insulin solution as the aqueous phase. These microemulsions displayed a 10-fold enhancement in bioavailability compared with a plain insulin solution administered orally to healthy rats [85]. The improved oral bioavailability of the w/o microemulsion system was also shown for a lecithin-based microemulsion of rh-insulin [86]. On the other hand, Kraeling and Ritschel [87] found that the oral pharmacological availability of insulin microemulsions as compared with intravenous insulin in beagle dogs was 2.1%, which further increased to 6.4% with the encapsulation of gelled microemulsions in hard gelatine capsules along with the protease inhibitor aprotinin and coating of the capsules for colonic release [87]. The improved oral delivery of insulin from a microemulsion system was also demonstrated by others [88]. A stable self-emulsifying formulation for the oral delivery of insulin was developed by Ma et al. [89]. It is composed of two non-ionic surfactants (polyethylene glycol-8-glycol octanoate/decanoate and polyglycerol-3 oleate). In diabetic beagle dogs, the bioavailability of this formulation was up to 15.2% at a dose of 2.5 IU/kg in comparison with the hypoglycaemic effect of native insulin (0.5 IU/kg) delivered by subcutaneous injection.

1.4.2.1.3. Solid-in-oil-in water (S/O/W) emulsions

S/O/W emulsions were also developed for the delivery of insulin whereby insulin was converted into a lipophilic complex by coating with surfactant molecules and dispersed in an oil phase of oil in a water emulsion to form the S/O/W emulsion [90]. The stability of this system was enhanced by lyophilization [91].

1.4.2.2. Mechanism of the absorption of lipid-based formulations

Suggested mechanisms of intestinal drug absorption, using lipid-based formulations include: an increase in membrane fluidity facilitating transcellular absorption, the opening of the tight junctions to allow paracellular transport (mainly relevant for ionized drugs or hydrophilic macromolecules), the inhibition of P-glycoprotein and/or cytochrome P450 to increase intracellular concentration and residence time, and the stimulation of lipoprotein/ chylomicron production [92].

2. Chitosan-fatty acid systems

2.1. Rationale

Chitosan nanoparticles prepared by ionotropic gelation or polyelectrolyte complexation dissociate easily in an acidic medium. This might be related to the fact that both insulin and chitosan have net positive charges at pH 1.2, that the columbic repulsive forces lead to the

dissociation of the complex and that the free insulin is subjected to degradation. For example, nanoparticles prepared from chitosan and poly (γ-glutamic acid) became unstable at pH 1.2 and broke apart [93] and nanoparticles composed of chitosan and tripolyphosphate rendered the protein more susceptible to acid and enzymatic hydrolysis [94]. In the present investigation, we benefited from the advantages of polyelectrolyte complexation between chitosan and insulin, its formulation in an aqueous environment without the need for heat or an organic solvent, and the solution of the shortcomings of burst release by the dispersion of nanoparticles in an oily phase. The oily vehicle was intended to reduce proteolytic degradation and improve absorption [95, 96]. In addition, the free chitosan amine groups may interact with any adjacent carboxylic acid groups of oleic acid, forming a protective hydrophobic coating layer at the surface of the dispersed phase, which may enhance stability in the GIT and promote lymphatic uptake. The particle size of the chitosan-oleic system is above 1 μm, due to the interaction. The reduction of the particle size of chitosan-oleic acid emulsion to nanosize was achieved by high pressure homogenization or else by the addition of surfactants. PEG-8 caprylic/capric glycerides (Labrasol) and polyglyceryl-6 dioleate were selected as surfactant and cosurfactant, respectively. Chitosan-insulin nanoparticles were solubilized in the inverted micelles. Chitosan plays an important role - as a matrix for nanoparticles and stabilizers of inverted micelles. In a previous work [97], we demonstrated the role of chitosan in the reduction of particle size of the w/o emulsion containing Labrasol, plurol oleique and oleic acid. This was attributed to the interaction of amine groups of chitosan with the surfactant-cosurfactant aggregates, resulting in the formation of a closer packing of surfactants at the interface, which leads to a reduction in particle size. This effect is more pronounced in 1:1 surfactant:cosurfactant rather than in 4:1 systems. Insulin is a water soluble protein that will be located inside the water droplets. Chitosan will be partly fixed near the surfactant head groups with the rest of it inside the water droplet. Interactions between chitosan, surfactants and insulin resulted in smaller microemulsion sizes [97]. Moreover, low molecular weight chitosans were chosen to prepare the nanoparticles, since their intestinal absorption is known to be significantly better than the high molecular weight candidates and showed a negligible cytotoxic effect on the Caco-2 cells [98].

The potential of chitosan-fatty acid or chitosan-fatty acid derivative nanoparticles as oral delivery carriers of insulin was investigated systematically.

2.2. Chitosan-fatty acid nanocarriers' development

Detailed descriptions of the compositions and preparation methods can be found in the relevant patents [99, 100]. Low molecular weight chitosans were obtained by the depolymerization of high molecular weight chitosan using 2 M hydrochloric acid. The resulting fractions were characterized by Fourier transformed infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD), nuclear magnetic resonance (NMR) and dynamic light scattering (DLS). The polyelectrolyte complexation method was utilized to prepare insulin-chitosan nanocomplexes. Chitosan was dissolved in deionized water and its pH was adjusted to 5.5 using 0.2 M NaOH. rh-insulin powder was

dissolved in 0.1 M HCl, followed by the addition of 1M Tris (hydroxymethyl)-aminomethane buffer pH 7. Chitosan-insulin complexes were prepared by adding chitosan solution to an equal volume of insulin solution in a glass vial under gentle magnetic stirring, and incubating for a further 15 minutes at room temperature. The parameters affecting the encapsulation efficiency were investigated (final pH of the complex, molecular weight of chitosan, DDA of chitosan, initial concentration of chitosan and insulin, and chitosan: insulin ratio). A phase diagram was constructed and the results were used as guidance to select the suitable percentages of surfactants, oil and aqueous phases suitable for the nanoparticle dispersion system. The nanoparticle dispersion system was prepared by mixing two phases - the aqueous phase and the oily phase. The oily phase consists of Labrasol® and plurol oleique® at a fixed weight (1/1) ratio and oleic acid. The aqueous phase composed of a chitosan-insulin complex. To prepare the dispersion system, 50 µl of the aqueous phase was added to 2.5 g of the oily phase during mixing with a vortex mixer (VELP Scientifica, Europe) for 1 min. at room temperature (25 ^{o}C). The preparation was characterized: viscosity, particle size, morphology and encapsulation efficiency were all determined. The chemical and immunological stabilities of insulin after entrapment into nanoparticles were studied. The suitability of the preparation to preserve insulin activity, to withstand gut enzymes and to maintain the stability of insulin upon storage was investigated [101]. The hypoglycaemic effect of the preparation after oral administration to streptozotocin-diabetic rats was evaluated. The parameters that influence the pharmacological availability were characterized. The bioavailability of the preparation versus subcutaneous injection was calculated together with the pharmacokinetic parameters. Moreover, human studies were conducted where twenty-five healthy volunteers participated in five studies using a two-phase, two-sequence crossover design with a washout period of one day [102]. Other chitosan fatty acid systems were also formulated, for example chitosan sodium lauryl sulphate nanoparticles [103] and chitosan-oleic acid nanoemulsion (particle size reduced by a high pressure homogenizer) and their hypoglycaemic effects were evaluated and compared to the chitosan-oleic acid-surfactants system.

2.3. Results and discussion

2.3.1. Depolymerization and characterization of chitosan

Most commercially available chitosans possess quite large M.wts. LMWCs are better amenable for a wide variety of biomedical applications due to their solubility in water [104-106]. In addition, chitooligomers were found to be non-mutagenic and non-genotoxic when orally administered to mice [107]. To generate low molecular weight chitosans from high molecular weight candidates, hydrolysis by hydrochloric acid was adopted due to its practicability and reproducibility. IR spectrum spectroscopy demonstrated that there was no structure change during depolymerization. DDA was determined by NMR and it was about 99%. [101]. The solubility of chitosan increased with decreasing molecular weight. Chitosan has a positive zeta potential and its value is affected by the pH, molecular weight, DDA and concentration [108]. LMWCs with an average molecular weight of 13 KDa and DDA ~ 99% were used for further studies.

2.3.2. Chitosan-insulin polyelectrolyte complexes (PECs)

Insulin was first complexed with chitosan through the interaction of negatively charged insulin with positively charged chitosan to form PEC before incorporation into the oily vehicle. This is because chitosan has many beneficial effects, such as penetration enhancement. Chitosan was also found to stabilize insulin when incubated at 50 ± 1 °C while shaking at 100 strokes/min in a water bath [101]. As shown in **Figure 3**, the insulin solution was almost degraded while the chitosan-insulin complex protected the insulin from degradation for at least 24 h. In addition, chitosan has a role in protecting insulin from those enzymes present in the small intestine. This was reflected in the partial protection of insulin from pancreatin, as depicted in **Figure 4**, and the protection increases with the increase of the chitosan ratio. Moreover, chitosan may also protect insulin from destabilization at the oil/water interface when the PEC was dispersed in the oily vehicle. The PEC formation process is influenced by a variety of parameters, including the system pH, chitosan molecular weight and DDA. The most important factor appears to be the system pH [101].

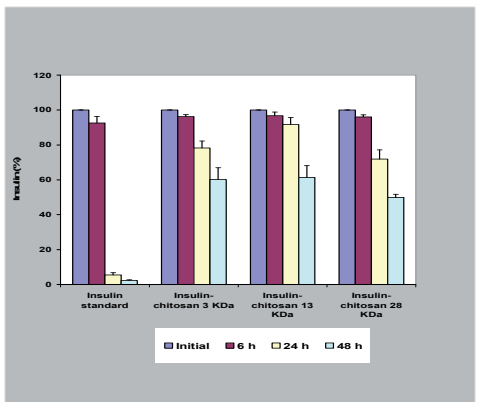


Figure 3. Effect of the temperature and shaking on the stability of the insulin

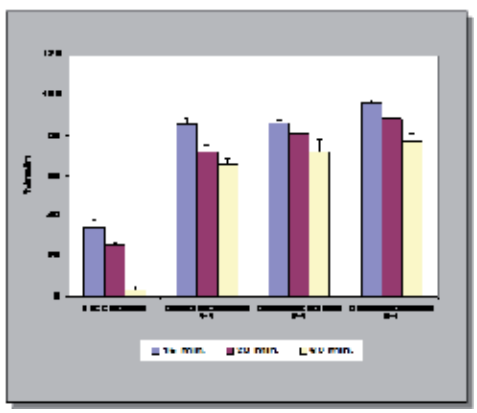


Figure 4. Effect of the chitosan:insulin ratio on the pancreatic degradation of insulin

2.3.3. Oily nanosystem preparation

Chitosan-insulin PECs were solubilized in an oily vehicle composed of a surfactant Labrasol, cosurfactant Plurol Oleique and an oily vehicle oleic acid. We attempted to formulate an oral insulin delivery system that combined the advantages of nanoencapsulation and the use of an oily vehicle. The nanoparticles were expected to translocate the intestinal epithelium, while the oily vehicle was intended to reduce proteolytic degradation and improve permeability [109, 110]. In addition, Labrasol and oleic acid are known penetration enhancers [111, 112]. Our expectation is that part of the chitosan will rest inside the water droplet of the inverted micelles where it forms PEC with insulin while the other part projecting near the surfactant head groups where it interacts with surfactants stabilizes the w/o microemulsion and resulted in smaller microemulsion sizes [97]. Chitosan at pH 6 will also interact with oleic acid to coat the particles with a hydrophobic layer. This interaction was studied by molecular mechanics, as illustrated in **Figure 5**. The structure of chitosan 13 KDa, oleic acid and chitosan-oleic acid

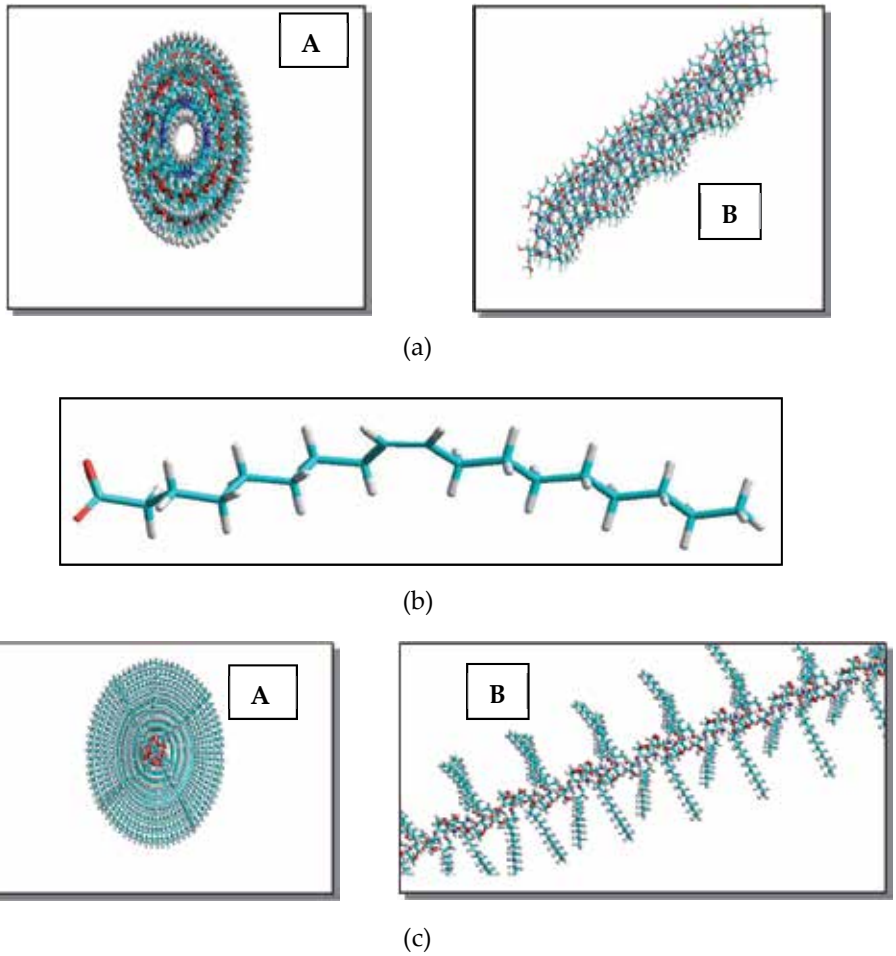


Figure 5. a. Top view (A) and side view of chitosan (B); b. The computed molecular geometries of oleic acid; c. Top view (A) and side view of the chitosan-oleic acid complex

complex, which were built up in Hyperchem®, was shown in **Figures 5.a**, **b** and **c**, respectively. The conformation of individual chitosan and oleic acid was the same as the complex.

It is obvious that the outer structure of the complex was hydrophobic due to presence of oleic acid while chitosan was embedded inside the structure. This is consistent with the immiscibility of the complex with water. The binding energy of the resulting chitosan oleic acid complex is calculated according to the following equation:

$$\mathbf{E\ binding} = \mathbf{E\ complex} - \left(\mathbf{Eoleate} + \mathbf{Echitosan}\right)$$

$$\mathbf{E_{complex}} = -\mathbf{539.94};\ \mathbf{E_{oleic}} = \mathbf{2131.64};\ \mathbf{E_{chitosan}} = \mathbf{4526.58};\ \mathbf{E\ binding} = -\mathbf{7198.2\ kcal/mol}.$$

The value of the binding energy suggested a high degree of interaction between chitosan and oleic acid. The chitosan-oleic acid interaction was also studied using statistical design [108]. The effects of three formulation variables (the aqueous chitosan solution to oleic acid ratio, the chitosan molecular weight, and the degree of deacetylation of chitosan) on the viscosity of the system and the length of the emulsified layer (%) were studied in a conventional 2^3 factorial design. It was found that chitosan-oleic acid interaction is significantly influenced by pH. At a pH of around 6.5, chitosan is almost 50% ionized (pK_a NH_3/NH_2 ~ 6.5) and the ionized amine groups in chitosan will interact with the carboxylate ion of oleic acid. However, at pH 1.5, chitosan will be available as chitosan hydrochloride, and so no interaction was observed. 50% oleic acid and 50% chitosan aqueous solution (2%) at different pH were mixed and their viscosities were determined, as depicted in **Figure 6**. A sharp increase in viscosity was noticed when the pH of chitosan was ≥ 5.5. This indicates that the rheological properties of this dispersion were notably influenced by other factors - apart from the disperse phase volume fraction - such as the interaction between chitosan and oleic acid. These results were consistent with the surface tension measurements of the chitosan-oleic acid system [97].

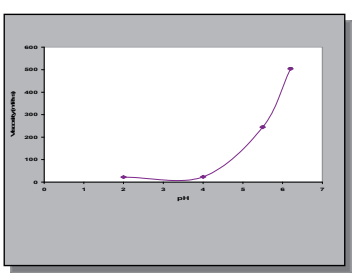


Figure 6. The effect of the pH of chitosan on the viscosity of oleic acid chitosan dispersions

2.3.4. Oily nanosystem characterization

The mean particle size was determined by dynamic light scattering - it was 111 ± 6.9 nm - and showed a unimodal particle size distribution. The particle size is affected by the molecular weight of chitosan, as shown in **Table 6**. An increase in the molecular weight of the chitosan polymer led to an increase in the dispersed phase particle size. The particles'

shapes were assessed by a transmission electron microscope (TEM) and it was spherical, as depicted in **Figure 7**. The viscosity of the nanosystem was measured by a Vibro viscometer - it was 52.25 ± 2.6 mPa s. Neither the particle size nor the viscosity of the nanosystem changed upon storage at 4 or 25 ºC for one month, indicating the physical stability of the nanosystem. The preparation procedure is mild and the insulin is chemically and immunologically stable, as illustrated by RP-HPLC and ELISA, respectively. About 90% of the insulin was recovered from the preparation after incubation with pepsin, indicating the protective ability of the preparation for insulin under conditions simulating the gastric environment [97]. In addition, short term chemical stability demonstrates that the chemical stability of the insulin was maintained for at least 30 days of storage at 4 and 25 °C, according to the HPLC method. Moreover, the biological activity was reserved after one month at storage temperatures of 4 and 25 ºC.

Chitosan M.wt (KDa)	Mean diameter (nm) ± SD
3	79 ± 2.5
13	111 ± 6.9
30	205 ± 2.6

Table 6. The mean diameter of the oily nanosystem prepared from chitosans with different molecular weights

Figure 7. TEM image of the oily nanosystem

2.3.5. In vivo studies

The preliminary screening of the biological activity of nanoparticles prepared from different grades of chitosan (different molecular weights and DDA) and administered orally to STZ diabetic rats revealed a maximum effect with nanoparticles prepared from chitosan with a molecular weight of 13 KDa and DDA~ 99%. **Figure 8** illustrates changes in the plasma glucose levels after the oral administration of the nanoparticles prepared from chitosan with a molecular weight of 13 KDa and DDA 99%. As expected, the insulin oral solution showed no hypoglycaemic effect compared to the control group ($P > 0.05$). In contrast, the blood glucose levels of the rats decreased remarkably after the oral administration of insulin-loaded nanosystem, achieving a significant decrease at 3 h when compared with the control group ($P< 0.05$). More interestingly, the hypoglycaemic effect was maintained without recovery at the baseline for 12 h. A pharmacological availability value of 29% was obtained for the dose 5 IU/kg. An explanation of this positive behaviour of the oily nanosystem could

be put forward in terms of the demonstrated ability of oily preparations to make the entrapped insulin more stable and protect it from degradation in the harsh conditions of the gastrointestinal tract as well as enhance its intestinal absorption.

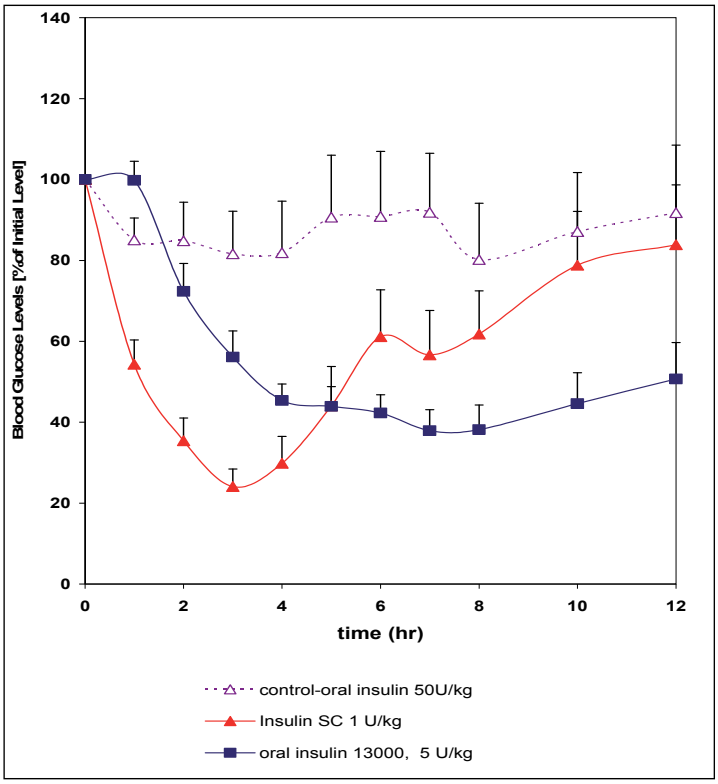


Figure 8. Changes in blood glucose level versus time profiles after a single oral administration of the oily dispersion of chitosan-insulin nanoparticles given at a dose level 5 IU/Kg (■to STZ-diabetic rats compared to a free insulin solution given orally (50 IU/Kg) as a control group (Δ) and a subcutaneous injection of a free insulin solution (1IU/Kg) (▲ The results are expressed as the mean ± S.E.M (n = 12 per group)

A concomitant increase in plasma insulin levels was observed after the oral administration of the 5 IU/kg of insulin-loaded oily nanosystem to diabetic rats, as depicted in **Figure 9**. The pharmacokinetic parameters were determined based on the insulin concentration plasma profiles of **Figure 9**, as shown in **Table 7**. The subcutaneous injection group exhibited a rapid increase in serum rh-insulin concentration up to 279.2 μIU/ml over 30 min of administration. Meanwhile, the intragastric administration of 5 IU/kg of nanoparticles exhibited slower absorption and sustained elimination, reaching a maximum after 2 h (102.22 μIU/ml). Moreover, the serum rh-insulin levels of the nanoparticle group were significantly different from that of the control group ($P < 0.001$). The $AUC_{0\text{-}12}$ of orally administered nanoparticles was 664.99 μg hr/ml for the 5 IU/kg dose and 626.02 μg hr/ml for the 1 IU/kg subcutaneous rh-insulin dose. The corresponding relative bioavailability was calculated to be 21.24% [108].

These results clearly show that rh-insulin absorption was markedly enhanced by the nanoparticles dispersed in oily vehicle. As a proof of concept, early clinical trials have been performed by Badwan et al. [102]. The pharmacokinetic, pharmacodynamic and absorption kinetics of insulin-loaded oily nanosystem preparations of different particle sizes (57-220 nm) were compared with those of subcutaneous formulation in 25 healthy individuals using a euglycaemic clamp technique. The dose used was either 1, 2 or 3 IU/Kg. The effective permeability ratio (Peff*) was higher for preparations with a particle size of 57 nm than for those with a larger particle size. The preparation with the lowest particle size also exhibited the highest ratio in the dimensional analysis of the glucose infusion rate as a pharmacodynamic effect, while the other insulin formulations that were tested showed similar ratio profiles. The calculated intestinal permeability coefficients (×10–4) of the insulin best test and reference formulations were 0.084 and 0.179 cm/sec respectively. The total fraction of the insulin dose absorbed (Fa) for the test and reference products were 3.0% and 19% respectively. From these small studies, it was concluded that oral insulin bioavailability is promising for the development of oral insulin products.

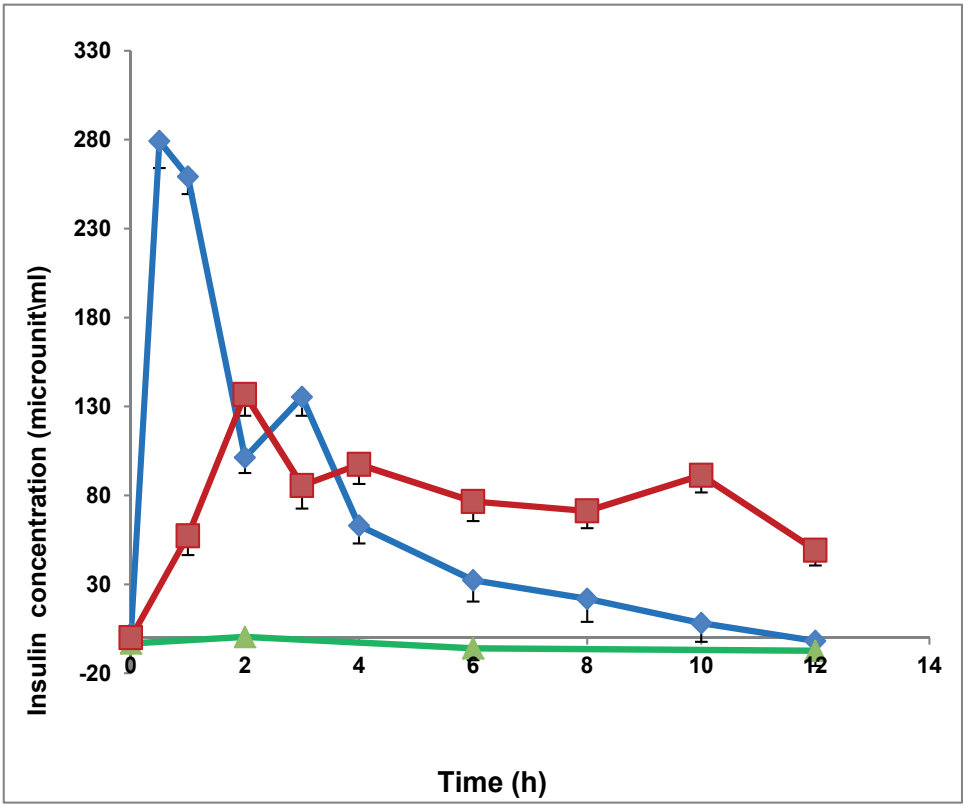


Figure 9. Insulin plasma levels profile after a single oral administration of the oily dispersion of chitosan-insulin nanoparticles (5 IU/Kg) (■) in fasted diabetic rats compared to the subcutaneous injection of free insulin (1 IU/Kg) (▲)and an oral insulin solution (50 IU/kg) as a control (▲) The results are expressed as the average of the three independent experiments (n = 18)

Preparation	Cmax [μg/ml]	Tmax [hr]	MRT [hr]	AUC_{0-12} [μg hr/ml]	F %
S.C injection (1 IU/kg)	**279.19**	**0.5**		626.02	
Oral formula (5 IU/kg)	**102.22**	**2**	**6.72**	664.99	21.24%.

Table 7. Pharmacokinetic parameters derived from the plasma level vs. the time profile for insulin

Other chitosan fatty-acid systems were also developed and compared to the above mentioned system. For example, chitosan-sodium lauryl sulphate nanoparticles dispersed in an aqueous vehicle elicited a pharmacological response after oral administration [103]. However, the pharmacological availability was poor - 1.1% versus 29% for nanoparticles dispersed in an oily vehicle. Another system was developed by dispersing PEC in oleic acid and the particle size was reduced using a high pressure homogenizer. The oral administration of this preparation resulted in a pronounced effect ($P < 0.001$) after 12 h of administration and the effect was sustained for 24 h (**Figure 10**). This indicates that chitosan-oleic acid nanoparticles were slowly absorbed in comparison with the preparation containing surfactants. This may be due to the permeation enhancing effect of surfactants [108]

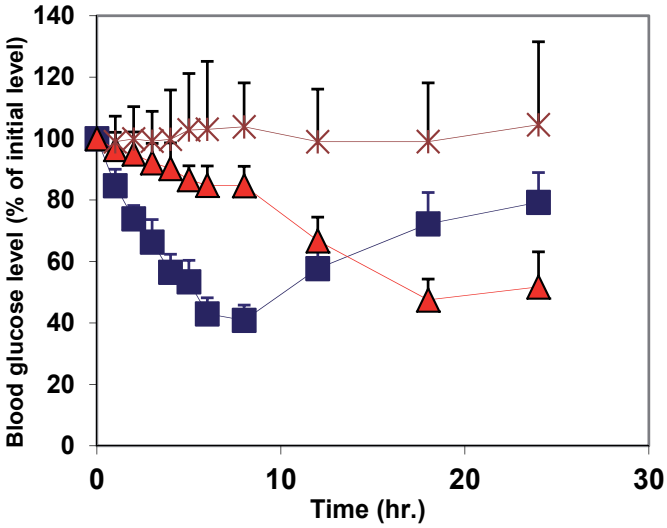


Figure 10. Changes in blood glucose level versus time profiles after a single oral administration of an oily dispersion of a chitosan-insulin oily nanosystem containing surfactant given at a dose level of 5 IU/Kg (▼) to STZ-diabetic rats compared to a chitosan-insulin oily nanosystem without surfactant (▲). The control was given a placebo (a formula containing all excipient) (x). The results are expressed as the mean ± S.E.M (n = 12 per group)

In conclusion, the use of the combination strategy of nanoencapsulation and an oily vehicle has shown considerable improvement in insulin delivery along the following lines:

1) A significant hypoglycaemic action with a maximum pharmacological availability of 29.0% was obtained; 2) The relative bioavailability was 21.2%; 3) The antidiabetic activity was prolonged for many hours; 4) The insulin in the preparation was chemically and biologically stable for a period of one month at storage temperatures of 4 °C and 25 °C. 5). The system could be considered as a platform technology for the delivery of other peptides, such as calcitonin.

3. Oral insulin formulations in clinics

Oral systems in different clinical phases and the companies who have invested on them were listed in **Table 8.** The modification of proteins' structures by the attachment of proper moieties which alter their biopharmaceutical properties have been investigated by Biocon (Bangalore, India) and Nobex Corporation (Research Triangle Park, NC, USA). Nobex developed an orally active amphiphilic human insulin analogue, methoxy (polyethylene glycol) hexanoyl human recombinant insulin. Biological activity is retained and this compound is readily absorbed from the gastrointestinal tract. The effect of a single oral dose of hexyl-insulin monoconjugate 2 (HIM2) on the rate of whole-body glucose disposal (*R*d) and endogenous glucose production (EGP) was investigated in healthy non-diabetic subjects using a euglycaemic clamp technique. Oral HIM2 suppresses EGP and increases tissue *R*d in a dose-dependent manner. The effects of HIM2 on EGP and *R*d persisted for 240 min. In patients with type 1 diabetes mellitus, a statistically significant effect of HIM2 on glucose excursion was observed [113]. The further development of the product was abandoned by the company. Biocon modified the hexyl insulin monoconjugate 2 (HIM2) developed by the Nobex Corporation. Conjugation with poly (ethylene glycol) improves the protein solubility, stability from enzymatic degradation and intestinal absorption [114]. The modified insulin (IN-105) was formulated as tablets and has a short duration of action (1.5-2 h). It was found that IN-105 reduced postprandial glucose excursion by 2 h in a dose-dependent manner and that it was readily tolerated by patients [115]. However, in Phase III studies, IN-105 did not meet the target of lowering the level of glycated haemoglobin by 0.7% compared to a placebo, as announced by the company. The level of glycated haemoglobin in the body is an indication of the effectiveness of a drug in controlling blood sugar levels. In contrast, the noncovalent interaction of macromolecules with small hydrophobic organic compounds, SNAC (n-(8-[2-hydroxybenzoyl]-amino) caprylic acid) and 5-CNAC (N-(5-chlorosalicyloyl)-8-aminocaprylic acid), was the technology developed by Emisphere's Eligen™. As a result of this interaction, the lipophilicity and absorption of the macromolecules increases and this is reflected in the rapid onset of action (about 10 minutes). Emisphere's oral insulin product was well tolerated and improved both glycaemic control and insulin sensitivity. However, a high dose (40 mg/day) is needed to decrease HbA1c significantly after 3 months of therapy [116]. ORMD 0801 is a capsule formulation of insulin developed by Oramed Pharmaceuticals (USA). An omega-3 fatty acid (carrier), a soya bean trypsin inhibitor (protease inhibitor) and sodium EDTA (absorption enhancer) were selected as adjuvants to protect the insulin from the harsh environment of the gastrointestinal tract and enhance its transport across the intestinal mucosa. ORMD is an

intermediate insulin product with a duration of action of 5-6 h. This product demonstrated improved absorption, as indicated by a 28% increase in post-prandial serum insulin and it is well tolerated by patients [117]. However, the onset of action is delayed by 2 h, which may be due to the enteric coating of the capsules. Another capsule formulation (Capsulin) was developed by Diabetology (Jersey, UK). The capsule contains a mixture of penetration enhancers and solubilizers which are generally considered to be safe. The administration of the oral insulin Capsulin preparation to sixteen persons with type 2 diabetes demonstrated a significant hypoglycaemic action over a period of 6 h and was associated with only a small increase in circulating plasma insulin concentrations. Significant falls in HbA1c, weight and triglycerides were also observed [118].

Technology	Example	Company	Current status	Ref
Chemical modification	Hexyl-insulin monoconjugate-2 (HIM2)	Nobex corporation	Phase II	[113]
Chemical modification	IN-105	Biocon	Phase III	[115]
Delivery agent	Sodium N-[8-(2-hydroxybenzoyl) amino] carpylate (SNAC)	Emisphere	Phase II	[116]
Soft-gel capsule with enhancers	Oramed insulin capsule	Oramed Pharmaceuticals (USA).	Phase II	[117]
Absorption enhancers (Axcess™ delivery technology)	Capsulin™	Diabetology (Jersey, UK)	Phase II	[118]

Table 8. Systems currently being studied for the oral delivery of insulin

4. Conclusions and prospects for further investigations

Oral delivery is a physiological route for insulin administration. Improved disease management, the enhancement of patient compliance and the reduction of long-term complications of diabetes could be achieved by oral application. However, the challenges for developing oral insulin dosage forms are significant. A number of reports have appeared in the literature seeking to enhance insulin delivery via the oral route; however, the bioavailability in humans has not exceeded 10%. Most systems evaluated the pharmacodynamics and pharmacokinetics of oral insulin preparations on animal models. However, a few reports studied absorption mechanisms. The absorption of insulin is the major obstacle. Therefore, more focus should be directed on studying the very small details of absorption, especially with the development of many instrumental technologies that will help in this area. Nanotechnology will contribute largely to the success of oral insulin delivery. The investigators should plan to search for safer, simpler and scalable methods using biologically acceptable polymers. Nowadays, researchers from both academia and industrial fields work on oral insulin. With these efforts, the dream of oral insulin will become real in the near future.

Nomenclature

Da	Dalton
KDa	Kilodalton
HDV	Hepatic directed vesicles
PCL	poly ε -caprolactone
PLGA	Poly (lactic-co-glycolic acid)
PL	Polylactides
PACA	Poly (alkyl cyanoacrylate)
PEG	Polyethylene glycol
LMWC	Low molecular weight chitosan
HMWC	High molecular weight chitosan
DDA	Degree of deacetylation
M.wt	Molecular weight
HPMCP	Hydroxypropylmethylcellulose phthalate
γ-PGA	poly-γ-glutamic acid
TPP	Tripolyphosphate
TMC	Trimethyl chitosan
LDS	Lipid-based delivery systems
SLN	Solid lipid nanoparticles
GIT	Gastrointestinal tract
rh-insulin	Recombinant human insulin
WGA	Wheat germ agglutinin binds
FTIR	Fourier Transformed Infrared Spectroscopy
DSC	Differential Scanning Calorimetry
XRPD	X-Ray Powder Diffraction
NMR	Nuclear magnetic resonance
DLS	Dynamic light scattering
RP-HPLC	Reversed-phase high performance liquid chromatography
ELISA	Enzyme-linked immunosorbent assay
TEM	Transmission electron microscope
PECs	Protein Polyelectrolyte Complexes
AE	Association efficiency
SLS	Sodium lauryl sulphate
LDS	Lipid-based delivery systems
BSA	Bovine serum albumin
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
W/O	Water in oil
O/W	Oil in water
W/O/W	Water-in-oil-in-water
S/O/W	Solid-in-oil-in water
RSD	Relative standard deviation

s.c.	Subcutaneous
PA	pharmacological availability
RBA	Relative bioavailability
STZ	Streptozotocin
Tmax	the time taken to reach the plasma peak level
Cmax	The plasma peak level
MRT	Mean residence time
AUMC	Area under the first moment curve
AUC	Area under the curve
F%	Relative bioavailability
AAC	Area above the curve
ANOVA	analysis of variance
MSE	Standard error of the mean
P	probability value
T_m	Melting temperature
GIT	Gastrointestinal tract
*R*d	Rate of whole-body glucose disposal
EGP	endogenous glucose production

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Chapter 11

Novel Mucoadhesive Polymers for Nasal Drug Delivery

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Additional information is available at the end of the chapter

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1. Introduction

The use of nasal cavity as a route of administration of drugs, specifically systemically acting drugs that pose a delivery challenge, have become an area of great interest to the pharmaceutical companies in the past decade. The physiology of the nasal cavity allows for variety of drug delivery possibilities and destinations which include local, systemic, vaccine, and access to the central nervous system (CNS)[1]

Anatomically, the nasal cavity can be divided into three functional regions (**Figure 1**):

1. Vestibular region having an area of 10 to 20 sq.cm and is situated just inside the nostrils. It is covered with stratified, keratinised and squamous epithelium.
2. Respiratory region having an area of about 130 sq.cm and occupies majority of the nasal cavity and consists of three turbinates namely inferior, middle and superior.
3. Olfactory region has an area of about 10 - 20 sq.cm. It is located in the roof of the nasal cavity and on the upper part of the nasal septum. It contains the receptors for the sense of smell. Local delivery of drugs in the nasal cavity can be used to treat allergies, congestion and infection. Systemic delivery of the drugs can be used for crisis treatments during a rapid onset of symptoms, daily administration of drugs for long-term treatment of disorders or delivery of peptides or proteins that may be difficult to administer. The nasal cavity can also be used to deliver vaccines including antigens (whole cells, split cells, and surface antigens) and DNA vaccines [1]. The nasal cavity also allows access to the CNS, thus allowing drugs to circumvent the blood-brain barrier (BBB) [1]. It has been suggested that there is free communication between the nasal submucosal interstitial space and the olfactory perinueronal space, which appears to be continuous with a subarachnoid extension that surrounds the olfactory nerve [2].

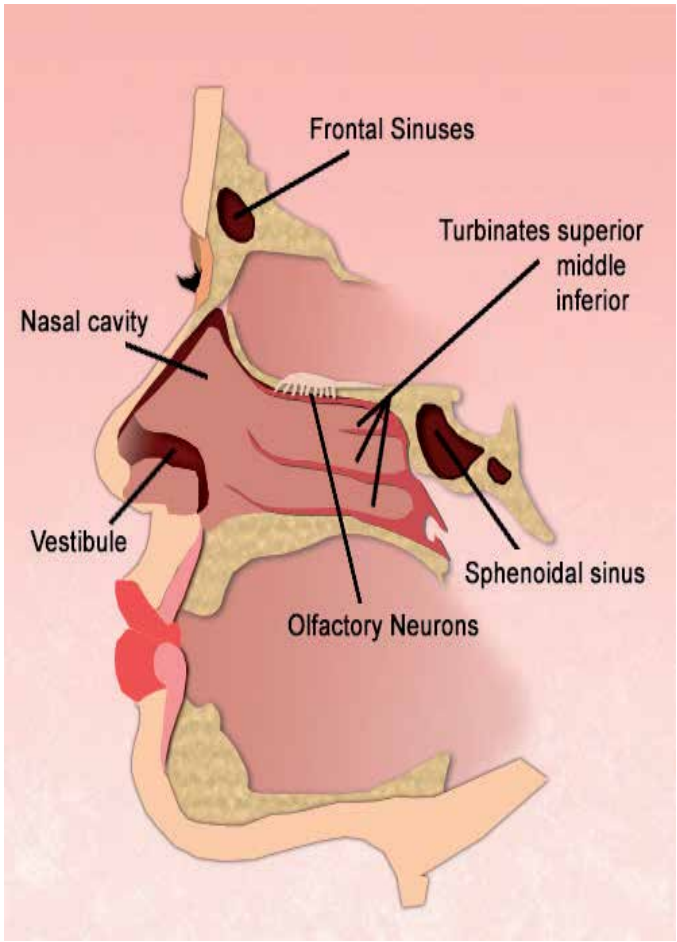

Figure 1. Nasal Anatomy and Physiology

The pharmaceutical companies are increasingly marketing drugs as nasal formulations, drugs such as sumatriptan, estradiol, buserelin, and calcitonin, all which have shown to have faster onset of action, improved bioavailability, and a better delivery method [3].

There are a number of advantages in using nasal cavity for administration of drugs. Some of the advantages are: avoidance of the gastrointestinal tract along with hepatic first pass metabolism, increased absorption and bioavailability of small and larger drug molecules[4]. Furthermore, drugs that have low oral bioavailability have been shown to be successfully delivered systemically using the nasal route; studies have suggested that the nasal route is a great alternative to parenteral route for delivery of protein and peptide drugs [4]. Also due to direct delivery of drugs to the systemic circulation, the onset of pharmacological action is rapid [5].

Administration of lower drug doses through the nasal route, may lead to lower side effects. The convenient delivery of drugs via the nasal route, especially in long-term therapies has proven to increase patience compliance compared to parenteral and injection methods [4].

Lipophilic drugs generally have no trouble being absorbed through the nasal cavity. In fact, the bioavailability of lipophilic drugs been shown to be very close to those of intravenous injection (100% bioavailability), for instance, fentanyl has been shown to have an 80% bioavailability for nasal administration [1].

Even though the nasal cavity has a large surface area along with extensive blood supply, it has been shown that the permeability of the nasal mucosa is low for polar molecules. The limiting-factor for nasal absorption of polar drugs such as peptides and proteins is epithelial membrane permeability. Drugs with molecular weights lower than 1000Da can generally pass the epithelial membrane via transcellular route, receptor mediated transport, vesicular transport, use of concentration gradient force [6], or travelling in the paracellular route through the tight junction between the cells. In fact tight junctions seem to have finite permeability to molecules with molecular radii less than or equal 3.6 A and are essentially impermeable to those with molecular radii greater than or equal to 15 A [7]. Despite the advantages of nasal drug delivery, some of the drugs may also cause inconvenience due to potential for nasal irritation. Pathological conditions such as cold and allergies may alter nasal bioavailability significantly, which can have an effect on the intended pharmacological action [8].

Another factor that plays an imperative role in low membrane transport of nasally administered drug therapeutics is rapid drug clearance by the mucociliary clearance mechanism. This problem is common with drugs that are not easily absorbed across the nasal membrane. It has been shown that drugs that do not readily cross the nasal membrane, whether liquid or power form are removed from the nasal cavity in 15-20 min [9]. Mucociliary clearance tends to decrease the residence time of the administered drug. This problem can be overcome using formulation strategies. Novel delivery platforms based on polymeric drug carriers along with variety of methods that can be used to improve the absorption of drugs through the nasal route will be discussed in the following paper. These delivery systems work by attaching themselves to the mucus layer and thus preventing clearance of the drug delivery system. Some of these delivery systems are still experimental, whereas others have advanced to clinical use. **Figure 2** summarizes the various mechanisms involved in mucoadhesion of the main drug carriers discussed in this chapter.

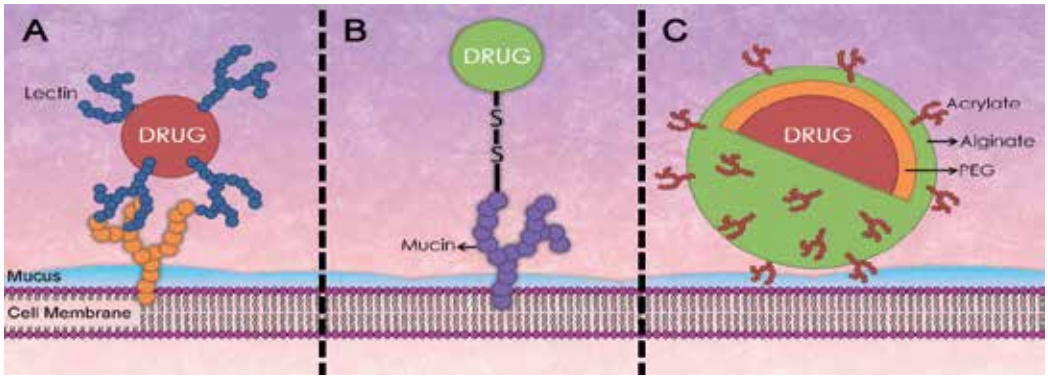

Figure 2. Mechanisms of mucoadhesion by lectins (A), Thiomers (B), Alginate Poly ethylene glycol acrylate (C).

2. Lectins

Lectins are classified as a group of structurally diverse proteins [10] that are found in plants as well as in the animal kingdom. They are also found in some microorganisms [11]. Lectins have the capability to identify and bind to specific sugar moieties. The sugar-binding moiety of most lectins is only a small part of the lectin, i.e., a major portion of lectin is not involved in the recognition and binding to the receptor[12]. Lectins also cause agglutination due to their ability to cross link sugar containing macromolecules. Primarily they identify only specific sugars like mannose, glucose, galactose, N-acetyl-glucosamine, N-acetyl galactosmaine, furose and N-acetyl neuramic acid [13]. The various lectins which have shown specific binding to the mucosa include lectins extracted from *Ulex europaeus* I, soybean, peanut and *Lens culinarius*. The use of wheat germ agglutinin has been on the rise due to its least immunogenic reactions, amongst available lectins [2]. Lectins have the ability to stay on the cell surface or become internalized via a process called endocytosis if the adhesion is receptor mediated. In this manner lectins offer twin functionality of not only allowing target specific attachment but also a means of delivering the drug through a controlled process to the cells by active cell mediated drug uptake [1]. Lectins have potential to be used in Nasal Drug Delivery, especially where internalization of the drug encapsulated nanoparticles is of particular importance such as DNA delivery[14]. Inspite of lectins offering significant advantages, it is worth noting that such polymers suffer at least in part from premature inactivation by shed off mucus. This phenomenon has been reported to be advantageous, given that the mucus layer provides an initial yet fully reversible binding site followed by distribution of lectin-mediated drug delivery systems to the cell layer[15].

There are three types of lectins - classified based on their molecular structure:

1. *Merolectins*: lectins which have one carbohydrate recognising domain [1].
2. *Hololectins*: lectins which have two or more carbohydrate recognising domains [1].
3. *Chimerolectins*: lectins with additional unrelated domains [1].

Lectins are involved in various biological processes: cell-to-cell recognition and communication, particularly in the mammalian immune system (transendothelial migration), adhesion and attack of infectious agents on host cells, and clearance of glycoproteins from the blood circulation [16]. Lectins are used in conjugation with other mucoadhesive polymers as drug delivery vehicles to the brain or systemic circulation through the nasal cavity. It was observed that negligible penetration of nanoparticles takes place between cells in the nasal epithelium when administered on their own. Secondly, mucociliary clearance reduced the residence time of the particles in the nasal cavity (particles cleared within the nose every 15 to 20min, thus, resulting in incomplete absorption of the formulation. Also it was found that, unmodified nanoparticles distributed in the nasal cavity without selectivity. This resulted in poor brain targeting efficiency of the formulation. To deal with these problems, novel lectin-modified nanoparticles were constructed. The lectin used was wheat germ agglutinin (WGA), which specifically binds to N-acetyl-D-glucosamine and sialic acid moieties, both of which were abundantly observed in the nasal cavity especially in the olfactory mucosa [17].

Factors like low ciliary irritation and high permeability among other considerations favour the potential use of lectins for nasal drug delivery [18]. Studies in animals concluded that lectins have minimal acute irritancy and can be thus be used for further in vivo studies[19]. However, many lectins are toxic or immunogenic especially those obtained from *Ricinus communis, Phaseolus vulgaris* and *Lycopersicon esculentum* and *Canavalia ensiformis[*5] .There is a probability that lectins promote the production of antibodies which could lead to the blockage of lectin-based delivery vehicles. These antibodies may also expose patients to the risk of systemic anaphylaxis on successive exposure. But, by using truncated varieties of lectin molecules as mucoadhesives this potential risk of toxicity may be triumphed over [20].

Mucoadhesive Polymer	Dosage Formulation	Active Ingredient	Reference
Odorranalectin	Liquid	coumarin-6	Wu H et al[21]
wheat germ agglutinin conjugated PEG-PLA nanoparticles	Liquid	coumarin-6	Liu Q et al[22]
Solanum tuberosum lectin-conjugated PLGA nanoparticles	Powder	coumarin-6	Chen J et al [18]

Table 1. Synopsis of the studies on the use of Lectins in formulations for nasal drug delivery

3. Thiomers

Thiomers are mucoadhesive polymers that have side chains carrying thiols which lead to formation of covalent bonds between the cystiene groups in the mucus and the polymer by thiol/disulphide exchange reactions or simple oxidation process. These bonds are also known as disulphide bridges. These bridges sometimes improve mucoadhesion by 100 folds. They also have permeability enhancing effect and ability to control the rate at which drugs are released. This property and increased mucoadhesion leads to higher residence time of the drugs administered in combination with thiomers hence improving their bioavailability [23]. Thiomers are also used in combination with other polymers like chitosan, poly acrylic acid, etc. Due to immobilization of thiol groups, mucoadhesive properties of these polymers are increased by 140 folds and 20 folds respectively [12]. Thus, thiomers are one of the most mucoadhesive polymers known at the present [24]. Thiomers enhance the permeability of drugs with the potential advantage of not being absorbed through the nasal mucosa compared to low molecular weight permeation enhancers. Thus their permeation enhancing effects can be maintained over a longer period of time while excluding systemic toxic effects.[25] Thiomers tend to cause reversible opening of the tight junctions with glutathione as permeation mediator [26]. Thiolated polymers display in situ gelling properties due to the oxidation of thiol groups at physiological pH-values, which results in the formation of inter- and intramolecular disulfide bonds[12]. This increases the viscosity of the formulation coupled with extensive crosslinking due to formation of disulphide bonds with the nasal mucosa, which increases the residence time of the formulation tremendously [27].

Other studies on thiomer combinations with other polymers by Bernkop-Schnurch led to formation of thiolated polycarbophil which increases the uptake of Leu-enkephalin from the nasal mucosa by 82 folds, thus a promising excipient for delivery of Leu-enkephalin through the nasal mucosa[28]. In another study, thiolated polyacrylate microparticles were generated for the nasal delivery of human growth hormone (hGH). The intranasal administration of this microparticulate formulation to rats resulted in a relative bioavailability of 8.11 ± 2.15% that represents a 3-fold improvement compared to microparticles comprising the corresponding unmodified polymer[29].

The nasal route is an attractive alternative to parenteral delivery for a number of therapeutic peptides such as calcitonin, insulin, desmopressin, buserelin and octreotide. However, membrane permeability is low for nasally administered peptides leading to low bioavailabilities, a short local residence time at the site of absorption and a high metabolic turnover in the epithelium. The three major approaches to increase the bioavailability of intranasally administered peptide drugs are (i) the use of permeation enhancers, (ii) incorporation of enzyme inhibitors and (iii) increasing local drug residence time using mucoadhesive polymers. Thiomers are capable of combining most of these strategies. Therefore, thiomers can be used as multifunctional vehicles for systemic nasal peptide delivery[30]. Due to their high molecular mass, thiomers are not absorbed from the nasal mucosa thus systemic toxic effects can be excluded. Ciliary Beat Frequency (CBF) studies with human nasal epithelium cells show that thiomers do not cause any alteration or impact on CBF. Thiomers have also been found in various studies to not cause any irritation to mucosal cells[31]. Table 2 summarizes nasal drug delivery studies with thiomers.

Mucoadhesive Polymer	**Dosage Formulation**	**Active Ingredient**	**Reference**
Thiomer (polycarbophil-cysteine)	Gel	Leu-enkephalin	Bernkop-Schnürch A et al.[28]
Thiomer (polycarbophil-cysteine /glutathione gel)	Gel	Human growth Hormone	Leitner VM et al.[32]
Thiomer (polycarbophil-cysteine)	Microparticles	phosphorothioate antisense oligonucleotide	Vetter A et al[33]
Thiolated chitosan	Microparticles	insulin	Krauland AH et al[34]

Table 2. Use of thiomers in mucoadhesive nasal formulations for systemic delivery

4. Alginate poly-ethylene glycol acrylate

Alginate Polyethylene glycol Acrylate is also known by the acronym Alginate-PEGAc. It has an alginate backbone with acrylated polyethylenglycol groups attached to it. This polymer meshes the properties of alginates (strength, simplicity and gelation) with characteristics specific to the acrylate functionality of PEG like mucoadhesion. PEG's have the ability to penetrate the mucus surface while the acrylate group of the polymer reacts with the sulphide group of glycoproteins present in the mucus. This results in a strong interaction

between the mucus and the polymer [35]. It is expected to be cross-linkable by two different paths: chemically via the acrylate end groups and physically through the alginate backbone [36]. Alginate is a mucoadhesive polysaccharide of 1 → 4 linked α-l-glucuronic acid and β-d-mannuronic acid which binds to the glycoproteins in the mucus through carboxyl–hydroxyl interactions [37]. It is anionic in nature. It is known to undergo ionic sol to gel transition (gelation) upon interaction with multivalent ions such as $Ca2^{+}$, $Fe2^{+}$ [38], thus reducing its adhesion to mucosal tissues [39]. On the other hand Poly-Ethylene Glycol (PEG) is an FDA approved polymer. It is non-toxic, non-immunogenic and non-antigenic. It has high solubility in water and rapid in vivo clearance. It also has the ability to form hydrogen bonds with sugar moieties on glycosylated proteins. This causes PEG to form strong bonds with mucus leading to increased mucoadhesion [25].

Poly Acrylic Acid forms hydrogen bonds between its carboxylic acid groups and sialic acid-carboxylic acid groups present in the mucus [40]. The most recent method for synthesis of Alginate PEG-Ac is a two stage procedure. First the synthesis of alginate thiol takes place. In the second stage, a Michael type addition reaction takes place where a nucleophilic addition between PEG-Diacrylate and alginate backbone occurs conjugating the two [25].

Modification of Alginates with addition of acrylic acid is done to optimize its shortcomings such as erosion in neutral pH. Addition of acrylic acid controls the release rate of drugs and also improves its adhesive properties [29]. Also it has been found that at physiological pH of 7.4 both poly acrylic acid and sialic acid undergo ionization, thus repelling each other. This leads to rapid removal of this polymer-based drug delivery system. Addition of PEG results in H-bonding with PAA enhancing the viscosity of the resulting drug delivery vehicle. Addition of PEG to the polymer increases the viscosity of the resulting polymer complex retarding disintegration and removal of the polymer from the mucosal surface thus increasing mucoadhesion [30]. The combination of the three functional moieties of Alginate Polyethylene glycol Acrylate leads to an improved novel polymer that can be used mucoadhesive nasal drug delivery.

5. Poloxamer (Pluronics)

There is a great interest in Poloxamer based formulations. A number of reviews have been published describing in detail poloxamer formulations like gels, poloxamer-coated nanoparticles, o/w and w/o emulsions, and solid polymer blends [41]. Poloxamers are made up of non-ionic difunctional triblock[42] copolymers containing a centrally located hydrophobic polypropylene oxide between hydrophilic polyethylene oxides [43,44]. Aqueous solutions of poloxamers are extremely stable in the presence of acids, alkalis and metal ions. These polymers are readily soluble in aqueous, polar and non-polar organic solvents. Hence, they are widely preferred choice as excipients in formulations [45]. Poloxamers are said to contain thermoreversible property and will convert from a liquid to a gel at body temperature, thus, causing in situ gelation at the site of interest [1] preventing the drug to be removed from the nasal cavity due to mucociliary clearance. This vastly improves the bioavailability of the drug administered. The formation of the gel can be explained as follows. When the poloxamer is

cooled, the hydration layer surrounds the poloxamer molecule and hydrophobic portions are separated due to hydrogen bonding. As the temperature increases, desolvation of the hydrophilic chains occurs as the result of breakage of hydrogen bonds. This results into hydrophobic interactions amongst the polypropylene oxide domains and gel gets formed. Hydroxyl groups of the copolymer become more accessible due to hydration[46]. Thus, desolvation caused by increase in temperature and subsequently micellization results in formation of a more closely packed viscous gel [47] Various combinations of poloxamers and other mucoadhesive polymers like polycarbophil and polyethylene oxide have been found to be more advantageous since their combination tends to reduce the gelation temperature of poloxamer. This can help in making a polymer that can gel at the temperature observed in the nasal mucosa[5]. Poloxamers are also known as Pluronics. Pluronics have also been chemically combined with poly(acrylic acid)s like Dihydroxyphenylalanine (DOPA)[31] to produce systems with enhanced adhesion and retention in the nasal cavity.

One of the most promising poloxamer is Poloxamer 407 (Pluronic F127) because of its low toxicity, high solubility, bioadhesion characteristics, and acceptability as drug delivery vehicle [48]. Some of the other investigated combination of poloxamers are polymer pluronic PF127 along with benzalkonium chloride which helped decreased the gelation onset temperature. This combination was prepared for the nasal delivery of Vitamin B12[49]. In another study, poloxamer 407 was combined with a mucoadhesive polymer or polyethylene glycol. The combination allowed the manipulation of the temperature at which the conversion of sol to gel would take place as well as decreasing and increasing the in vitro release of the drug respectively[50]. But work still needs to be done on reducing irritability which is one of the major limitations of these formulations[51]. Controlled release nasal formulations of propranolol have been made using a combination of poloxamers and other mucoadhesive polymers for such as Carbopol 934P. By controlling the release of the drug and by increasing its residence time in the nasal cavity, there was a significant increase in bioavailability of the drug[52]. Poloxamer properties are said to be affected by addition of various additives. Concentration can greatly affect the thermodynamic properties of poloxamer. Water soluble additives also affect the thermodynamic properties. The range at which gelation occurs increases with polymer concentration, whereas addition of sorbitol and PEG 15000 narrows the gel range. Significant enthalpy change occurs in gels containing sorbitol and PEG, indicative of interactions with the polymer during the phase transitions. As the concentration of the polymer increases, the aqueous gels display non-Newtonian characteristics. The hydrophobic interaction of Benzalkonium Chloride and pluronic produces a gel with higher viscosity. Addition of PEG 15000 helps achieve desired gelation characteristics for increased drug loading and use of desired formulation additives [41]. Poloxamers at low concentrations, when dispersed in liquid exist individually as monomolecular micelles. As the concentration of the pluronic in the system increases, it forms multi-molecular aggregates[53]. Different aggregate forms of poloxamers are seen depending on the molecular weight, solvent composition, and temperature[54]. Micellar behavior changes with changes in solvent composition and temperature. Various salts and additives like surfactants, polymers, cosolvents have marked effect on the micellar properties, clouding and solubilization characteristics of pluronic solutions[55]. Salts in the

order of Na_3PO_4 > Na_2SO_4 > NaCl have been found to lower the critical micellar temperature significantly[56]. Pluronic block copolymers are amongst the most potent drug targeting systems. Recent research on pluronics has generated new findings indicating immuno-modulation and cytotoxicity-promoting properties of Poloxamer 407 revealing significant pharmacological interest. Human trials are in progress based on these results [57]. Along with these new findings and favourable properties, poloxamers have generated immense interest as one of the most promising novel mucoadhesive drug delivery systems. Table 3 shows most of the published studies involving nasal drug delivery with this polymer.

Mucoadhesive Polymer	**Dosage Formulation**	**Active Ingredient**	**Reference**
Poloxamer 188 or 407	Nanovescicles	olanzapine	Salama HA et al[58]
Poloxamer-Chitosan	Gel	(32)P-siRNA dendriplexes	Perez AP et al[59]
poloxamer 407/ hydroxypropyl-β-cyclodextrin/chitosan	Gel	fexofenadine hydrochloride	Cho HJ et al[47]
chitosan-poloxamer 188	Spray	fentanyl	Fisher A et al[60]
PLGA: Pluronic F68	Nanoparticles	plasmid DNA	Csaba N et al[61]
Pluronic F127 (PF127)/ Carbopol 934P	Gel	sumatriptan	Majithiya RJ et al[62]
poloxamer 407/Polyethylene glycol	Liquid	metoclopramide hydrochloride	Zaki NM et al[63]
Poloxamer 407 /PEG 4000	Liquid	Radix Bupleuri	Chen E et al[64]
Poloxamer 407	Liquid	tetracosactide	Wüthrich P et al[65]
Pluronic F127	Microspheres	Bordetella bronchiseptica multiple antigens containing dermonecrotoxin	Kang ML et al[66]
Pluronic F127 (F127)/ chitosan	Liquid	tetanus toxoid	Westerink MA et al[67]
Pluronic PF 127	Gel	Vitamin B(12)	Pisal SS et al[68]
poloxamer 188	Liquid	isosorbide dinitrate	Na L et al[69]

Table 3. Application of Poloxamer (pluronics) in formulations for nasal drug delivery

6. Future prospects

Although several novel strategies are currently used for nasal drug delivery using bio-and muco-adhesion strategies, the potential exists to improve these methods using other strategies such as nanoparticles, bacterial adhesion, altered amino acid sequence, and antibody mechanism. A graphic representation of these methods is shown in Figure 3. Each of these methods is discussed below.

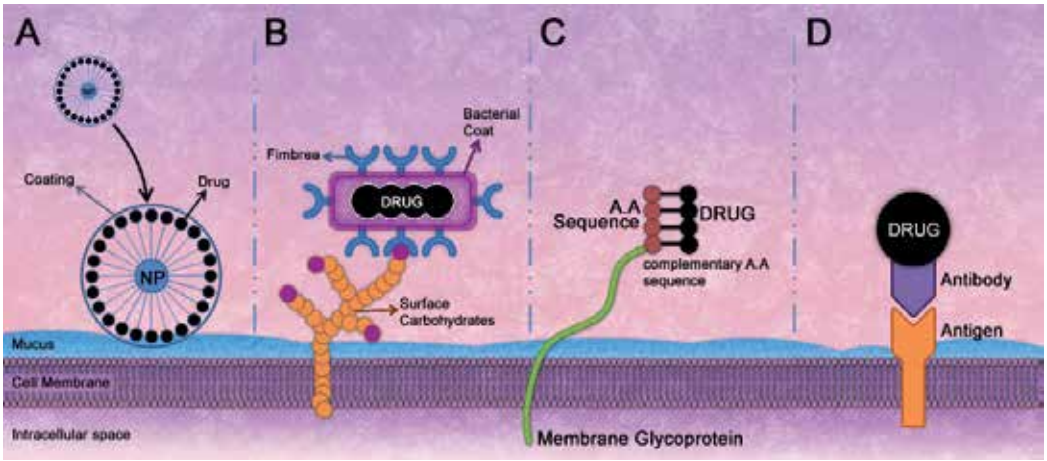

Figure 3. Potential future novel strategies for muco-/bio-adhesive drug delivery using Mucoadhesive Nanoparticles (A), Bacterial Adhesion (B), Altered Amino Acid Sequence (C) and Antibody mechanism (D).

7. Mucoadhesive nanoparticles

Nanoparticles generally vary in size from 10-1000nm. Biodegradable nanoparticles have been used frequently as drug delivery vehicles due to its better encapsulation efficiency, control release and less toxic properties[70]. They offer enhanced biocompatibility, superior drug/vaccine encapsulation, and convenient release profiles for a number of drugs, vaccines and biomolecules to be used in a variety of applications in the field of medicine[71]. The average pore size of viscoelastic mucus is around 150 ± 50 nm. Thus, formulations of mucoadhesive nanopolymers can lead to effective drug delivery to the target site. Commonly used materials for formulating nanoparticles are poly(lactide-co-glycolide) (PLGA) and Pluronics [55]. PEG coatings have been widely used in the development of polymeric drug carriers, including particles composed of biodegradable polyesters and polyanhydrides. PEG coatings reduce aggregation and enhance the blood circulation times of biodegradable nanoparticles designed for drug delivery[72]. Nanoparticles up to 200 nm in diameter that are coated with a dense layer of non-mucoadhesive PEG polymers, including drug carriers composed entirely of Generally Regarded As Safe(GRAS) components, readily penetrate nasal mucus. The development of polymeric particles with improved sinus mucus penetration capability should encourage the commercial development of new generations of nanoparticle-based intranasal drug delivery systems [46].

8. Bacterial adhesion

Non-denatured bacterial cell envelopes, also known as bacterial ghosts, are produced as a result of plasmid-encoded lysis gene *E* of bacteriophage in gram-negative bacteria[73]. Due to its hydrophobic nature, gene *E* product integrates into the inner membrane, resulting in fusion of inner and outer membrane. This leads to the formation of a trans-membrane tunnel[74]. The generated trans-membrane tunnel ranges between 40-80 nm in diameter,

through which all cytoplasmic contents are expelled[75]. It is imperative to note that the process of Protein E-specific lysis does not result in physical or chemical denaturation of bacterial surface structures. The bacterial ghosts have been suggested to be a great alternative method for inactivated non-living whole-cell vaccination [76]. Depending on the site-directed sequences included in the fusion, a variety of foreign proteins can be expressed within or on the cell envelop of bacterial ghosts [77]. The advantage of using bacterial ghosts is, bacterial ghosts can be produced in large quantities, do not require the cold-chain-storage system, and are stable for a long time. Further, the size of the foreign protein insert can be very large (>600 amino acids) thus allowing the presence of multiple epitopes simultaneously[78].

The attachment of synthetic or natural macromolecules to mucus or epithelial surface is defined as bioadhesion. Bacteria are capable of adhering to the epithelium surface with aid of fimbriae, which are long, lectin-like proteins found on the surface of many bacterial strains[79]. There is a correlation between the pathogenicity of bacteria and the presence of fimbriae, thus, the adhesion of bacteria to epithelial surfaces can be used as an efficient method of efficient drug-delivery[80].

Bacterial ghosts have been shown to display bio-recognitive abilities which allow their attachment to different surfaces of numerous body tissues depending on the species chosen[81]. Many of these bacterial ghosts are able to bind to surfaces due to the presence of long fimbriae which facilitate the penetration of the mucus covering epithelial tissues[65]. Thus, as a result of the properties of fimbriae a bioadhesive drug delivery system has been developed by using the ghost bacteria with a therapeutic agent coupled to *E. coli* K99 fimbriae[62]. This strategy may be applied to nasal drug delivery with the intention of reducing mucociliary clearance.

9. Altered amino acid sequence and antibodies

Certain amino acid sequences can be used to promote binding of drug molecules to specific cell surface glycoproteins due to the amino acids having complementary sequences present to these glycoproteins[82]. In certain disease conditions the sequence of glycoproteins is altered. This altered state can be used as a target by complementary amino acid sequences by attaching them to a drug delivery device [66]. Antibodies can be produced against selected molecules present on mucosal surfaces. Due to their high specificity, antibodies can be a rational choice as a polymeric ligand for designing site-specific mucoadhesives. This approach can be useful for targeting drugs to tumour tissues[66] or even normal cells.

10. Conclusion

There is no question that the nasal route has a great potential for systemic drug delivery. The physiology of the nasal cavity creates a variety of opportunities for drug companies to develop local and systemic drugs. As nose- to- brain delivery makes it possible to by-pass the blood-brain-barrier for certain drugs; administration of drugs via this route for treatment of

neurological diseases presents exciting opportunities. Despite the advantages of nasal drug delivery, the absorption and permeability of polar drugs through the nasal mucosa remains a challenge. The mucociliary clearance system compounds the problem by limiting how long the drug stays in the nasal cavity for absorption to take place. Hence several strategies have been developed to enable the drug molecules to attach onto the mucus or epithelial layer, thus preventing them from being cleared from the nasal cavity. The application of lectins, thiomers, alginate poly-ethylene glycol acrylate and poloxamers were discussed in this chapter. These polymers are not the only polymers used for nasal delivery. However, they are among the least reviewed polymers for systemic drug delivery via the nasal route. Other bioadhesive strategies including nanoparticles, bacterial adhesion, altered sequence and antibody strategies can further improve the bioavailability of polar drug molecules delivered via the nasal route. However, a lot of work remains to be done in this area.

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Chapter 12

Amphiphilic Cyclodextrins, Synthesis, Utilities and Application of Molecular Modeling in Their Design

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Additional information is available at the end of the chapter

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1. Introduction

Drug delivery systems that traditionally are used by the patient do not respond the drug delivery's needs of the world. According to the large number of the hydrophilic and hydrophobic drugs, design and synthesis of new drug delivery system seems to be necessary. With the traditional drug delivery systems practically there is no control over the time, location and rate of drug release, in addition to the drug concentration was fluctuated in the blood frequently and may even go beyond the therapeutic dose and less effective and cause more side effects. With the new drug delivery systems that called controlled released drug delivery system, we will be able to control and determine the rate, time and location of drug release. CDs are potential candidate for such a role, because of their ability to change physicochemical and biological properties of guest molecules through the formation of inclusion complexes (Uekama et al,1998). The most common pharmaceutical application of cyclodextrins is to increase the stability, solubility and bioavailibity of drug molecules and other pharmacological benefits,such as the reduction of unwanted side effect (Hedges, 1998).

Cyclodextrins (CD) are macrocyclic oligosaccharides composed of D-(+)-glycopyranosyl units linked α (1→4). CDs are classified as α-,β- and γ-CD according to the number of glucose units: six, seven and eight, respectively. Cyclodextrins have a truncated cone shape with a hydrophilic exterior and a hydrophobic cavity. A guest molecule of appropriaty size and shape is incorporated into hydrophpbic cavity in aqueous media (Szejtli, 1998).

However, the potential use of CDs in biological system needs amphiphilic properties because natural CDs have relatively low solubility both in water and organic solvents, thus limits their uses in pharmaceutical formulations. Amphiphilic or ionizable cyclodextrins can

modify the rate or time of drug release and bind to the surface membrane of cells, that may be used for the enhancement of drug absorption across biological barriers.

Amphiphilic cyclodextrins can be obtained by the introduction of lipophilic groups at primary and or secondary face of the CD. Amphiphilic CDs have been shown to form monolayers at the air-water interface (Parot-Lopez, 1992; Greenhall et al, 1995) and micelles in water (Auze´ly-Velty et al, 2000). Different self-organized amphiphilic CDs, such as nanospheres (Skiba et al, 1996), solid-lipid nanoparticles (Dubes, 2003) , liquid crystals (Ling et al, 1993) and vesicles (Ravoo & Darcy, 2000) were prepared with varying length of hydrophobic chains for their promising properties for farmaceutical applications.

Hydrophilic-hydrpphobic balance, molecular shape and solvation have all been enunciated as important criteria for formation of distinct lyotropic assemblies (Israelachvilli, 1985; Fuhrhop & Koning, 1994).

A particulary interesting example of self-assembly of amphiphilic CDs in water is bilayer vesicles. CDs vesicles consist of bilayers of CDs, in which the hydrophobic ″tails″ are directed inward and the hydrophilic macrocycle″head groups″ are facing water, thereby enclosing an aqueous interior. Recently, vesicles composed entirely of nonionic, anionic, and cationic amphiphilic CDs was described (Falvey et al, 2005).

Nano capsules and nanospheres were prepared using amphiphilic β-and γ-CDs modified on the secondary face by nanoprecipitation and emulsion/solvent evaporated techniques avoiding the use of additional surfactant (Woussidjewe et al, 1996).

The first amphiphilic cyclodextrin were synthesized in 1986 by Kwabata et al. The primary OH-groups of β-CD were made lipophilic with alkyl sulfunyl groups with various length. This amphiphilic CD could form monolayer at the air-water interface (Kawabata et al, 1986).

2. Synthesis of amphiphilic cyclodextrins

β-CDs in synthesis of amphiphilic cyclodextrins was used more than α- and γ CDs. Common synthetic path to amphiphilic cyclodextrins was shown in Fig. 1.

2.1. Alkylated, arylated and lipid –Conjugated CDs

Synthesis of alkylated α- and β-CDs and their treatment at air –water interface, have been described by Jurczak et al (Wazynska et al, 2000). Silyl-protected α- or β-CDs was alkylated then desilated to achievement amphiphilic per-(2,3-di-O-alkyl)-CDs (5) (Fig. 2).

Compound (10) which contain amphiphilic chains, was synthesized from per-amino-β-CD using peptide chemistry (Imamura et al, 2002) (Fig. 3). Reason of suitable complexation of anilinonaphthalene sulfonic acid can ralativ to presence of Adipic-glucamine chains that enhance extension of the cavity in this structure. γ-CDs derivatives of this family, can complex with two molecules of anthraquinone-2-sulfonate (Ling & Darcy, 1993) (Fig. 4).

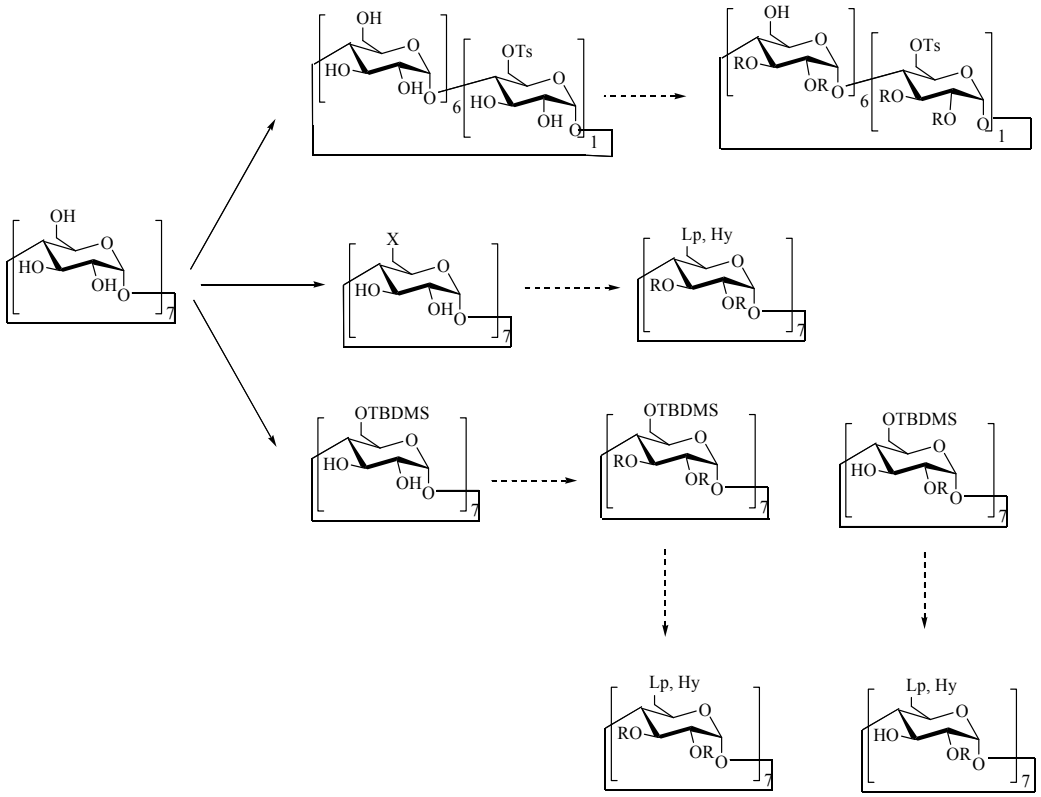


Figure 1. Common synthetic path to amphiphilic cyclodextrins

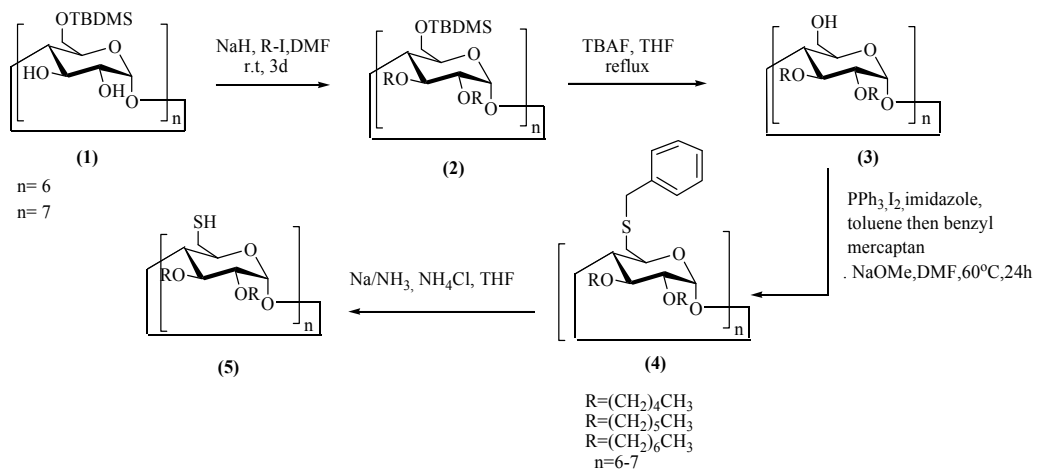


Figure 2. Synthesis of alkylated cyclodextrins

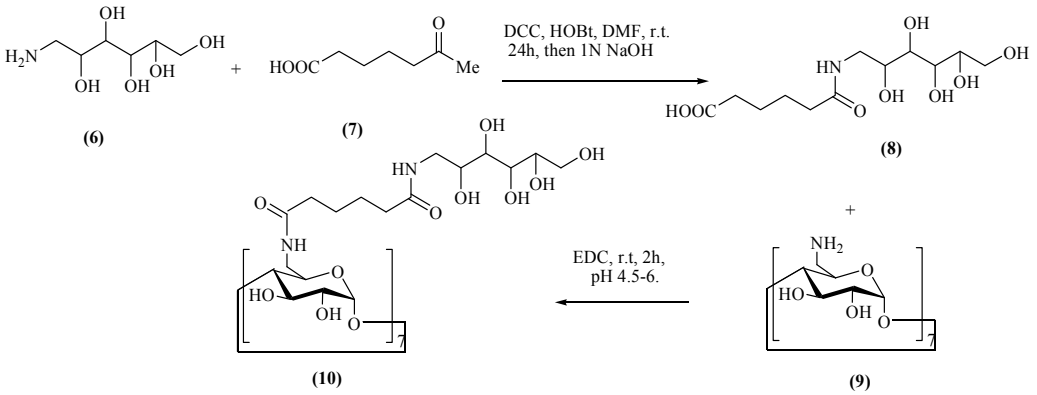
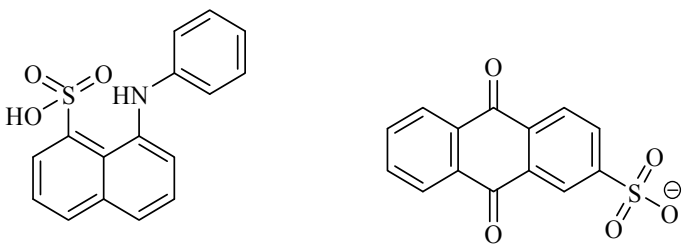


Figure 3. Peptide chemistry was used for synthesis of amphiphilic cyclodextrins



8-Anilino-1-naphtalenesulfonic acid

(11)

Anthraquinone-2-sulfonate

(12)

Figure 4. Structures of two guest molecules for complxation with compound (10)

Wu and coworkers in 2010 reported the synthesis of new amphiphilic biodegradable β-cyclodextrin/poly (L-leucine)(β-CD-PLLA) copolymer by ring-opening polymerization of N-carboxy-L-alanine anhydride in N,N-dimethylformamide(DMF) initiated by mono-6-amino-β-cyclodextrin (Zhang et al, 2010). These compound could self- assemble into nano-micelles in water and could be expected to find application in drug delivery systems (Fig. 5).

A novel thiolated carboxymethyl chitosan-g-β-cyclodextrin (CMC-g- β-CD) drug delivery carrier was synthesized by Gong et al (Prabaharan & Gong, 2008).

Thiolated CMC-g- β-CD was prepared using two steps. First ,carboxymethyl- β-CD (CM β-CD) was grafted onto carboxymethyl chitosan (CMC) using water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as the condensing agents. Next, the resultant product was further grafted with cycteine methyl ester hydrochloride (CMEH) (Fig. 6).

The drug release showed that thiolated CMC-g- β-CD tablets provided a slower release of the entrapped hydrophobic model drug, ketoprofen, than the chitosan control, and the release behavior was influenced by the amounts of thiol groups present on the polymer chains. These results suggest that thiolated CMC-g- β-CD with improved mucoadhesive

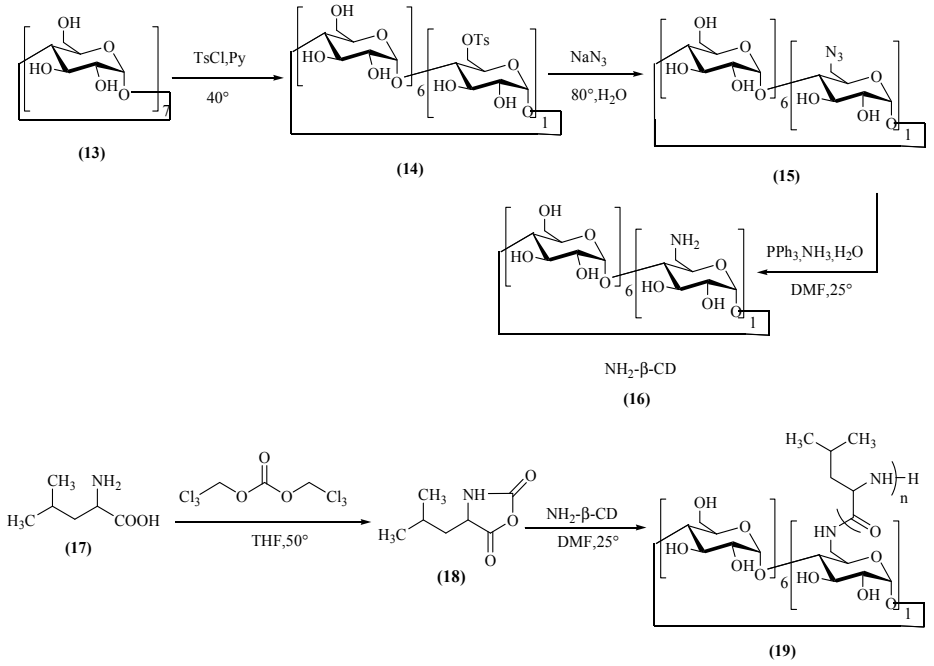


Figure 5. Synthetic pathway to amphiphilic biodegradable β-cyclodextrin/poly (L-leucine)(β-CD-PLLA) copolymer

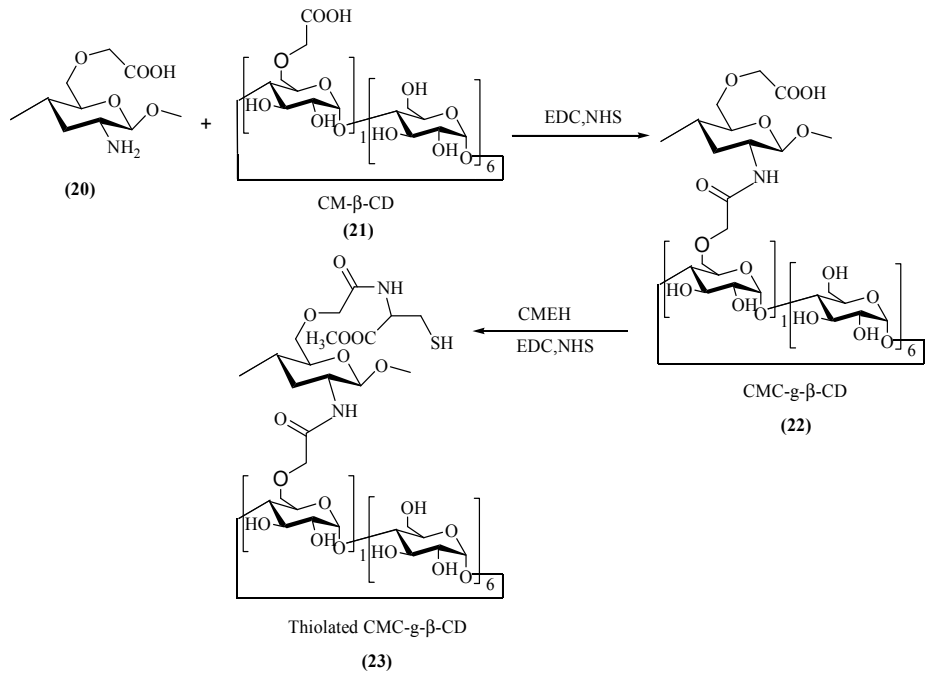


Figure 6. Preparation of thiolated CMC-g- β-CD

properties may potentially become an effective hydrophobic drug delivery system with controlled drug release capability.

Chitosan-cyclodextrin nanosphere were prepared by in situ formation through Michael addition between N-maleated chitosan (NMC) and per-6-thio- β-cyclodextrin sodium salt in an aqueous medium (Wang et al, 2011). This facil preparation method did not involve any organic solvent and surfactant. Through adjusting the preparation conditions, the nanosphere with a relatively narrow size analyzer. Doxorubicin hydrochloride (DOX-HCl), a water soluble anticancer drug, was loaded in the nanosphere with a high encapsulation efficiency (Fig. 7).

Chloesteryl derivatives of CDs were mostly investigated by Pilard and coworkers. Synthetic pathway of one of them was shown below (Auze´ly-Velty et al, 1999) (Fig. 8).

In 2007, Mallet and coworkers synthesized new derivatives of amphiphilic CDs (Collat et al, 2007). These compound were obtained from reaction between carboxylic acid that derivative of chlosterol with di-amino CDs in presence of DCC and 1-hydroxy benzo tri azol(HoBt). These compounds can act similar to biological membrans (Fig. 9).

2.2. Oligo(ethylene oxide) amphiphilic CDs

The first Amphiphilic CD to form bilayer vesicles were reported by Ravoo and Darcy in 2000 (Ravoo & Darcy, 2000; Mazzaglia et al, 2001; Falvey et al, 2005). Synthesis of them initiated from per-6-bromo and at the result of nucleophilic substituation with the sodium or potassium salt of alkyl thiols, per-alkyl thio CDs were prepared then hydroxyl groups that bind to C-2 of these compounds reacted with an excess of ethylene carbonat and finally average of two units of ethylene glycol were located in this position (C-2 of the CD) (Ravoo & Darcy, 2000; Mazzaglia et al, 2001) (Fig. 10).

This reaction was performed with α- and γ-CD subsequently (Falvey et al, 2005). In these derivatives, the cavity size and hydrophilic of the cyclodextrin headgroup increase. Oligo(ethylene oxide) amphiphiles can form bilayer, vesicoles, and nanoparticles.

2.3. Cationic CD amphiphiles

Donohue and coworkers reported the synthesis of CDs in which hydroxyl groups of oligo(ethylene oxide) were substitude with amine groups and their hydrochloride salts were used in gene delivery studies (Donohue et al, 2002) (Fig. 11).

2.4. Anionic CD amphiphiles

With alkylation of CDs by Declercq and coworkers (Leydet et al, 1998), achievement to versatile groups and so new structures were possible. For example Kraus et al with oxidation by osmium tetroxide in presence of 4-methyl morpholine prepared novel alcoholic stractures of mentioned allylic derivatives (Kraus et al, 2001). They converted the resulting diastreoisomeric diols to carboxylated CDs with oxidation (Fig. 12).

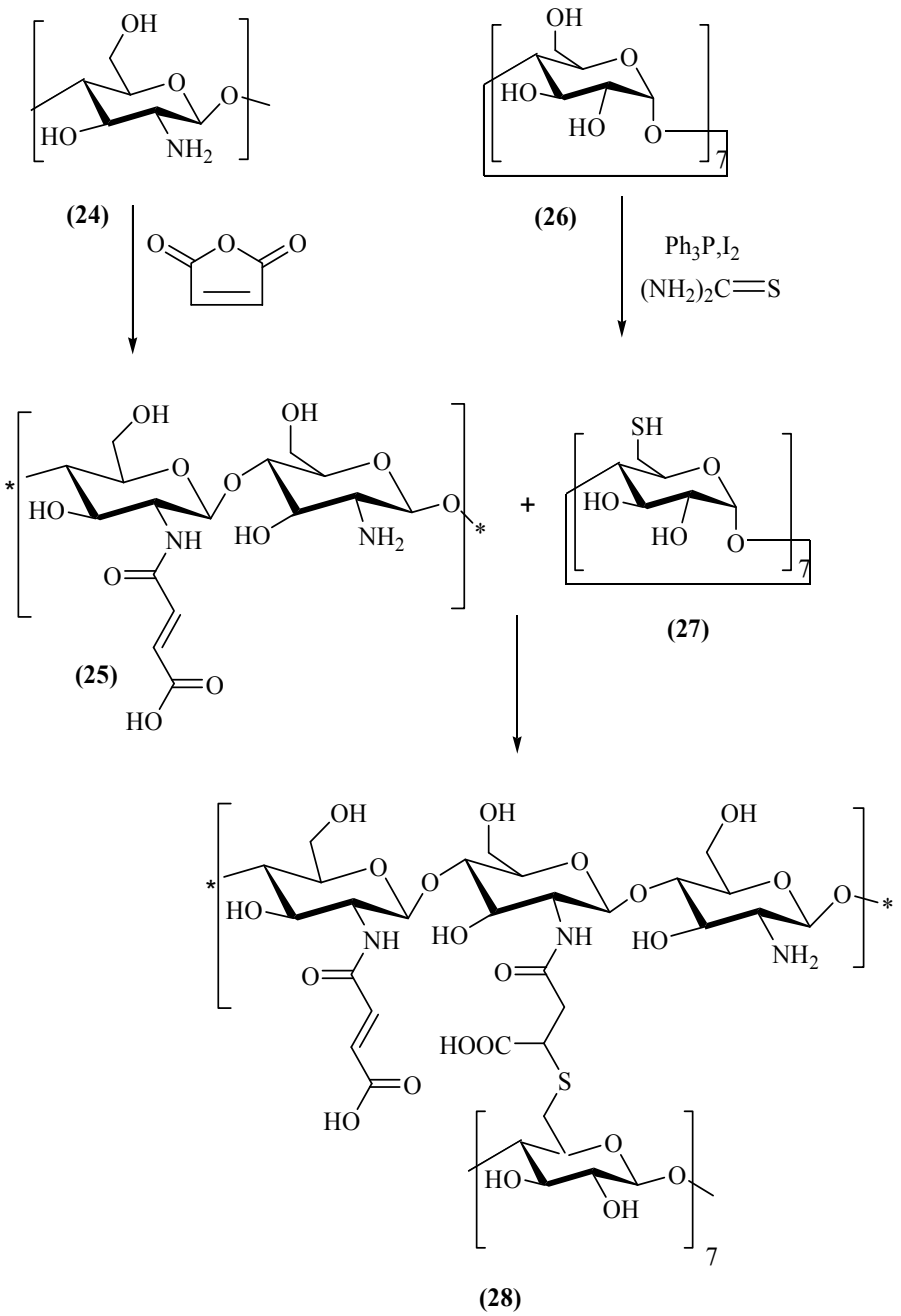


Figure 7. Synthetic path to Chitosan-cyclodextrin nanosphere

The first sulfated amphiphilic CD synthesized by Dubes (Dubes et al, 2001, 2003). They produced compound (48) via esterification of silyl-protected CDs (45) with hexanoic anhydride at position 2 and 3. After removal of the silyl groups, the primary hydroxyl groups were sulfated by SO_3.pyridine complex (Fig. 13).

2.5. Flurinated CD amphiphiles

Granjger and coworkers prepared the per tri fluromethyl thio-β-CD derivative (Granger et al, 2000), which formed monolayer at the air-water interface despite the short hydrophobic chains that applicable in oxygen delivery. Mono- di and per fluoro alkyl thio-CDs (Péroche et al, 2003, 2005) were made subsequently and demonstrated to self-organise into nanosphere in aqueous media, unlike their analogous alkylated derivatives that formed flat particle under same condition (Fig. 14).

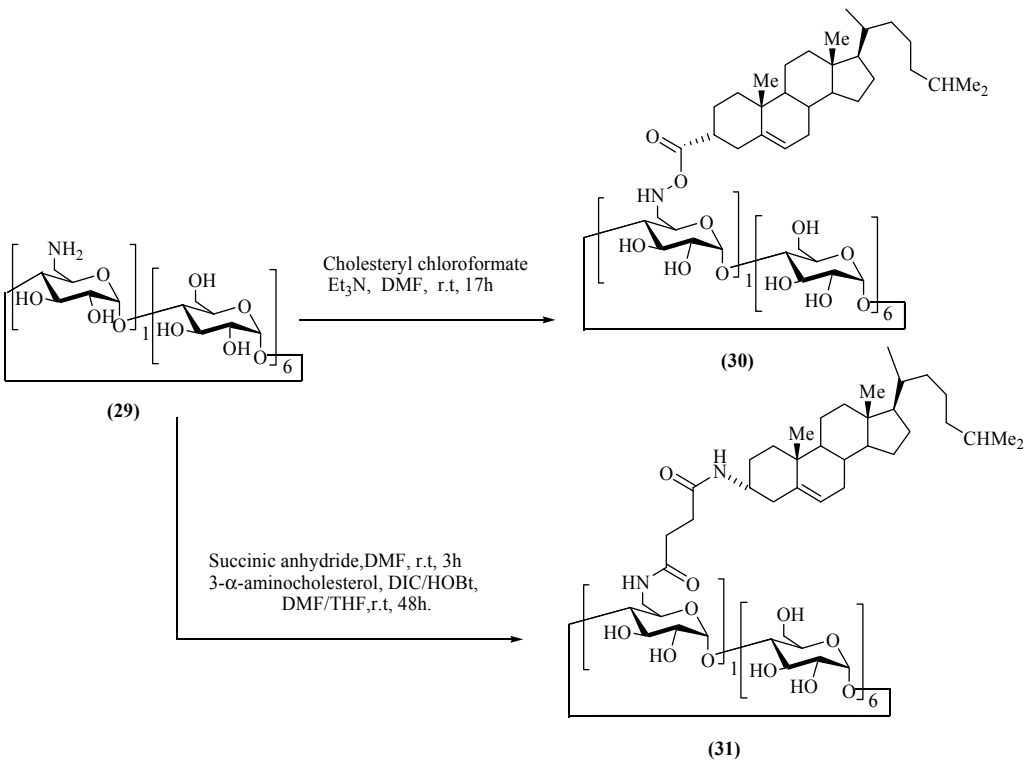


Figure 8. Synthesis of one of chlosteryl derivatives

The synthesis of a γ-CD 6-per fluroalkyl ester was reported by Lim et al (Lim et al, 2006). CD was easily reacted with an excess heptafluoro butanoic acid and finally, compounds were formed that complexes with many substances for example surfactants.

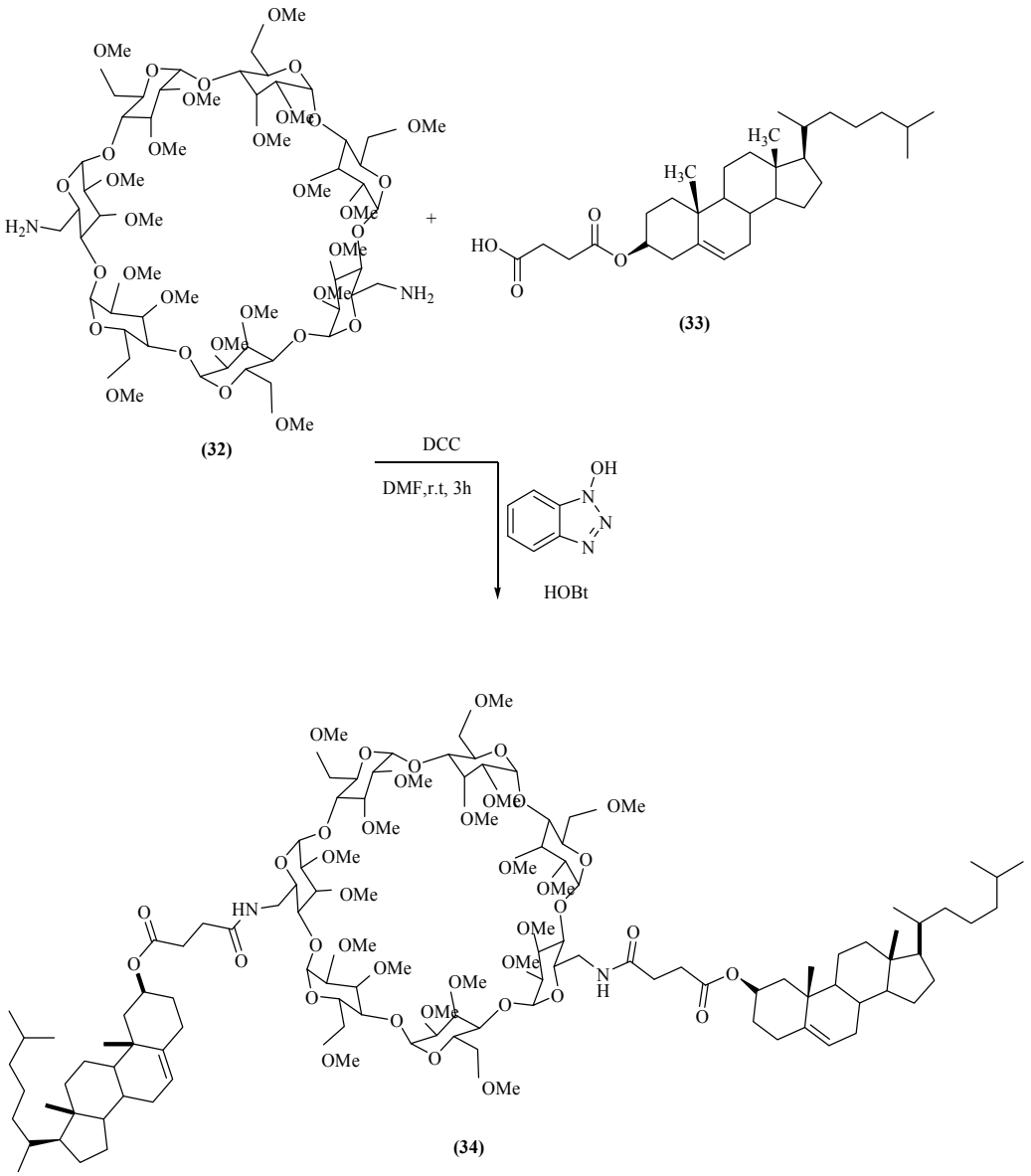


Figure 9. Synthesis of amphiphilic cyclodextrin that can act as similar to biological membrans

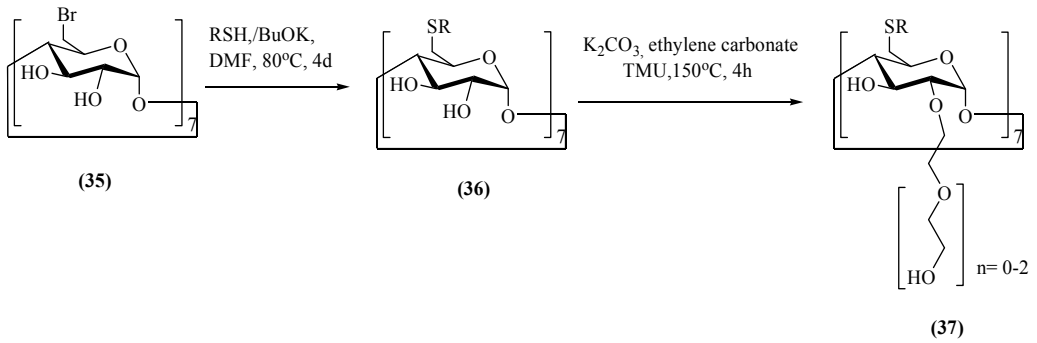


Figure 10. Synthesis of the first oligo(ethylene oxide) amphiphilic cyclodextrin

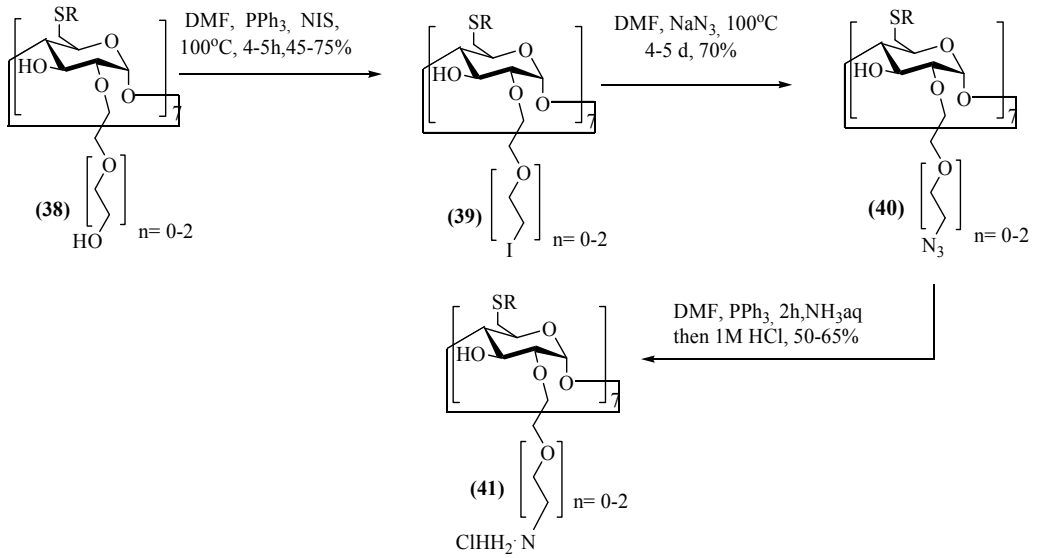


Figure 11. One of synthetic path to cationic cyclodextrin amphiphiles

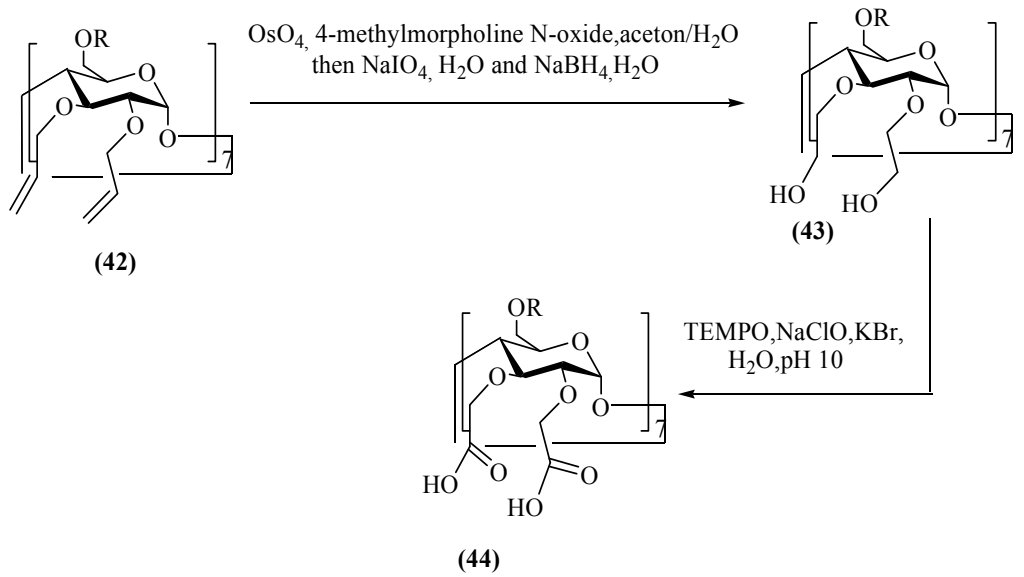


Figure 12. Osmium tetroxide beside to 4-methyl morpholine were used for synthesis of anionic cyclodextrin amphiphiles

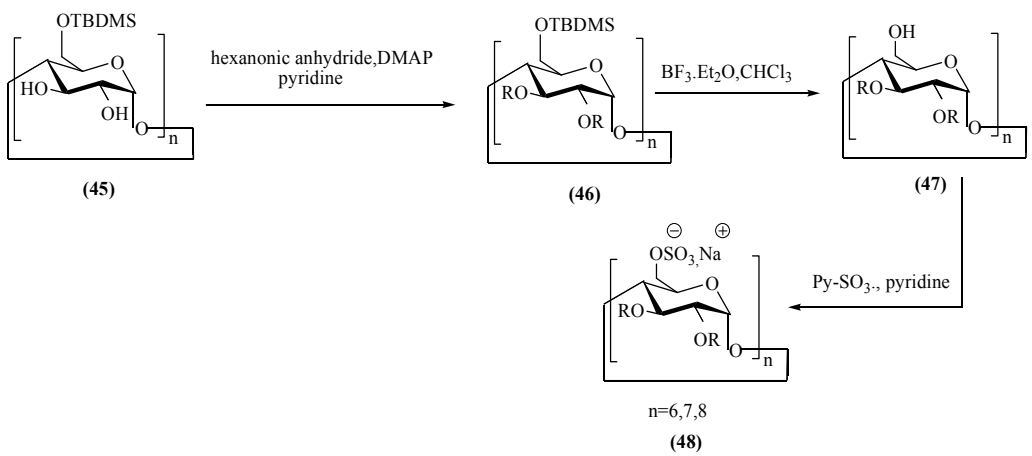


Figure 13. Synthesis of the first sulfated amphiphilic cyclodextrin

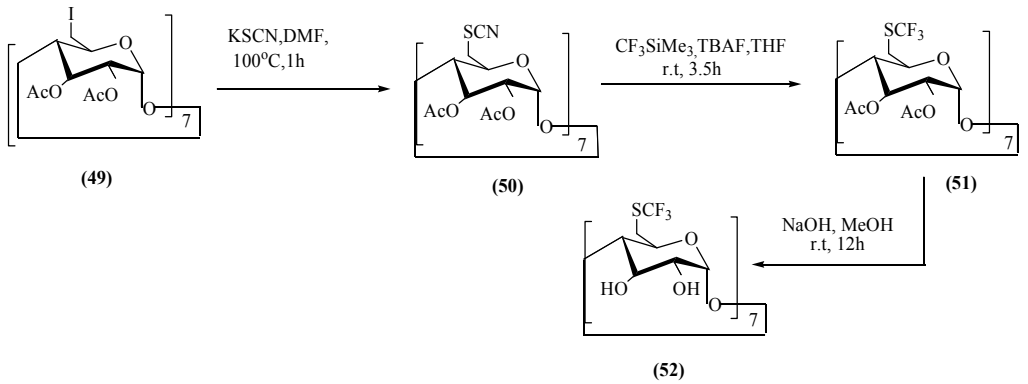


Figure 14. Synthesis of the per tri fluromethyl thio-β-CD derivative by Granger et al

2.6. Glycosylated CD amphiphilic

Sallas and coworkers prepared CDs esterified on the C-2 and C-3 and glycosylated on the C-6 position of glucose units (Sallas et al, 2004) (Fig. 15).

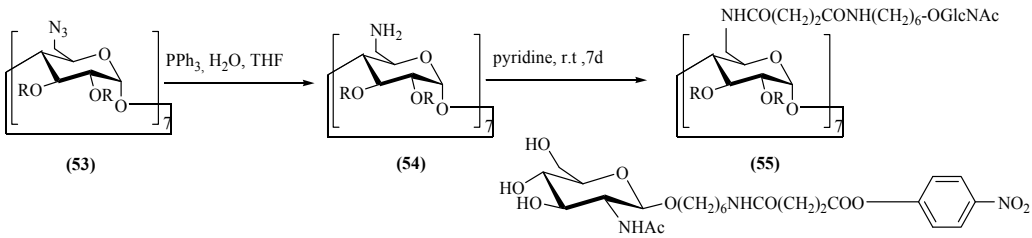


Figure 15. Preparation of glycosylated cyclodextrin amphiphilic

Glycosylation was occurred when a glucosamine having either a terminal amino group or an active ester group was reacted with amino CDs (Fig. 16).

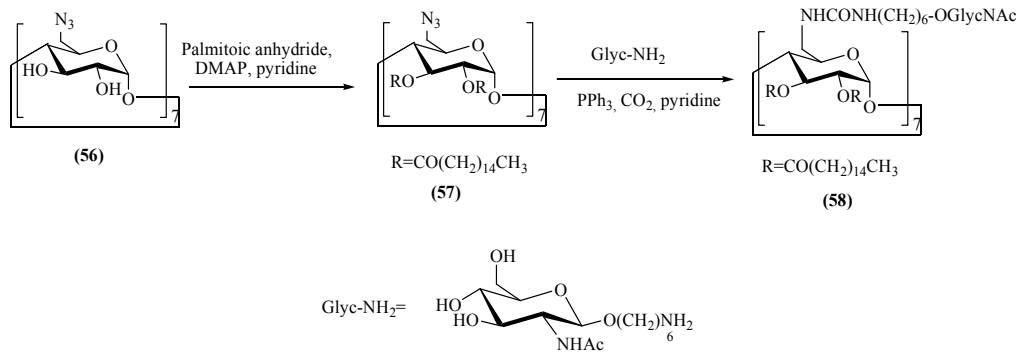


Figure 16. Glycosylation of amino cyclodextrins

A Staudinger reaction in pyridine and in presence of carbon dioxide led to the glycosylated CDs that having a urea function.

Other type of glycosylated amphiphilic CDs have been prepared from oligo(ethylene oxide) CD amphiphile by Mazzaglia et al (Mazzaglia et al, 2004). Despite the existence of seven glycosyl groups, these compound aggregrated in water into vesicles (Fig. 17).

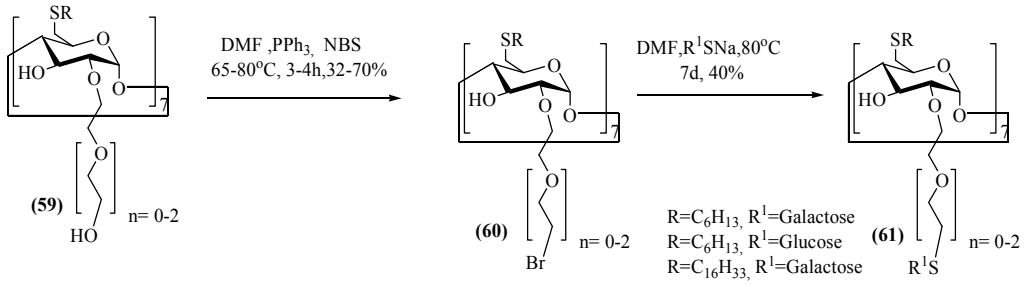


Figure 17. Glycosylation of oligo(ethylene oxide) cyclodextrin

Photochemical addition of sugar thiol to allylic groups on the CDs was another pathway for glycosylated CDs (Fulton & Stoddart, 2001). The hydroxyl groups can be protected with silyl groups, then allylated the hydroxyl groups (on the position 2). This pathway has been further developed to create a variety of glycosylated amphiphilic CD such as (66) (Nicholas, 2005) (Fig. 18).

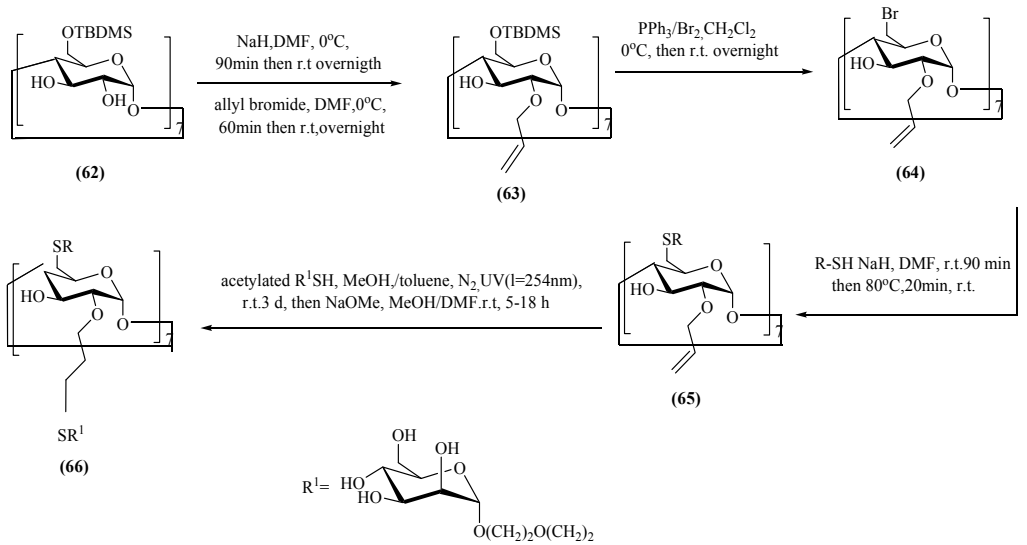


Figure 18. Photochemical addition, another path for synthesis of glycosylated cyclodextrin

3. Molecular modeling in designing of host molecules

Prediction of host-geust binding affinity could be made by calculating the binding energy. It could be down in molecular modeling software such as HyperChem (http://www.hyper.com/). In such software we can design and optimize the geometry of host and gust molecule separately and then by introducing the guest molecule in the cavity

of host molecule, the geometry of the combined structure is optimized. Then the total energy of the host, guest and host-guest structures are separately calculated by single point command under one of the molecular calculation methods: molecular mechanics, semi-empirical, *ab-initio* or DFT. By subtracting the single point energy of host-guest complex from sum of the host and guest energy, we can determine the binding energy. In this procedure there is some incompetency: Construction of the best geometry depends on oreintention loaded into the cavity of the host molecule. For example in fig.18 we can see two opposite and acceptable oreientaions of the dopamine in the cavity of the Heptakis [6-*O*-(*N*-acetyl-L-Valyl)]-*α*-cyclodextrin in the screen of the hyperchem software (Fig. 19). For this case, binding energy of both models must be separately calculated. The orientation with less energy is the preferable binding model for analyzing.

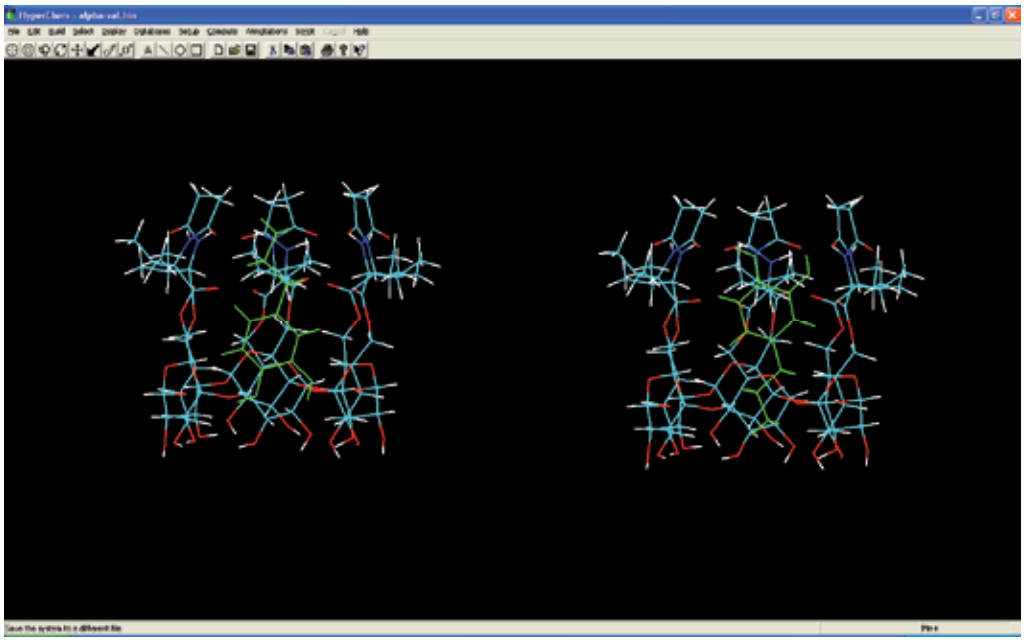


Figure 19. Stick view of the two binding model of dopamine loaded in the cavity of Heptakis [6-*O*-(*N*-acetyl-L-Valyl)]-*α*-cyclodextrin in the screen of the hyperchem software.

In docking software, there is not such a mentioned limitation. In this software we can search all of the possible oreientation and rise to further acceptable binding conformers. This kind of molecular modeling operates based on the two combined calculation methods: local search and genetic algorithm known as Lamarckian Genetic Algorithm (LGA) (Morris et al, 1998). In this procedure the best binding conformation of the gust molecule based on the intermolecular interactions such as hydrogen binding, electrostatic force and … is achieved.

AutoDockTools, also known as ADT, is an easy-to-use graphical front-end to the automated docking software packages AutoDock and AutoGrid (Phyton, 1999). ADT provides menus to:

- set up a ligand (the 'moving' molecule to be docked) and write out a PDBQ file;
- set up a macromolecule (the 'fixed' molecule being docked to) and output a PDBQS file with solvation parameters;
- assign Kollman United Atom charged to a protein or peptide, or DNA or RNA;
- compute Gasteiger PEOE partial charges for small molecules or cofactors;
- add polar hydrogens, or 'merge' non-polar hydrogens;
- set up a grid box for atomic affinity grid maps and electrostatic potential maps around the target macromolecule (the box is a cubic space with definable center and dimension length);
- set various docking parameters;
- write GPF and DPF files (grid parameter and docking parameter files);
- launch AutoDock and AutoGrid calculations;
- read in DLG files (docking log files) and visualize AutoDock results;
- cluster and re-cluster docking results in various ways.

The ADT software is programmed for modeling of ligand-protein interactions. For small part of a protein (active site pocket) we set a restricted region as grid box, where docking operation performs in it. For host molecules with less than 4-5 KD, it could be defined so that all of the molecular structures located there (Fig. 20).

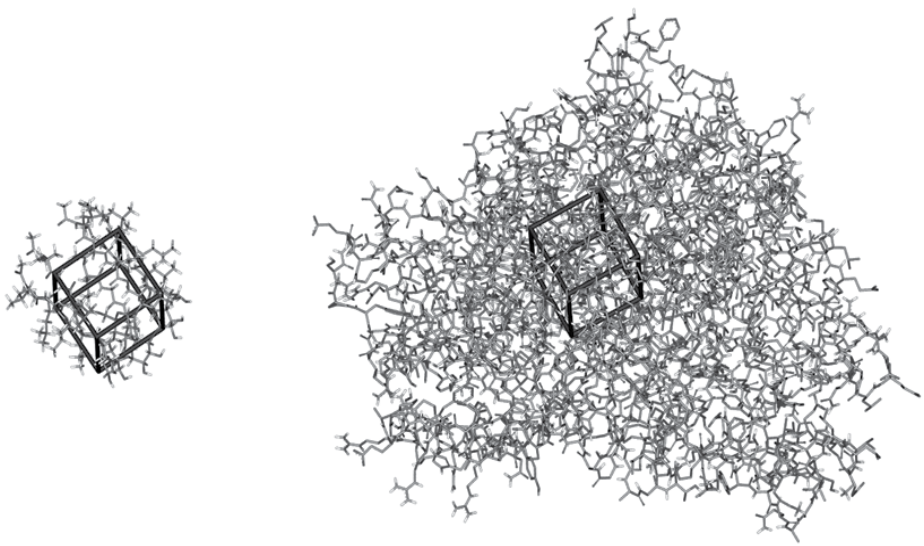


Figure 20. Comparison between the two host molecule grid boxes with the same size in ADT software: soybean 15-lipoxygenase enzyme (right) and a suggested host molecule with 2.6 KD molecular weight.

For reducing the formation of unfavorable binding conformer of gust molecules it is better to limit the size of the grid box around the internal space of the host molecule cavity. By doing the mentioned work, the external binding conformers will not originated by software and population of suitable conformers is increased.

In modeling project, for a series of homolog guest molecules, it ought to be the docking results compare with the experimental data (extraction content, binding affinity, phase transferring ability with considering of lipophilic factors such as logD, and …). This comparison help us to reach the best binding conformation and it able us to predict the new structure of host molecule for better scavenging results. It lead us about how structure modifications, such as grafting of electronegative atoms, increase or decrease of lipophilic properties by introducing of hydrophobic substituents, could be made. Recently we have shown the ability of the mentioned calculating methods for a new series of peptide - βcyclodextrins as suitable host molecules for phase transferring of glucosamine (Seyedi et al, 2010).

3.1. Amphiphilic peptide-cyclodextrins: Synthesis, designing and their utility as drug carrier: A novel experience

Among the amphiphilic cyclodextrin, amphiphilic peptide-cyclodextrins (APCs) is one of the new and interesting case for studying in drug delivery field. In the aforementioned molecules, hydrophobic residues are aligned above the CD ring *via* amid or ester bonds and the cylindrical structure shape is observed in which the outer part is hydrophobic (duo to hydrophobic amino acids) while the head (CD ring) possess the hydrophilic nature. The structure was stabilized by cross intermolecular hydrogen bonds of amide groups. This molecular structure has ability for introducing the polar molecules into its cavity and transfer of them across the lipophil phases (Fig. 21).

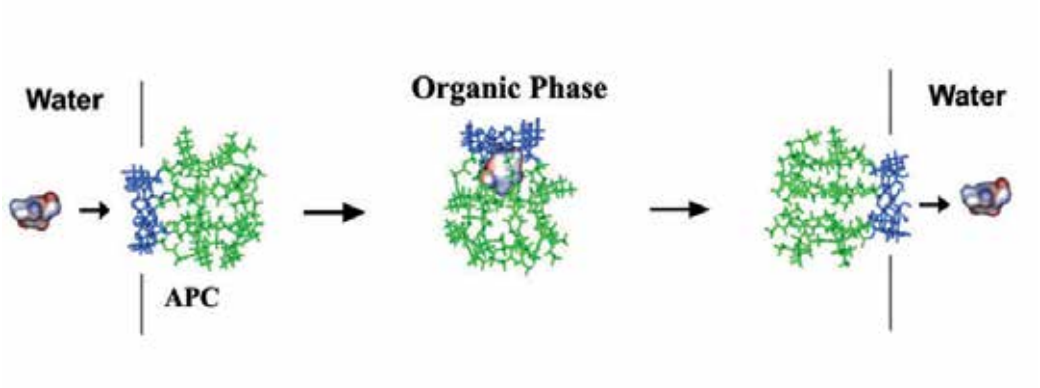


Figure 21. Transport of drug molecules across the organic phase by APC. The cyclodextrin ring and peptide moieties are distinguished by blue and green color respectively.

Our interested in design and synthesis of β-CD peptide derivatives emerges from the work of Imamura and co-workers in which preparation of amphiphilic compounds from per-amino-β-CD using peptide chemistry was reported. The desired amphiphilic structures have been produced from ester linkage between all the C-6 of β-CD and the carboxyl group of *N*-acetylated resides: H_2N-Leu-COOH, H_2N-Leu-Gly-COOH, H_2N-Leu-Gly-Leu-COOH and H_2N-Leu-Gly-Leu-Gly-COOH (Fig. 22).

In this molecular designing, L-leucine (Leu), the most lipophilic amino acid, was selected for extension of the cavity of *β*-CD and organizing an external lipophilic structure. Due to the inhibiting effect of pH on the amphiphilic property of the compounds, the *N*-acetyl form of the aforementioned residues was used. To prevent steric hindrance adjust to leucine, Gly was selected as the preferred connector of the two residues.

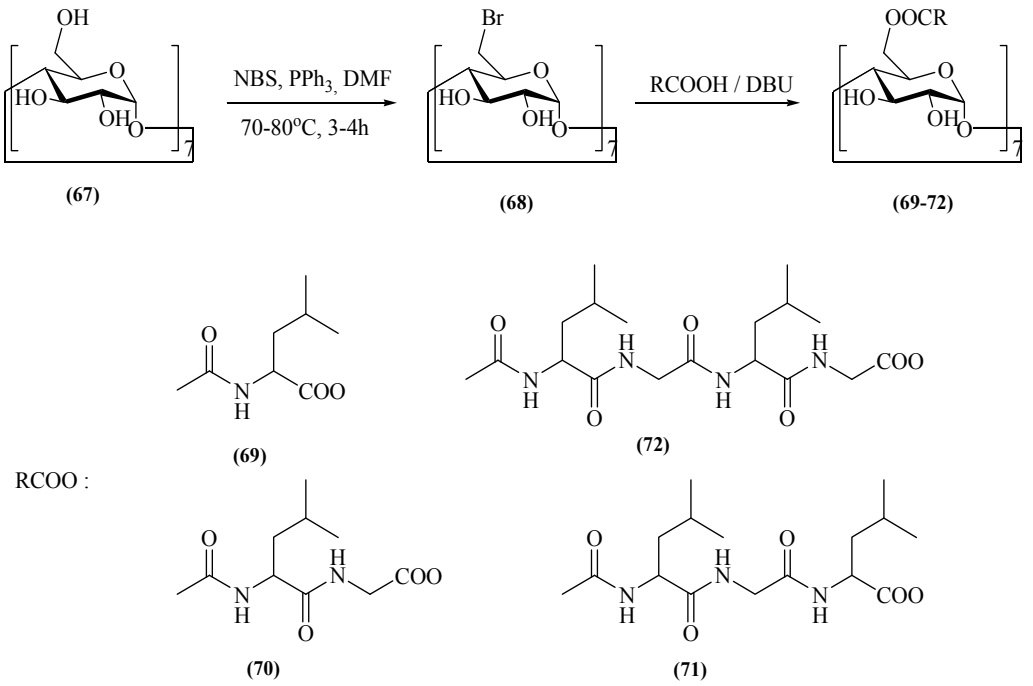


Figure 22. General procedure for the synthesis of amphiphilic peptide β-CDs (69-72).

Binding behavior was studied using glucosamine as a guest molecule. The relative binding affinity of 69-72 toward glucosamine was measured using a phase extraction method. the decrease in concentration of aqueous glucosamine (pH 7.4) after shaking for 12 h with a lipid phase (octanol) containing 69-72 and *β*-CD was determined and reported as extraction content (%E).

the equilibrium constant of extraction could be defined as the binding constant (K_b):

$$K_b = ([\text{host-Glu}])/([\text{Glu}][\text{host}] \quad (1)$$

$$\text{host} + \text{Glu} \rightleftarrows \text{host-Glu}$$

The observed glucosamine binding constants (K_b) of 69-72 are outlined in Table 2.

In the other experiment, phase transfer of glucosamine by 69-72 and *β*-CD was determined. In this work the tendency of each compound for glucosamine transferring between two aqueous phases, separated by octanol, was measured (Fig. 23). Two stirred aqueous phases (pH 7.4), in which one of them (primary phase) contained glucosamine (25 mM), were

connected through octanol, containing test compound (69-72 and β-CD; 5 mM), without any solvent diffusion. After 24 h the glucosamine concentration of the intact aqueous phase (secondary phase), was determined. The ratio of glucosamine concentration of the primary and secondary phases was recorded as phase transferring extent (%T).

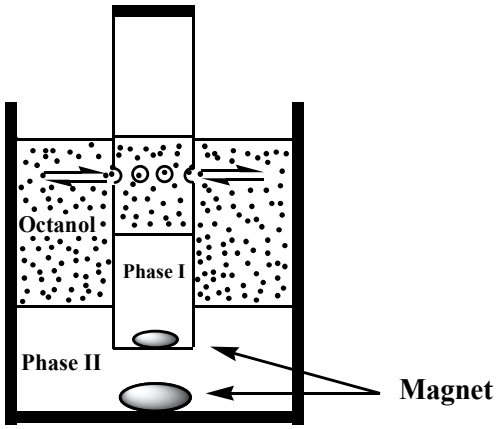


Figure 23. Schematic presentation of the apparatus used for phase transfer experiment

Case	%E	%T
69	24.4 ± 0.9	1.62 ± 0.3
70	23.8 ± 2.2	1.50 ± 0.2
71	28.6 ± 1.8	3.49 ± 0.3
72	26.3 ± 1.2	1.63 ± 0.4
CD	0.40 ± 0.2	0.74 ± 0.1
Ctr.	0.21 ± 0.1	0.43 ± 0.1

Table 1. Extraction and phase transferring percentage of glucosamine (%E and %T respectively) by compounds 69-72.

Among the tested compounds, a modest phase transfer was observed for 71, while the experiments with 69-70 and 72 showed worse results (Table 1). Phase transfer of glucosamine by β-CD was significantly lower.

To explore the origin of these effects, the octanol-water disturbtion coefficient (logD) of 69-72 was measured using shake flask method (Griffin et al, 1999). On the other hand by considering the logD values, the phase transfer results of 69-72 could be rationalized. It was concluded that more lipophilic host molecule (71: logD 1.57) has a higher capability for transferring glucosamine in determined period time.

To complete the study, the binding affinity of glucosamine toward 69-72 was calculated and reported as estimated binding free energy (ΔG_b). In this section, the 3D structures of 69-72 were modeled *via* grafting the desired residues on all primary hydroxyls of *β*-CD crystal structure followed by geometry optimization (PM3 methods). In the modeled molecules, residues are aligned above the *β*-CD ring and the cylindrical structure shape is observed in which the outer part is hydrophobic while the head (*β*-CD ring) possess the hydrophilic nature. The structure was stabilized by cross intermolecular hydrogen bonds of amide groups. The binding affinity was estimated in AuoDockTools software using autodock4.0 program (Python, 1999). 100 docked conformers of glucosamine were generated in ADT software for each of 69-72. The detailed assessment of all 100 docked models revealed as followings: 100% of docking results had nearly identical orientations in the *β*-CD ring with average ΔG^o_b of -5.81 kcal/mol for **69**; while for **70**, 68% of docking results had nearly identical orientations in the *β*-CD ring and 32% in the pocket formed by the residues with average ΔG_b of -6.13 and -6.25 kcal/mol respectively; for 71, 58% of docking results had nearly identical orientations in *β*-CD ring and 42% in the mentioned pocket with average ΔG_b of -5.62 and -5.45 kcal/mol respectively and finally for 72, 69% of docking results had nearly identical orientations in *β*-CD ring and 31% in the mentioned pocket with average ΔG_b of -6.14 and -6.08 kcal/mol respectively (table 2). Due to the orientation mentioned above, the docked conformers displayed hydrogen bonds with hydroxyl and amides of the sugars and residues in 69-72. Considering $\Delta G^o_b = -RTlnK_b$ and equation **(1)**, the high similarity between experimental and theoretical results (ΔG^o_b and ΔG_b respectively) was anticipated.

Compd.	ΔG_b	ΔG^o_b	K_b	logD
69	**-5.81 ± 0.13**	**-2.62**	**84.2**	**1.17 ± 0.07**
70	**-6.17 ± 0.22**	**-2.60**	**80.8**	**1.05 ± 0.04**
71	**-5.55 ± 0.26**	**-2.79**	**110.8**	**1.57 ± 0.05**
72	**-6.11 ± 0.29**	**-2.70**	**95.6**	**1.11 ± 0.09**

Table 2. Octanol-water disturbtion coefficient (logD), experimental free energy of binding (ΔG^o_b), binding constant (K_b) and average of estimated free energy of binding (ΔG_b) for compounds 69-72.

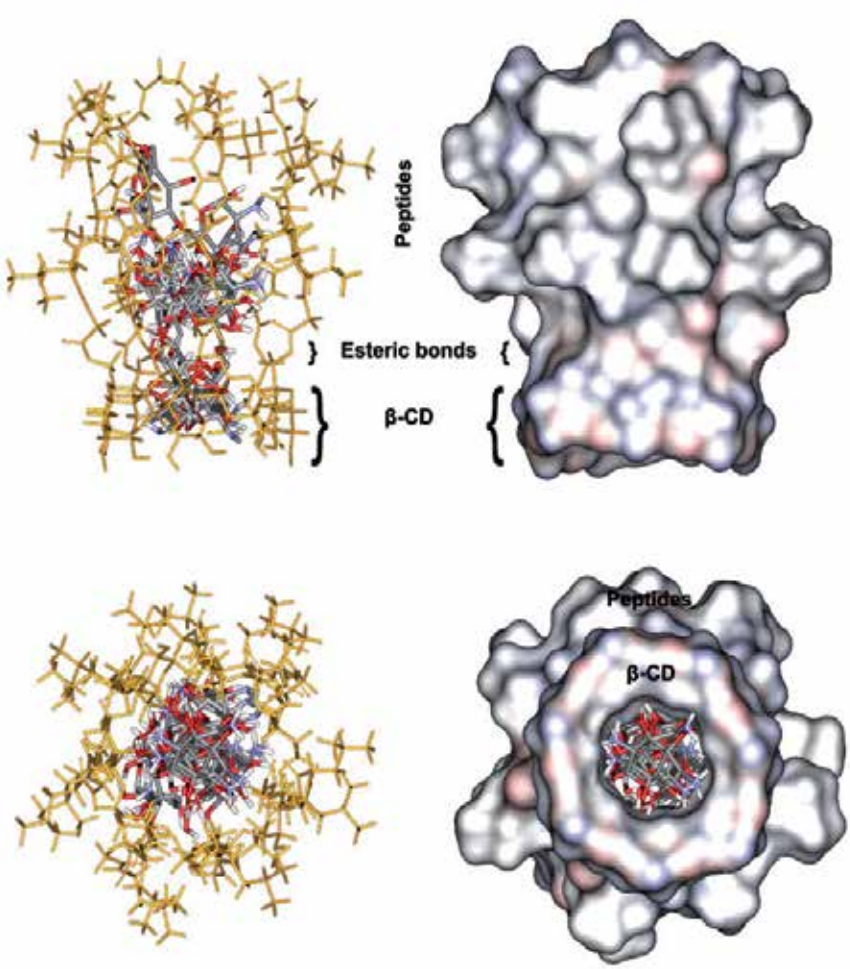


Figure 24. All of the Docked models of glucosamine in the cavity of 72. The below figure represents the cross view of docked models in 3d cavity.

Docking results of 69-72, showed that the binding free energy of glucosamine for all of the host molecules are almost equal and the docked models are mainly located in β-CD cavity. This could explain why the extraction contents and K_b of 69-72 are similar.

Phase transfer properties of 69-72 is mainly depend on the external lipophilic character of their cavity while the host molecules have similar binding affinity toward glucosamine, the more lipophilic one (71), would transfer higher quantities of the guest molecule.

This kind of molecular modeling method operated based on the two calculation methods: local search and genetic algorithm. In this It could be useful for further design of host molecules. Recently we have show the ability of the menthioned calculating methods for a new series of peptide-α-cyclodextrins as a suitable host molecules for extracting of dopamine (Seyedi et al, 2011).

4. Conclusion

In summary, we have designed and synthesized a new series of hydrophobic peptide β-cyclodextrins as a phase transfer carrier for glucosamine. These types of cage-molecules could be a precursor for designing and synthesis of other similar molecules as carriers for desired biological compounds in the future. Considering the external hydrophobicity and terminal hydrophilicity of these molecular structures, it would be hypothesized, that the natural capacity of these compounds to locate in the bilayer membrane of the cells, act as a channel for transferring special compounds.

By using of the molecular modeling techniques, down by the molecular interaction stimulator softwares, we can design a suitable host APC for recognition and scavenging a desired guest molecule. The mentioned method helps us for predicting which of the cyclodextrin types (α, β or γ type), how long of the peptide chains and what kinds of the amino acids could be suitable for the synthesis of an efficient host APC. In this chapter we discussed about the principles of the APCs modeling, host-guest interactions, synthetic methods and the application of them in drug delivery systems.

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Chapter 13

Processing and Templating of Bioactive-Loaded Polymeric Neural Architectures: Challenges and Innovative Strategies

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Additional information is available at the end of the chapter

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1. Introduction

The chapter highlights the current drug delivery strategies and phenomenon involved in the design of neural architectures employed for neuropharmaceutical applications attempting to replicate the nervous tissues. We seek to give an overview of the current drug delivery approaches such as degradable/diffusion-based delivery systems, affinity-based delivery systems, and immobilized drug delivery systems used to generate bioactive-release from scaffolds for neural tissue engineering applications. We talk about the combinatorial approach being employed in recent years in the form of "new generation of multifunctional biomaterials" which are - able to mimic the molecular regulatory characteristics by providing a three-dimensional architecture representing the native extracellular matrix and also able to sequester and deliver biomolecular moieties in highly specific manner. A special focus will be given to the micro- and nano-structured scaffolds which have been proved to be effective in axonal repairing, in guiding functional neurogenesis and in controlling stem cell differentiation. Emphasis will be given to the processing requirements for synthetic and natural polymers and biomaterials for producing potential scaffold materials as well as to the templating of the target drugs with their benefits and drawbacks. A detailed discussion will be provided for certain specialised and recently developed architectures in the form of injectable matrices, electrospun nanofibers, and hydrogels. Injectable scaffolds provide a very unique advantage of being a non-invasive approach for neural tissue engineering as further damage to the soft neural tissue due to surgery can be prevented. Current injectables in tissue engineering consists of gelation methods based on in situ chemical polymerization and crosslinking, photo-initiated polymerization and crosslinking, thermogelling injectable systems, ionic crosslinking, and self-assembling. Injectable scaffolds also provide opportunities

for drug delivery in the form of composite materials with embedded micro- and nanoparticles. Additionally, the easy customization of the injectable architectures by modification of solidification, porosity, biofunctionalization, mechanical properties, and biodegradation is an added attribute. The electrospinning process, on the other hand can be used to engineer neural architectures having micro to nanoscale topographical cues as well as micromeritic properties similar to the extracellular matrix. Enhanced cell attachment, drug loading, and mass transfer properties can be easily obtained by changing the surface-to-volume ratio and by controlling the bulk mechanical properties of electrospun scaffolds where the fibers can also be oriented or arranged randomly, to regulate the biological response to the scaffold. Drugs ranging from anti-inflammatory and antibiotic agents to growth factors, proteins, DNA, and RNA, and even living cells can be incorporated into electrospun scaffolds. Interventionally, the current tissue engineering and therapeutic approaches pertaining to neural injuries will be analysed and critiqued in detail with respect to various strategies, perspectives, challenges and expanding opportunities. Considerable efforts have been carried out to enhance the properties of tissue engineering architectures via surface engineering and surface functionalization for providing an extracellular matrix mimicking environment for better cell adhesion and tissue in-growth. These modifications can be carried out through plasma treatment, wet chemical method, surface graft polymerization, and co-fabrication of surface active agents and polymers. These modifications can further be designed and customised to release bioactive molecules, such as growth factors, DNA, or drugs, in a sustained manner to facilitate tissue regeneration (**Figure 1**) [1]. Target molecules are usually loaded on the surface of scaffolds by physical adsorption, nanoparticles assembly on the surface, layer-by-layer multilayer assembly, and chemical immobilization. Tissue engineering scaffolds can deliver bioactives via certain signals such as interspersed signals, immobilized signals and signal delivery from cells and hence act as specialised and desired controlled release matrices.

Material choices for the fabrication of neural architectures will be elucidated among, but not limited to, synthetic polymers such as poly(lactic acid), poly(glycolic acid) and co-polymers; poly(lactic acid-co-ethylene glycol); synthetic polyesters (PLA, PLGA) and collagen blends; aliphatic/aromatic degradable polyesters; polyfumarates; poly(ethylene terephtalate)–poly(butylene terephtalate); polyhydroxyalkanoates; poly(glycerol sebacate); hydroxyl group containing polymers; amine group containing polymers; poly(amido-amines)s; N-succinimidyl tartarate monoamine–poly(ethylene glycol)-block poly(D,L-lactic acid); poly(depsipeptide-co-lactide); poly(urethane)s; pluronic F-127 (PEO–PPO–PEO); tyrosine-derived polycarbonates; polyorthoesters; polyphosphazenes; polyanhydrides; polypyrrole; and poly ether ester amides and natural polymers such as alginates, chitosan, hyaluronans, and carageenans as well as self-assembling peptides.

Specific and representing examples cited, but not limited to, are as follows: Incorporation of protein-eluting microspheres (poly(lactide-co-glycolide) (PLGA 50/50)) into biodegradable nerve guidance channels (chitin tubes) for controlled release of growth factor; sodium tripolyphosphate cross-linked chitosan microspheres for controlled release of bioactive nerve growth factor; multifunctional, multichannel bridges that deliver neurotrophin encoding lentivirus (encoding the neurotrophic factors NT-3 or BDNF) for regeneration following spinal

cord injury (combining gene delivery with biomaterials); poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) nerve guidance channels as nerve growth factor delivery vehicles; regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann cells; and sustained release of dexamethasone from hydrophilic alginate hydrogel matrices using PLGA nanoparticles for neural drug delivery.

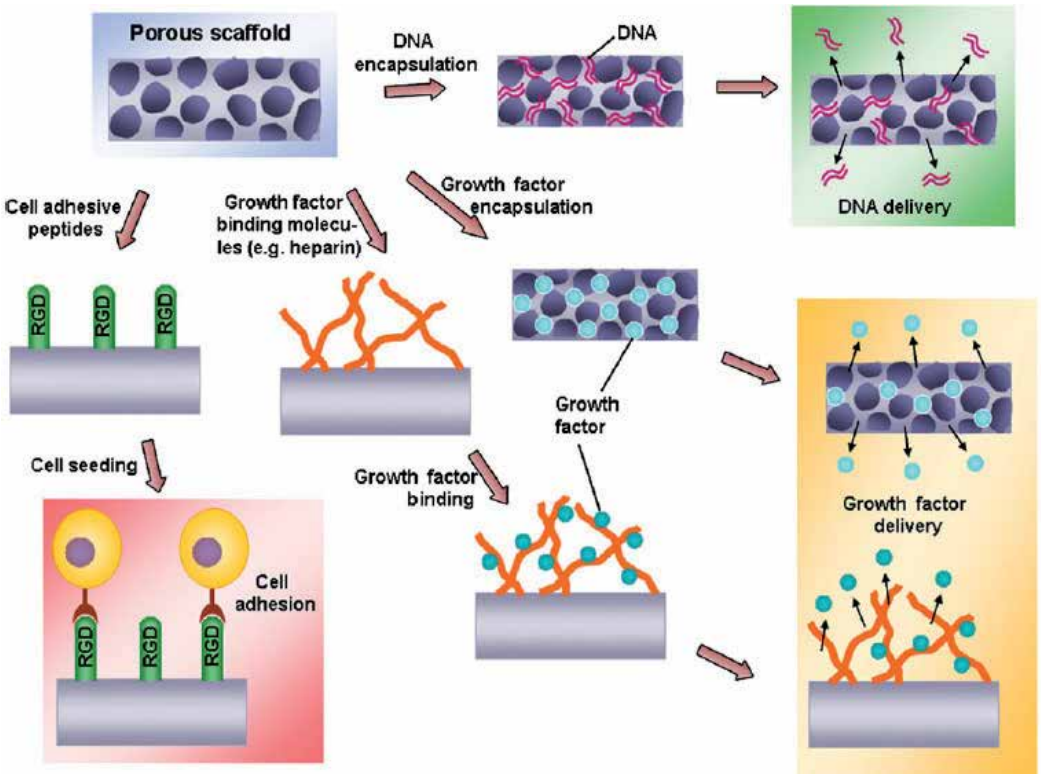

Figure 1. Surface engineered and growth factor releasing scaffolds for tissue engineering: Scaffolds can be either immobilized with cell specific ligands for cell adhesion, or encapsulated with growth factors or DNA to promote cell proliferation and morphogenesis [Adapted from Ref. 1 with permission © Elsevier Science BV].

2. Meta analysis of leading research reports

2.1. Local drug delivery for neuroprotection and tissue repair

In a series of studies, Shoichet et al., (2006, 2009a, 2009b) demonstrated the potential of hyaluronic acid-methyl cellulose (HAMC) blend as an injectable scaffold for the delivery of bioactives to an injured spinal cord, via an intrathecal injection, in conjugation with providing necessary support and microenvironment for the proliferation of neural cells. The studies were conducted in the backdrop of three most common strategies for localized intrathecal delivery viz. a bolus injection, a minipump delivery, and an injectable gel that localizes release to the site of injection. The first method pose a challenge in terms of the

effects being short-lived due to washing-out of the therapeutic molecule by cerebrospinal fluid and the second one being invasive and may lead to complication ranging from device blockage to infection. The novel HAMC blends' strategy provided a potential solution (**Table 1**) through the design of a shear-thinning based gel with an inherent property of increase in gel strength with an increase in temperature fast gelling [**Figure 2**].

S. No.	Design criteria	Advantage
1.	Fast gelling	localized delivery to the site of injection; no spread-out with the cerebrospinal fluid (CSF) flow
2.	Injectable through 30G needle	minimally invasive surgery
3.	Non-cell adhesive	minimization of scar formation in the intrathecal space
4.	Degradable	no need for scaffold removal afterwards
5.	Biocompatible	minimized foreign body reaction

Table 1. Design criteria for an injectable scaffold architecture

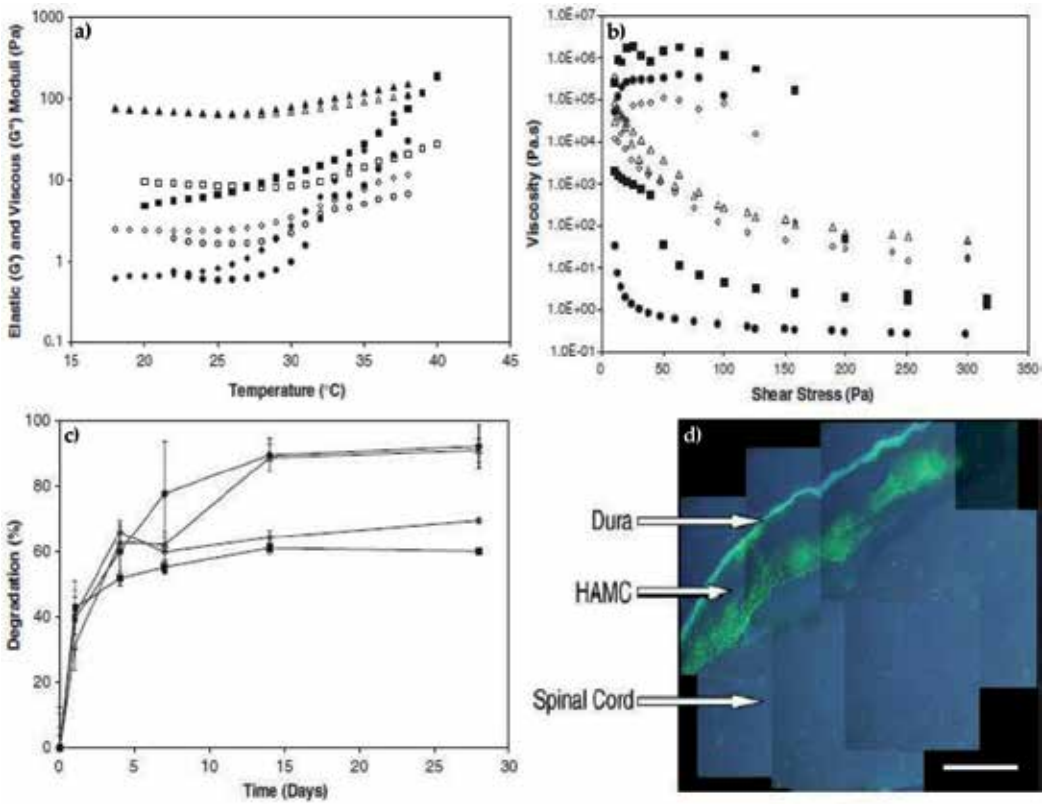

Figure 2. A) Elastic and viscous moduli of injectable gels at 1Hz using a rheometer with a cone and plate geometry (●) 7%MC G′, (○) 7%MC G″, (■) 9%MC G′, (□) 9%MC G″, (▲) HAMC G′, (Δ) HAMC G″, (▼) acet-HAMC G′, and (◊) acet-HAMC G″. B) Thixotropic loop of injectable gels at 37°C (●) 7%MC, (■)

9%MC, (Δ) HAMC, and (◊) acet-HAMC. C) *In vitro* degradation in aCSF of (●) 7%MC, (■) 9%MC, (Δ) HAMC, and (◊) acet-HAMC determined by change of dry mass over time. D) Parasagittal section of rat spinal cord rostral to site of injection shows that the fluorescent HAMC is localized in situ in the intrathecal space where it was injected [Adapted from Ref. 4 with permission © Elsevier Science BV].

In a typical experiment, 2% HA was blended with 7% MC and the gelation mechanism, degradation profile, and cell adhesion were studied in vitro. Additionally, in vivo studies were carried out for testing the injectability, biocompatibility and therapeutic efficacy (2006). Furthering the study, the potential of HAMC blend for the delivery of neuroprotectant such as nimodipine and erythropoietin were investigated experimentally. Specifically, nimodipine-loaded gels yielded particle size-dependent biphasic release profiles suggesting the accelerated delivery of poorly soluble drugs with tuneable release rates. In an another report, in vitro erythropoietin release studies revealed a 16 h release profile with the implant showing reduced cavitation after spinal cord injury along with enhanced neuronal number proving the potential of HAMC in neuroprotection after neural injury [2-4].

2.2. Multifunctional, multichannel bridges that deliver neurotrophin encoding lentivirus

Tuinstra at al., 2012, addressed the neural injury regeneration challenge by proposing a "gene delivery-biomaterial combination" strategy to surpass the "multiple barriers limiting regeneration". Spinal cord injury induced neuron and oligodendrocyte cell death, demyelination, inflammation, and deposition of a glial scar are attributed to insufficient trophic factor support, and up-regulation of axonal growth inhibitors. To overcome the deficiency of trophic factors, peripheral nerve implants are preferred due to their biograft-architecture and secretion of trophic factors by cells such as Schwann cells. However, the source limitation and the ill-defined contribution of the architecture and cell-secreted factors led to the requirement of systems and strategies capable of recapitulating their effects. To overcome the challenges, the delivery of lentiviral vectors (encoding neurotrophin-3 (NT-3) and brain derived neurotrophic factor (BDNF)) from multiple channel bridges was proposed as a combinatorial approach to promote regeneration in the injured SC. Interventionally, the lentivirus was immobilized to hydroxyapatite nanoparticles and then loaded into poly(lactic acid-co-glycolic acid) (PLGA) bridges for implantation into a rat spinal cord lateral hemisection and the axonal growth and myelination were characterized as a function of time, treatment, and location within the bridge. The strategy was aimed at (a) providing mechanical stability to the injured tissue, (b) channels directing the axonal elongation, (c) supporting cell infiltration, (d) preventing cavity formation secondary to the initial injury, (d) limiting scar formation. Interestingly, the cells were aligned with the major axis of the bridge providing a directional signal for regenerating axons [5].

2.3. In situ gelling hydrogels for local delivery of BDNF after spinal cord injury

Over the last decade, a research group led by Ravi V. Bellamkonda at Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory

University, Atlanta, USA, has emerged as a leading authority working towards the development of neurological biomaterials and therapeutics especially focusing on neural injuries. Jain et al., 2006, developed an agarose in situ gelling three-dimensional (3D) scaffold capable of initiating axonal growth in 3D. To this 3D scaffold was added neurotrophic factor BDNF-loaded lipid microtubule (40μm in length and 0.5μm in diameter) for local release of the bioactive. The individual benefits incurred by the components are structured in **Table 2**. Spinal cords treated with agarose/BDNF displayed no axonal bulbed-up morphology and the axons crossed the interface into the hydrogel scaffold based on the NF-160kDa staining data analysis where NF-160 kDa intensity was significantly greater than the agarose-treated spinal cords at the interface [6].

Agarose scaffold	Brain derived neurotrophic factor
Biocompatible: does not cause an adverse reaction when implanted in vivo,	encouraged neurite growth into the scaffolds
can be optimized for maximum axonal outgrowth through manipulation of the porosity and mechanical properties,	reduced the reactivity of the astrocytes and the production of chondroitin sulfate proteoglycans (CSPGs)
Support cell migration	enhanced the ability of regenerating fibers to enter the permissive hydrogel scaffold
can be utilized as part of a trophic factor delivery system with embedded sustained release vehicles,	acted as a chemo-attractant for the axons to cross the interface
can be used to bind protein to its backbone for spatial control	reduces the minimal inflammatory response agarose gels generate in vivo

Table 2. Salient features of the in situ gelling hydrogel components

2.4. Neurite extension in anisotropic LN-1 scaffolds

An in vitro study by Dodla and Bellamkonda, the importance of directional cues for neural regeneration was proposed. The hypothesis was based on the performance outcomes of autografts where the researchers theorized that "anisotropic hydrogel scaffolds with gradients of a growth-promoting glycoprotein/extracellular matrix protein, laminin-1 (LN-1), may promote directional neurite extension and enhance regeneration". The scaffolds were fabricated via photochemical coupling approach wherein LN-1 was immobilized onto three-dimensional (3D) agarose scaffolds in gradients of differing slopes. A significant increase in the Dorsal root ganglia (DRG) neurite extension rates were observed in case of anisotropic scaffolds as compared to their isotropic analogues proving the importance and potential of built-in directional cues for guided tissue or nerve regeneration [7]. In line with the functionalized scaffold properties developed above, Crompton et al., 2007, described the potential of Polylysine-functionalized chitosan as an in vitro substrate, scaffold for cortical cells, and for neural tissue engineering as an injectable scaffold. The functionalized polysaccharide polymer was rendered thermoresponsive by ionic gelation mechanism of glycerophosphate. In first of its

kind study of chitosan-GP network gels on neural tissue, the 3D culture model showed that the survival of cells on chitosan/GP was less than the PDL-modified chitosan/GP justifying the inclusion of PDL as functionality for neural applications [8].

2.5. Topical delivery of Tx-MP-NPs into the contusion injured spinal cord

The delivery of bioactives on the site of neural injury can be achieved by various means including intravenous delivery eventually passing through blood brain barrier and reaching the site of action or through intrathecal/intracranial injection for localized delivery. Targeted delivery is an additional option but not much tried and tested for CNS injuries. The i.v. strategy encompasses the probability of inefficiency as compared to the localised delivery but invasiveness is a drawback for the later intervention. Chvatal et al., 2008, and later Kim et al., 2009, reported the therapeutic effectiveness of nanoparticle-mediated localized methylprednisolone (MP) delivery for spinal cord injury intervention. These studies tested the therapeutic effect of MP-loaded PLGA nanoparticles, incorporated into an agarose gel, in in vivo models of spinal cord injury. The first study by Chvatal et al, 2008, concentrated on the spatial distribution of MP along with the acute anti-inflammatory effect. In a typical fabrication, the drug was first conjugated with Texas-red cadaverine (Tx, Biotium) allow its visualization in and around an injured spinal cord. Thereafter, the PLGA nanoparticles were prepared using a double emulsion method followed by their incorporation into the agarose gel. Two different types agarose gel were used: denser gel without nanoparticles (SeaKem) and gel containing nanoparticles (SeaPlaque) as shown in **Figure 3**.

According to **Figure 4**; the drug-indicator conjugate revealed a slow release over six days and the data was used to determine the amount of Tx-MP released from the gel-embedded NPs showing a continual release pattern. The release was probably due to the gradual degradation of PLGA polymeric structures. In this way, a continual delivery of MP can be provided to the injured spinal cord thereby exerting its anti-inflammatory action with a single administration and hence can provide much needed lipid-peroxidation inhibition during the initial and acute phase of secondary injury. To further simulate the in vivo environment, the in vitro release studies of Tx-MP from NPs embedded in agarose hydrogels was examined by co-incubation with LPS-stimulated primary rat microglia. The quantification of nitric oxide (NO) release from the microglia at different time points, 24, 72, and 96 h, showed that the cells treated with Tx-MP-NPs had significantly reduced NO production as compared to the cells treated with Saline NPs (as a negative control) further proving that the hydrogel-based delivery system (i.e., NPs embedded in agarose gel) can be used as an MP depot.

The quantification of spatial distribution of Tx-MP in the contusion injured spinal cord revealed that MP diffused up to 1.5mm deep and up to 3 mm laterally into the injured spinal cord within 2 days. Other significant observations of this study included decreased early inflammation, reduction in the number of ED-1þ macrophages/activated microglia, diminished expression of pro-inflammatory proteins such as Calpain and iNOS, and finally reduced lesion volume within 7 days after contusion injury [9].

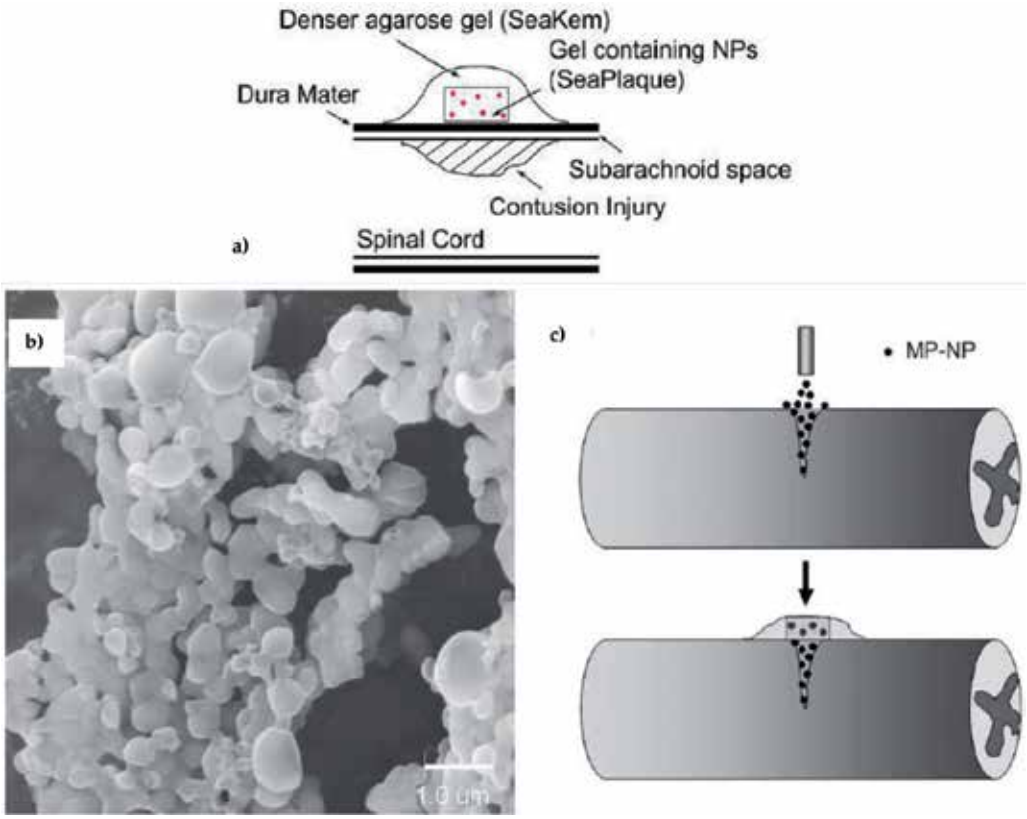

Figure 3. a) Schematic showing the Tx-MP-NP embedded gel is placed directly onto the injury site, on top of the dura. The denser gel is injected on top and quickly cooled to hold the NP embedded gel in place and minimize outward diffusion of Tx-MP. b) Methylprednisolone-encapsulating PLGA nanoparticles (MP-NP): SEM image of the lyophilized MP-NPs. c) Schematic of topical and local delivery of the MP-NPs onto dorsal over-hemisection lesioned spinal cord [Adapted from Ref. 9 with permission © Elsevier Science BV].

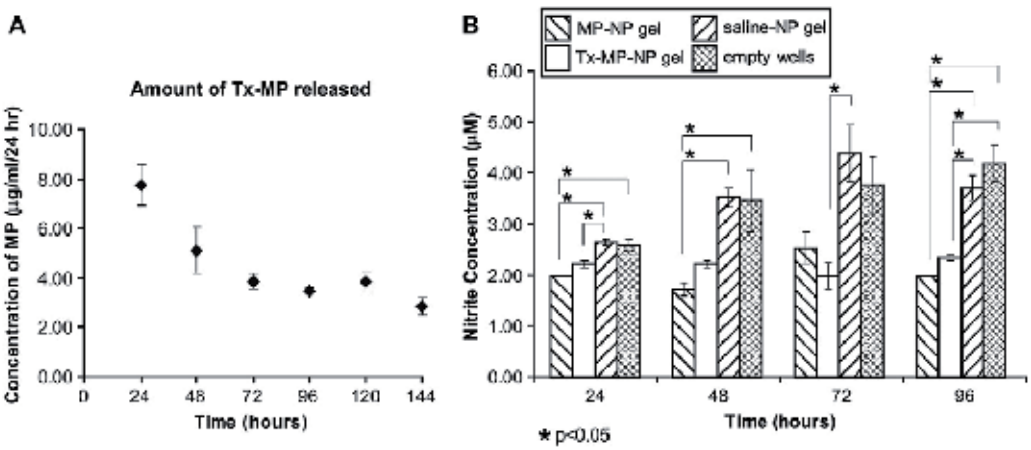

Figure 4. In vitro characterization of Tx-MP-NPs. (A) Release profile of Tx-MP over 6 days in vitro. Plotted is the amount of MP released every 24 h. (B) Bioassay showing the amount of nitrite in the

media surrounding LPS-activated microglia that had been treated with gels containing either MP-NP, Tx-MP-NP, Saline-NP, or nothing. This represents the amount of NO produced by microglia in each of these conditions for 4 days [Adapted from Ref. 9 with permission © Elsevier Science BV].

2.6. Nanoparticle-mediated local delivery of methylprednisolone

Further extending the above results, Kim et al., 2009, conducted a detailed histological and behavioural study of the developed local and sustained MP delivery through the use of biodegradable polymer-based nanoparticles. The following histological parameters were recorded:

1. reduced the reactivity of pro-apoptotic proteins (Calpain and Bax)
2. increased reactivity of anti-apoptotic protein (Bcl-2) at the lesion site
3. reduced iNOS (a key mediator of inflammation and neurotoxic effects expression
4. ratio of Bcl-2 to Bax, a key determinant of neuronal commitment to apoptosis, was increased indicating that MP treatment reduced the mitochondria-mediated cell death pathway.

These results suggest that sustained MP delivery by slow release MP-NP can reduce the reactivity of the injury-related proteins. Furthermore, MP-NP treatment produced significantly improved functional outcomes within one week after injury wherein MP-NP-treated groups performed significantly better in the beam walking test.

In conclusion, following advantages of MP-NP local delivery over conventional systemic delivery were proposed:

1. Better therapeutic effect as compared to systemic MP delivery;
2. More efficient, targeted delivery to the injury site as the MP delivery via systemic administration is influenced by the short pharmacokinetic half-life of intravenous MP (2.5–3 h) and P-glycoprotein-mediated exclusion of MP from the spinal cord necessitating a high-dose MP regimen. However, a significantly lower dose was required using nanoparticle-encapsulated MP (approximately 400 mg/ animal) and delivering this dose locally onto the target tissue. Additionally, this significantly enhanced the therapeutic effectiveness of MP by increasing the local dose levels at the target site;
3. Potential to adjust delivery rate or duration since the MP release profile from nanoparticles can be controlled through the composition of the biodegradable polymer, the rate, amount, or duration of delivery can be adjusted;
4. Stable formulation in form of injectable and lyophilized fine (submicron) powder formulation (unlike cell-based therapies). The formulation can be stored as a lyophilized fine powder and then easily suspended in saline or embedded in hydrogel and locally delivered onto the lesion site [10].

2.7. Sustained delivery of thermostabilized chABC

Furthering their study on the involvement of various axonal growth promoters and inhibitors, Bellamkonda and co-workers targeted Chondroitin sulfate proteoglycans, a major

class of axon growth inhibitors that are up-regulated after spinal cord injury and contribute to regenerative failure, via the use of Chondroitinase ABC (chABC) capable of digesting glycosaminoglycan chains on CSPGs thereby overcoming the CSPG-mediated inhibition. The formulation problem concerned with chABC is the loss of its enzymatic activity 37 °C, necessitating the use of repeated injections or local infusions for a prolonged period making it clinically inefficient. Lee et al., 2010, designed a thermostabilized chABC and developed a system for its sustained local delivery in vivo thereby resolving the problem to a great extent as the novel thermostabilized chABC retained its biological activity at 37 °C in vitro for up to 4 weeks [Figure 5].

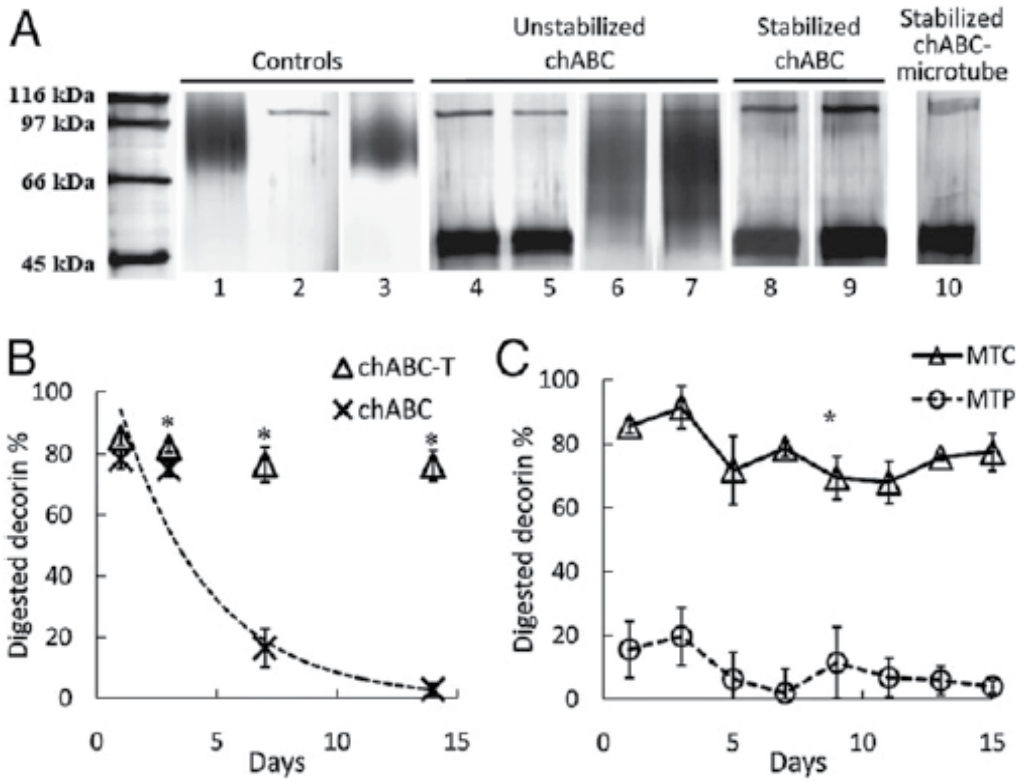

Figure 5. Enhanced thermal stability of chABC with trehalose, and sustained chABC release with lipidmicrotubes. (A) SDS-PAGE assay of enzymatic activity of chABC. (B) Kinetic analysis of chABC deactivation by DMMB assay. The dotted line represents the calculated deactivation curve of chABC in 1× PBS. (C) Enzymatic activity of post released chABC (Δ) and penicillinase (○) with trehalose/microtubes [Adapted from Ref. 11 © Proceedings of National Academy of Science (PNAS)].

Briefly, chABC was thermostabilize using the sugar trehalose and a hydrogel-microtube based delivery system was used for the sustained local delivery of chABC in vivo [11]. Key finding of the study included:

a. Trehalose Significantly Enhances chABC Thermal Stability and Prolongs Enzyme Activity

b. Temperature stabilization of chabc by trehalose was due to conformational stability
c. Thermostabilized chABC (TS-chABC) retains its ability to digest CSPGs in vivo 2 weeks post-injury,
d. CS-GAG levels remain significantly depleted at the lesion site for at least 6 weeks post-SCI.
e. Enhanced axonal sprouting and functional recovery is observed after topical delivery of agarose-microtube-chabc scaffolds after SCI
f. Animals treated with thermostabilized chABC in combination with sustained neurotrophin-3 delivery showed significant improvement in locomotor function and enhanced growth of cholera toxin B subunit– positive sensory axons and sprouting of serotonergic fibers.
g. chABC thermostability facilitates minimally invasive, sustained, local delivery of chABC that is potentially effective in overcoming CSPG-mediated regenerative failure.

2.8. Sustained delivery of activated Rho GTPases and BDNF

In the next stage of their Chondroitin sulfate proteoglycans targeting, Bellamkonda and co-workers fabricated constitutively active (CA) Rho GTPases, CA-Rac1 and CA-Cdc42, and BDNF loaded 1,2-bis-(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine lipid microtubes for the sustained release of bioactives to the lesion site for the alleviation of CSPG-mediated inhibition of regenerating axons. The lipid tubules were further loaded into an *in situ* gelling agarose-protein architecture similar to previous studies where nanoparticles were incorporated into an agarose injectable gel. The sustained release characteristics of the developed system were characterised both in vitro and in vivo BDNF was conjugated to rhodamine for its in vivo quantification. As depicted in **Figure 6**, 3 mg of BDNF was released within the first 24 h followed by a cumulative release of 4mg within the first 3 days. After this initial burst release, approximately 3ng/day of BDNF was released for the subsequent 11 days. For in vivo release study, microtubes loaded with BDNF/Rhodamine embedded in the agarose were injected into the spinal cord cavity. Spatial quantification of BDNF/Rhodamine displayed its presence in the spinal cord tissue between 1 to 2 mm proximal to the lesion site (Fig. 1B). An overlap of ED-1+ cells (green) with the BDNF-Rhodamine demonstrated co-localization and subsequent engulfment of the BDNF-Rhodamine by the ED-1+ cells. Additionally, BDNF-Rhodamine was double labelled with DAPI to visualize cell nuclei confirmed the presence of neurotrophins [12]. In conclusion, the study utilized the hydrogel/microtubule scaffold delivery system to deliver BDNF, CA-Cdc42, or CA-Rac1 to decrease the sensitivity of growth cones to CSPGs, and promote axonal growth through CSPG-rich regions at the lesion site based on following three phenomena:

1. The use of the in situ gelling hydrogel allows the lesion cavity to be conformally filled.
2. The lipid microtubes allow slow release of therapeutics from the hydrogel over time.
3. This strategy allows neuroprotective or axonal migration stimulators to be delivered locally over time.

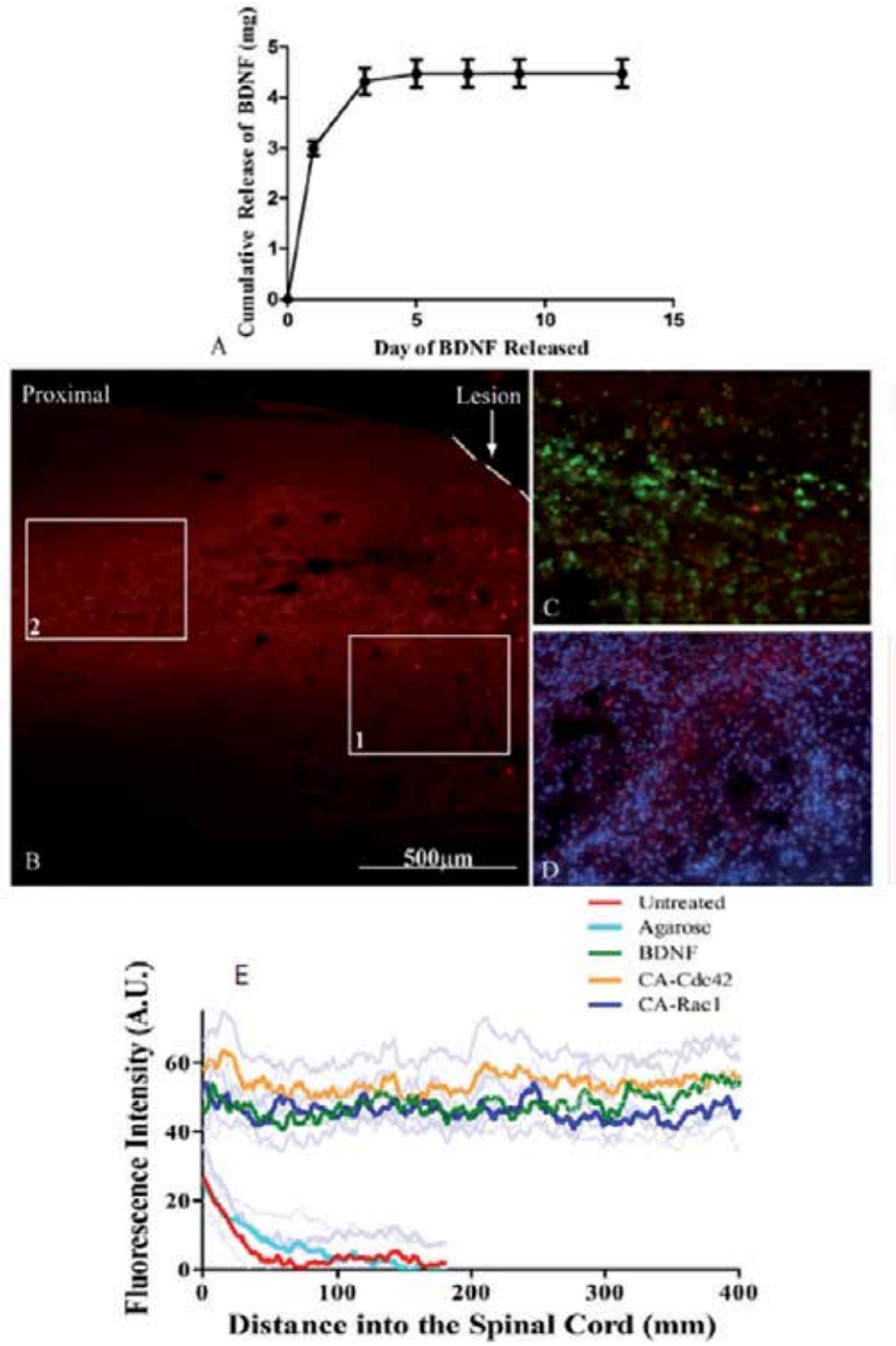

Figure 6. In Vitro and In Vivo Diffusion of BDNF. A. In vitro release assay of BDNF over the first 2 weeks. The graph shows that an initial burst released 4 mg of BDNF within the first 3 days. An average

of 3 ng/day of BDNF was released for the following 11 days. The data represent mean 6 SEM B. An image of the proximal region of a spinal cord section after delivery of BDNF/Rhodamine at 46. The 206images labelled 1 and 2 are outlined with white boxes in A and demonstrate that BDNF/Rhodamine diffused approximately 2 mm proximal to the lesion site. The dashed line represents the interface between the spinal cord and scaffold. White arrows indicate BDNF/Rhodamine. C. A 206image of ED-1+ cells (green) and BDNF-Rhodamine (red). The image shows some overlap demonstrating engulfment of the BDNF-Rhodamine by the ED-1+ cells. D. A 206image of DAPI (blue) and BDNF-Rhodamine (red) demonstrates that not all of the BDNF-Rhodamine is taken up by resident and migrating cells. Quantitative analysis of NF-160 intensity for the stained spinal cords. The spinal cords treated with CA-Cdc42, CA-Rac1, and BDNF had significantly higher fluorescent intensity and also had extended further into the scaffold filled spinal cord cavity than the untreated and agarose controls [Adapted from Ref. 12 © PLoS ONE].

2.9. Multiple-channel scaffolds to promote spinal cord axon regeneration

Moore et al., 2006, introduced a proposition regarding the use of a conjugation of molecular, cellular, and tissue-level treatments for spinal cord injury to elicit functional recovery in animal models or patients. In a tri-component study, multiple-channel biodegradable scaffolds were tested as a platform to investigate the effects scaffold architecture, transplanted cells, and locally delivered molecular agents on axon regeneration. Biodegradable polymers such as PLGA can provide a tissue scaffold, a cell delivery vehicle, and a reservoir for sustained drug delivery. Therefore, PLGA scaffolds were fabricated using injection moulding with rapid solvent evaporation.

The scaffolds so formed have characteristic plurality of distinct channels running parallel along the length of the scaffolds with various customized channel sizes and geometries. Inherent to the channels were void fractions (as high as 89%) with accessible void fractions as high as 90% through connections 220mm or larger. Scaffolds degradation and bioactive release in vitro accounted for over a period of 30 weeks and 12 weeks, respectively. The degradation profile of PLGA scaffold demonstrated typical polyhydroxy acid characteristics as the scaffold molecular weight decreased steadily from 100% of the initial molecular weight down to 5% by 26 weeks of degradation. Interestingly, minimal mass loss was observed for the first 20 weeks. The next ten weeks culminated into a more precipitous mass loss generating a sigmoidal mass loss behavior typical for PLGA degradation. The negligible change in mass during initial phase corroborated well with the slight changes in morphology. However, the morphology of the degrading scaffolds changed drastically during the precipitous phase. The model drug, Fluorescein isothiocyanate (FITC-D), was incorporated using, mechanical mixing within the polymer scaffolds. However the method was quite effective for scaffold loading, an inhomogeneous dispersal appeared in the final fabricated scaffolds. A drug release-scaffold degradation comparison in **Figure 7** compared the mass and molecular weight degradation profiles with model drug release. An initial burst over the first 48 h was followed by a period of more steady release for 4 weeks. The sustained release continued for next 8 weeks after which little or no release was detected for the remainder of the degradation period.

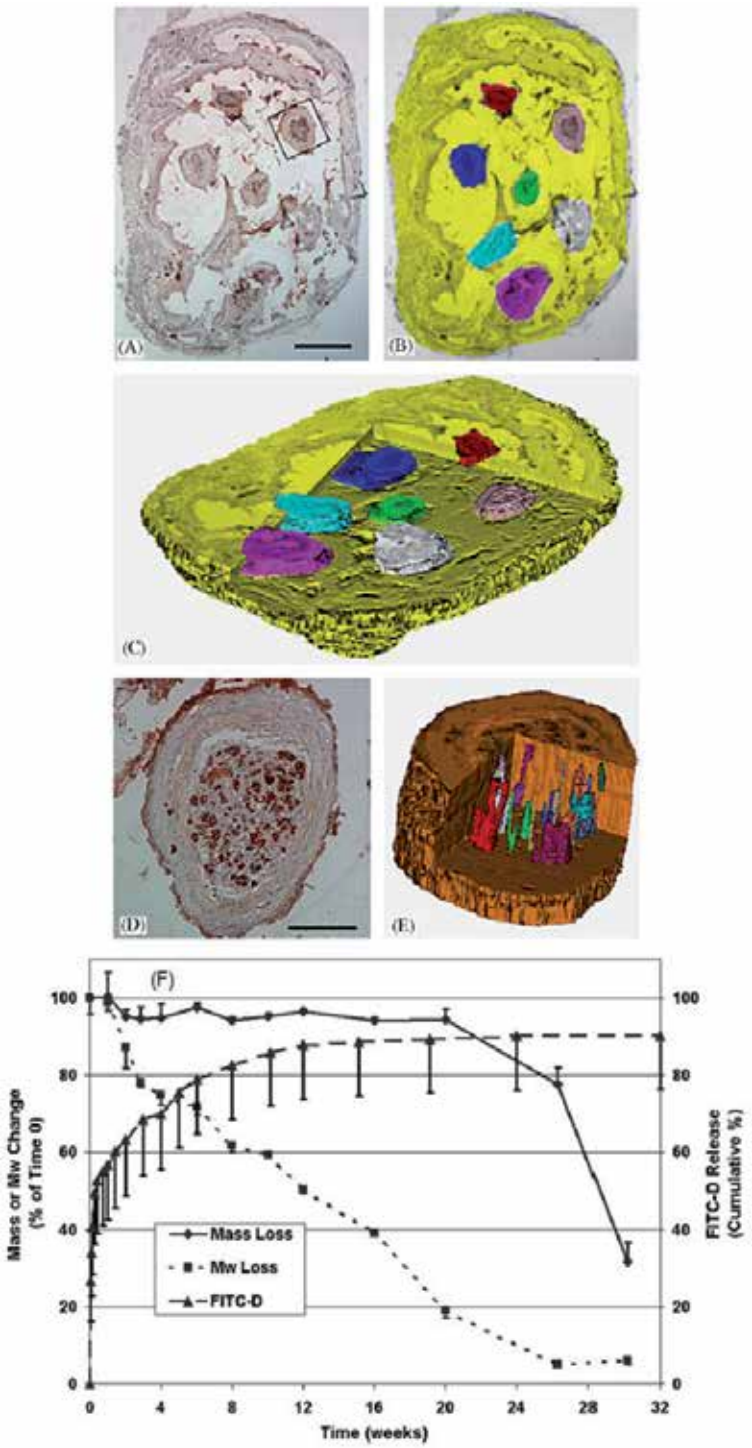

Figure 7. Histological images of tissue cables within scaffold channels. (A) Histological cross section near rostral end of scaffold showing tissue cables within all seven channels. Boxed region is shown in

greater detail in D. (B) Histological cross section shown in A with channels segmented and displayed in color. (C) Orthographic, wedged view of 16 serial slices registered, segmented, and reconstructed to reveal 3-D structure of channels. (D) Histological cross section of boxed region in A; (E) Orthographic, wedged view of reconstructed channel shown in D, with segmented axon bundles displayed in color. (F) Degradation and FITC-D release profile. Mass or molecular weight change is indicated as a percentage of initial value at time zero. FITC-D release is indicated as a cumulative percentage of loaded drug [Adapted from Ref. 13 with permission © Elsevier Science BV].

The release of the drug progressed in corroboration with scaffold degradation. However, the inhomogeneity played its role as a very high variability was observed. The polymeric architecture was designed to act as a template for the arrangement of cells that may attract and guide regenerating axons rather than for guiding the regenerating fibers by providing an oriented surface. The delivery system was proposed to be flexible enough to provide incorporation of various drugs/biomolecule/bioactives that may add tropic attraction, neuroprotection, or reduce the inhibitory effects of secondary injury. Another important aspect of the study was to attest the effect of incorporation of Schwann cell in the scaffold as their harvesting, suspension, and distribution into scaffold channels is of critical importance in this field. Results indicated that Schwann cells were successfully harvested, suspended in Matrigel, and loaded into distinct channels, and that cells survived in culture under physiologic conditions within the scaffold. Axon regeneration was visualized by 3-D reconstruction of serial histological sections, an approach that appears useful for evaluation of regenerating tissue architecture as shown in **Figure 7**. The 3-D reconstructions demonstrated the arrangement of the seven channels containing regenerating axons, as well as live axon bundles within the channels. This novel depiction corroborated well with the channels which were found distributed as arranged by the scaffold, with scaffold and tissue in the spaces separating the channels, approximately. An important finding revealed that channels generally contained a centralized core of tissue containing axons and capillaries, surrounded by circumferential fibrous tissue. Furthermore, macrophages could be identified, due to their engulfment of neurofilament-stained material [13].

2.10. Laminin and nerve growth factor-laden three-dimensional scaffold

Yu et al., 1999, engineered Leminin-functionalized agarose hydrogel scaffolds for the stimulation of neuronal process extension based on the hypothesis that "substrate-bound neurite promoting extracellular matrix (ECM) proteins and chemo-attractive diffusible trophic factors influence and stimulate axon guidance and neurite extension". The study indicated that the effect of LN-modified agarose gels on DRG/PC12 cell neurite outgrowth involves receptors for YIGSR/integrin/31 subunits, respectively. Additionally, 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine based lipid micro-cylinders were loaded with nerve growth factor (NGF), and embedded into agarose hydrogels which resulted in a directional neurite extension from DRGs in agarose hydrogels due to the presence of trophic factor gradients. The NGF-loaded lipid micro-cylinders released physiologically relevant amounts of NGF for at least 7 days *in vitro* based on a PC12 cell-based bioassay confirming

that the agarose hydrogel scaffolds can be used as biosynthetic 3D bridges capable of promoting regeneration across severed nerve gaps [14].

2.11. Agarose and methylcellulose hydrogel blends for nerve regeneration applications

Taking leads from the research done by Shoichet and co workers and from Bellamkonda and co workers, Martin et al., 2009, designed injectable scaffold consisting of agarose and methylcellulose (MC) as the responsive polymers. The following rationale was cited in support of using a blend of MC and agarose instead of using agarose alone: the low melting point of agarose makes it difficult to use *in vivo* and hence need to be cooled after injection wherein blowing of gases onto nervous system tissue could potentially be harmful to neurons that survived the initial injury. The release of bioactive from such gelling mixtures was related to the dissolution of the scaffold itself under physiological conditions. In an interesting experiment the percentage of hydrogel dissolved was measured on day 1, 4, 7, 14 and 28. Plain agarose dissolved completely when left in PBS in a 37 ◦C incubator for only 7 days, making it difficult to record the percentage dissolved due to hydrogel instability. However, the blends were approximately 70% dissolved by the end of 28 days in the cycled PBS solution. Pristine 7% methylcellulose proved to be a weaker hydrogel that dissolves more rapidly than the hydrogel blends, dissolving over 75% in 28 days. This confirmed the applicability of the blend in comparison to individual polymers. Furthermore, the authors pointed that "the ease of creation, simplicity of delivery and use of natural biocompatible components that solidify naturally without the use of ultraviolet light or cross linkers add to the advantages of utilizing this blend" [15].

2.12. An injectable, biodegradable hydrogel for trophic factor delivery

Piantino et al., 2006, devised a new method for an injectable based drug delivery system for the delivery of trophic factors to the injured neural tissues. The method utilized an injectable liquid polymer solution that crosslinks into a biodegradable, water-swollen hydrogel when photo-activated under visible light. The investigators described the uniqueness of their system over other systems as follows:(1) the system can provide constant and tailorable delivery of one or more growth factors to the precise site where needed; (2) reduce the possibility of host–graft rejection, as may occur with the use of live tissue or cell preparations; (3) does not involve viral vectors, which may induce an inflammatory response and which may require prolonged delays in achieving high levels of gene expression; and (4) does not involve the use of devices that can malfunction or cause infections (e.g., pumps or catheters). Briefly, degradable poly(ethylene glycol) hydrogels are formed from the radical polymerization of acrylated poly(lactic acid)-b–poly(ethylene glycol)-b–(poly lactic acid) (PLA-b–PEG-b–PLA) macromers. The network degradation and subsequent molecular release properties can be controlled by altering the network chemistry of these injectable hydrogels such as the number of degradable units and network crosslinking density by changing the macromer concentration). The degradation of the

hydrogels was postulated to be due to hydrolysis in the network crosslinks. The release profile of NT-3 *in vivo* was determined using ELISA and a peak was reported at the site of injury as early as 3 h after hydrogel administration. High concentrations over a distance of 1 cm and more were cited site on day 6 followed by a decline to approx. one-third of maximum after 14 at 14 days. The NT-3 levels within the first few millimetres of the lesion site were in the range of 50 ng/ml/g of tissue when averaged over the entire tissue sample. In vitro release studies corroborated well with the inv vivo data with a burst release of ~40% over first 24 hours and a sustained release thereafter. Interventionally, NT-3, delivered via hydrogel in the vicinity of injured nerve terminals, was presumed to be transported retrogradely to cortical pyramidal cells thereby promoting axonal branching along with promoting plasticity of the raphespinal tract and finally promoting the outgrowth of descending pathways involved in locomotor control leading to the lesion size being slightly smaller in NT-3-treated vs. control animals as early as 1 day and 1 week post-lesion [16].

2.13. Hydrogel scaffold and microspheres for supporting survival of neural stem cells

To develop a biomaterial composite for promoting proliferation and migration of neural stem cells (NSCs) and to rescue central nervous system (CNS) injuries, Wang et al., 2011 constructed a NSCs-cultured delivery system based on crosslinked hyaluronic acid (HA) hydrogels, containing embedded BDNF and vascular endothelial growth factor (VEGF)-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres for controlled delivery as well as angiogenesis on the materials. The study was designed on the drawbacks of previous studies that have shown an intrinsic ability of neural stem cells (NSCs) to regenerate in various neurological diseases' intervention. The most important among these were the low viability and undesired differentiation of the grafted NSCs and also the glial scar formation. In addition to these, the deficiency of neurotrophic factors and growth factors following the CNS may also contribute to failure of neural regeneration. The in vitro release kinetics study by ELISA measured the loading of BDNF and VEGF in microspheres as 0.10 and 0.34 μg/mg microspheres, respectively, corresponding to 52% and 84% loading efficiency making the loading efficiencies of HA gel composites in the range of 17.4 and 56.6 ng/mg, respectively, when embedded with microspheres. Interestingly, no burst release characteristic to the PLGA microspheres was within the period of 6 days wherein bioactives were released from the microspheres constantly and cumulatively. The thorough washing of the microspheres was believed to overcome the burst release of the proteins from the surface and provided a slow and linear release profile during the test period. During the first week of the study, about 20–30% of the loaded proteins were release possible by diffusion from areas near the surface as the degradation of PLGA was insignificant during initial phases. In contrast, the release of growth factors from the hydrogel embedded with microspheres revealed an initial "burst" followed by a stable release phase. This was attributed to the fact that released factors might have remained in the hydrogel during the cross-link processing leading to the diffusion through the hydrogel matrix. However, the cumulative amount of bioactive release was reduced to 12% and 13% of total loading as compared to 20-30% in case of

microspheres alone which was postulated to be due to the presence of the surrounding HA hydrogel as a delayed or deposited effect on the release profile of the biofactors [17].

2.14. Controlled release of Neurotrophin-3 from fibrin-based tissue engineering scaffolds

Pathologically, the ability of chronically injured supraspinal axon tracts to regenerate decreases if intervention is delayed for too long. The barriers to axonal regeneration in case of a chronically injured spinal cord are reported to be overcome interventionally by using a combination of diffusible growth stimulating molecules and a favourable growth stimulating extracellular matrix such as the use of a continuous transgenic delivery of neurotrophic factors from engineered cells that also secrete extracellular matrix (ECM) is conducive to regeneration [18]. However, there is a limitation of this strategy as that the growth is limited to the regeneration of axons into the transplant site, the source of the growth stimulating neurotrophins, and the axons fail to regenerate back into host tissue. Sakiyama-Elbert and co workers, 2009, coined the need for temporary controlled release of growth-stimulating neurotrophins that not only promote axon growth into the lesion but also foster growth into healthy spinal cord tissue thereby connecting the newly grown tissue to the neural tissue that survived the injury. The intervention consisted of delayed treatment of SCI with controlled release of neurotrophin-3 (NT-3) from fibrin scaffolds providing a supportive regenerative environment, reducing the accumulation of reactive astrocytes surrounding the lesion and enhancing the presence of neural fibers within the lesion. The heparin-based delivery system (HBDS) developed earlier by the investigators [19], was utilized as it forms non-covalent interactions between neurotrophins, heparin, and a covalently linked bi-domain peptide (ATIII peptide), which can be incorporated into the fibrin scaffold and allow for the controlled release of growth factors from the scaffolds. The controlled released of the biofactor from the fibrin scaffold was assessed in terms of quantification of neural fiber density, astrocyte density, and chondroitin sulfate proteoglycan density along with macrophage/microglia immune profile analysis. The final results demonstrated that controlled release of NT-3 promotes increased neural fiber density within the lesion and decreased reactive astrocyte staining around the lesion of subacute SCI (2 weeks after the injury) at a dose of 500 ng/mL of NT-3 with HBDS [20]. These results were in corroboration with the earlier reports where the NT-3/HBDS was employed in an acute SCI (immediately after the injury) model wherein the it promoted a significant increase in the density of neural fibers sprouting [21-23].

2.15. Nerve guidance channels as drug delivery vehicles

However, safe and effective use of pro-regenerative molecules requires a localized, controlled and sustained delivery to the site of neural injury. However, there are many challenges pertaining to the various approaches available in the neural arena as described in Table 3.

To overcome the challenges shown in the table above, Piotrowicza and Shoichet, 2006, developed a liquid–liquid centrifugal casting process which enables the formation of nerve

guidance channels (NGCs) from acrylate-based hydrogels such as poly(hydroxylethylmethacrylate-co-methylmethacrylate) or P(HEMA-co-MMA). The NGCs themselves were fabricated by a liquid–liquid centrifugal casting process and the protein incorporation strategies were compared in terms of protein distribution and nerve growth factor (NGF) release profile. The centrifugal casting process is based on the following phenomenon: Phase separation of the polymer phase from the monomer formulation during polymerization in a rotating cylindrical mould and hence the denser polymer phase is pushed to the periphery by centrifugal forces, where it gels forming a tube. The NGCs thus formed were semipermeable, soft and flexible, and matched the modulus of the nerve or spinal cord.

S. No.	Approaches	Challenge
1.	Systemic administration	short half-lives and high potency of many biomolecules, and delivery to the CNS is further limited by the blood–brain barrier
2.	osmotic mini-pumps	infections or tissue damage, pump failure due to catheter dislodgement or occlusion, as well as drug instability in the pump reservoir
3.	local administration as growth factor solutions	factors lose bioactivity in solution
4.	local administration as matrices saturated with growth factors	easily leak from the channel during the implantation procedure
5.	NGCs prepared by dip-coating or solution casting	the creation of a non-symmetric growth factor concentration profile within the lumen of the NGC

Table 3. Challenge inherent to various bioactive administration approaches

The investigators formulated three different approached for the delivery of nerve growth factor (NGF) using Nerve guidance channels (NGCs constituted with of poly(2-hydroxyethyl methacrylate-co-methyl methacrylate), P(HEMA-co-MMA) as the polymeric platform to facilitate regeneration after transection injury to the spinal cord: (1) NGF was encapsulated (with bovine serum albumin (BSA)) in biodegradable poly(D,L-lactide-co-glycolide) 85/15 microspheres, which were combined with a PHEMA polymerization formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique; (2) pre-formed NGCs were imbibed with a solution of NGF/BSA and (3) NGF/BSA alone was combined with a PHEMA formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique. The channels imbibed with NGF showed no sustained release of NGF as was achieved from NGCs with either NGF-loaded microspheres or NGF alone incorporated into the inner layer with values of 1040 pg/cm, 220 pg NGF/cm, and 8624 pg/cm, respectively, after 28 days [**Figure 8**]. As a key finding, the release of the bioactive from the conduits was dependent on the slow-degrading characteristic of the microspheric system as well as on the "maximum amount of microspheres" that could be incorporated into the cylindrical structure. Another

important finding of the report was that the liquid–liquid centrifugal casting process demonstrated potential towards localized and controlled release of multiple factors key to tissue regeneration after neural injuries [24].

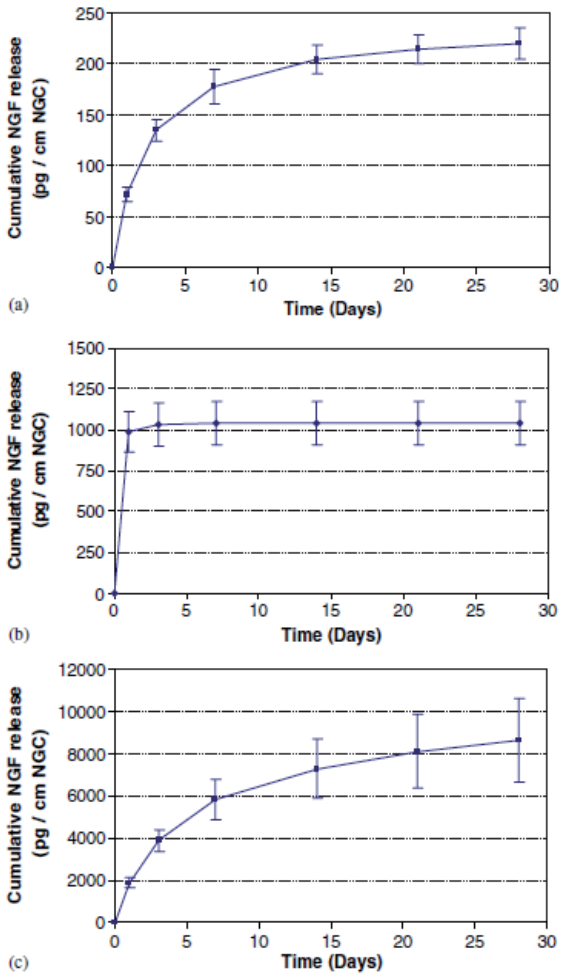

Figure 8. Cumulative release profile of NGF (pg/cm of NGC) over a 28-day period from NGCs (a) coated with an inner layer of PHEMA containing NGF-loaded PLGA 85/15 microspheres; (b) imbibed with NGF; and (c) coated with an inner layer of PHEMA containing directly entrapped NGF [Adapted from Ref. 24 with permission © Elsevier Science BV].

2.16. Self-assembling peptide nanofiber scaffold enhanced with RhoA inhibitor

In a recent study, Zhang et al., 2012, focused on two major targets for axonal regeneration after a spinal cord injury:

a. The reconstruction of the gap in the injured spinal cord
b. Inactivation of RhoA to ameliorate the hostile microenvironment perilesion

To achieve these targets, Zhang et al., 2012, transplanted self-assembling peptide nanofiber scaffold (SAPNS) based local delivery of RhoA inhibitor to the lesion sites after acute spinal cord injury centred on their previous reports wherein the SAPNS had shown multiple regeneration-facilitating properties to improve the recovery of the injured spinal cord and brain and that SAPNS has potential for controlled bioactive release. The study was based on the hypothesis that this novel combination might act by providing regrowth-promoting scaffold reducing the physical obstacles after injury (characteristic SAPNS capability of filling the cavities and alleviating glial scarring) and the RhoA inhibitor released from the combination implants may exert a therapeutic effect conducive for axonal regrowth.

As depicted in **Figure 9**, the SAPNS implants, with or without CT04 incorporation, reconstructed the injured spinal cord whereas a significant gap was developed in the lesion site in the saline group. Additionally, the implants integrated closely with the surrounding host tissue with no cavities or gaps occurring between the implants and host tissue. Another striking finding was the presence of the cells such as fibroblasts, which mainly clustered in the centre of the lesion area and formed collagen fibers, along with lymphocytes, macrophages, astrocytes, and microglias scattered among the fibroblasts. The growth of such cellular mass may further hindered the cavity or cysts formation- a major cause of anatomical disconnection after SCI. On the drug delivery note, SAPNS delivery of CT04 significantly improved the axonal regrowth. Based on fluorescent characteristics of dextran, it was incorporated into SAPNS to track the spread-out and diffusion of the bioactive in the lesion site. Seven days after the transplantation of the SAPNS incorporated with Dextran, significant fluorescence was observed into the surrounding host cord from the SAPNS, while the implant still retained high fluorescent signal confirming the release of chemicals from SAPNS and indication that SAPNS can effectively serve as a platform for further controlled release of exogenous therapeutic molecules of interest. In conclusion, the SAPNS delivery system proved to be capable of minimal risk of carrying biological pathogens or contaminants, providing 3-dimensional environment constructed for cell growth and migration, minimal cytotoxicity to the host, no apparent immune response, easy conformity to the various shape of lesion cavities, and immediate haemostatic and controlled drug release properties [25].

2.17. Sustained delivery of chondroitinase ABC from hydrogel system

As explained earlier in this report, chondroitin sulfate proteoglycans (CSPGs) are among the principal factors responsible of axon growth inhibition and they contribute to regenerative failure, promoting glial scar formation in an injured spinal cord. Chondroitinase ABC (chABC), although capable of digesting these proteoglycans leading to the degradation of glial scar and favoring axonal regrowth, suffers from delivery drawbacks such as administration being invasive, infection-prone and clinically problematic. Rossi et al., 2012, developed an agarose-carbomer (AC1) hydrogel, which they previously used in SCI repair strategies [26-28], as a delivery system capable of an effective chABC administration based on following considerations: ability to include chABC within its pores and the possibility to be injected into the target tissue; and release kinetics and the maintenance of enzymatic activity can be

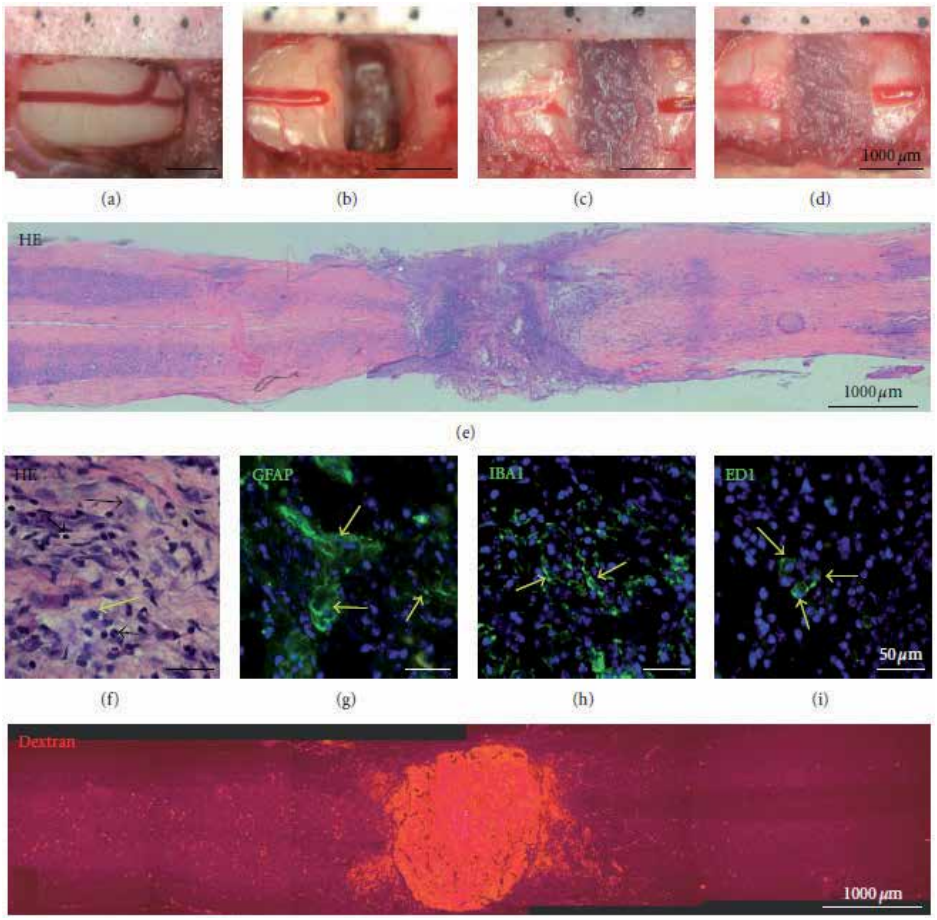

Figure 9. The implants of various SAPNS reconstructed the injured spinal cord. (a–d)Macroscopic observations of lesion sites immediately after surgery. (a) Sham surgery; (b) saline group; (c) SAPNS with vehicle group; (d) SAPNS+CT04 combination group. (e) HE staining shows the lesion area was reconstructed 12 weeks after SAPNS transplanted. (f) HE staining shows various cells in the grafted scaffold, which include fibroblast (black arrow), macrophage (yellow arrow), and lymphocyte (arrow head). (g–i) Immuno-staining shows the GFAP-positive astrocytes (g), IBA1 positive microglias (h) and ED1 positive macrophages (i) in the grafted scaffold. (j) shows the Dextran, a red fluorescent dye, was partially diffused from the mixture of SAPNS and Dextran at 7 days after transplantation [Adapted from Ref. 25 © Hindawi Publishing Corporation].

positively assessed. Additionally, being a hydrogel, AC1 had its own advantages in terms of (I) the ability to retain water; (II) mimicking living tissues; (III) high biocompatibility; and (IV) the possibility to allow precisely controlled release rates. As demonstrated in Figure 10, AC1 appeared to be a chemical gel synthesized through a statistical block polycondensation between Carbomer 974P and agarose. chABC was loaded before the sol/gel transition resulting in its physical entrapment within the three-dimensional polymeric network of the gel. Macroscopically, a highly entangled nanostructure was confirmed by ESEM analysis proving its ECM mimicking nature. In brief, Carbomer 974 P provided the main cross-linking

properties via its carboxylic groups which in turn reacted with hydroxyl groups from agarose leading to the formation of a three dimensional matrix. The in vitro release kinetics displayed typical hydrogel characteristics wherein a rapid initial release of Tx was observed during the initial phases due to a high concentration gradient possible caused by: molecules placed near solvent-hydrogel interface that may rapidly escape into the supernatant solution; molecules diffusing through the large pores of the hydrogel in comparison to the molecules diffusing through smaller pores. The burst release was followed by slower release and eventually forming a plateau. After this initial burst, the release became slower and reached a plateau - steady state condition - after 7 days. Importantly, the whole amount of Tx loaded was release eventually confirming the absence of any stable bonds between hydrogel polymeric network and loaded Tx. Furthermore, it confirms the hydrogel's capacity to deliver even those molecules that are deeply entrapped in its core [29].

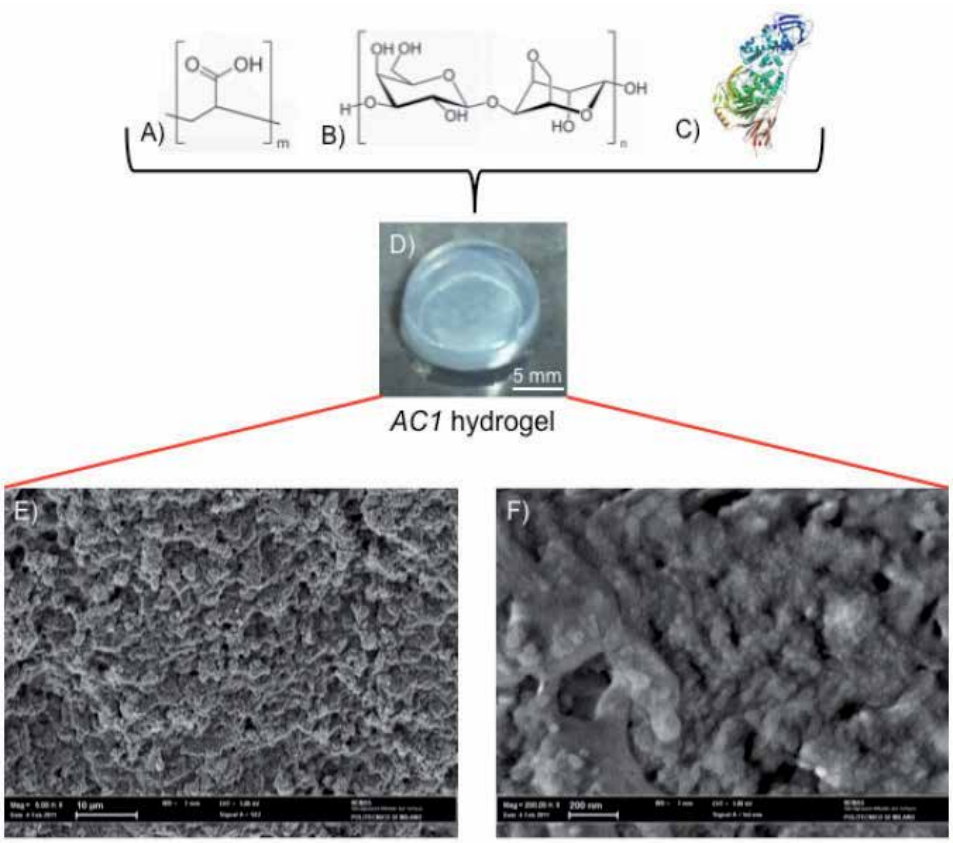

Figure 10. Carbomer 974P (A); agarose (B) were chemically cross-linked to form hydrogel in phosphate buffer saline solution. Esterification, hydrogen bonding and carboxylation bring polymer chains statistically closer, thus creating a stable heterogeneous structure. The gelling solution was homogenized together with chondroitinase ABC (chABC); (C) above sol-gel transition temperature. From a macroscopic point of view the resulting material appears as in (D). From a microscopic point of view the hydrogel is densely structured, as observable by ESEM analysis. [Adapted from Ref. 29 © MDPI Publishing]

2.18. Injectable functionalized self-assembling scaffold

Self-assembling peptides (SAPs) perfectly mimic the extra-cellular matrix's structural and conformational characteristics; can be injected into the lesion site; are reabsorbable; and have sites for biofunctionalizations making them a perfect nanomaterial for application in regenerative. Cigognini et al., 2011, explored the biofunctionalizability of self-assembling peptides to enhance the *in vitro* neural stem cells survival and differentiation. This injectable nanofibrous gel was composed of RADA16-I functionalized with a bone marrow homing motif (BMHP1) which was further optimized via the insertion of a 4-glycine-spacer for amelioration of scaffold stability and exposure of the biomotifs. The scaffold was injected immediately after contusion in the rat spinal cord was evaluated for its putative neuroregenerative properties in terms of early effects by semi-quantitative RT-PCR and the late effects by histological analysis. The axon regeneration/sprouting across the cyst was quantified by the presence of GAP-43 positive fibers and the relative value of GAP-43 immunopositive area was represented as percentage of the total cyst area (Figure 11Bi). Interestingly, a significantly greater synthesis of GAP-43 in 4G-BMHP1 group (12.9462.03% of the whole cyst area) in comparison with saline (6.3361.7% of the whole cyst area) and SCI control (5.8461.29% of the whole cyst area) groups was originated (Figure Bii). The chronic inflammatory response, evaluated by counting macrophages into the lesion site (Figure 11Ci), revealed that several infiltrating CD68 immunopositive cells in all groups, indicating that at the late phase of the injury the scaffold didn't significantly affect the host immune response (Figure Cii). Similarly, cyst and cavities extent, measured on hematoxylin-eosin stained sections (Figure 11Ai), was similar in all groups (Figure Aii) wherein no significant differences were observed among treatment and both control groups when the cavities size within the cyst excluding strands of connective tissue (trabeculae) were measured (Figure Aiii). Conclusively, the injectable SAP based scaffold demonstrated potential for enhanced matrix remodelling, release of trophic factors, cell migration and basement membrane deposition leading to an increased number of regenerating/sprouting axons after incomplete SCI. Apart from the neuro-compatibility, the scaffolds were able to fill the cavities at SCI site with implications leading to a good matrix for the in vivo delivery of growth factors and/or stem cells into the injured CNS [30].

2.19. Effects of dibutyryl cyclic-AMP on survival and neuronal differentiation of neural stem/progenitor cells

Enthused by the poor control over transplant cell differentiation and survival, Kim et al., 2011, introduced a combinatorial approach wherein the cell transplantation therapy was adjoined with a bioactive release platform. Firstly, dibutyryl cyclic-AMP (dbcAMP) was encapsulated within poly(lactic-co-glycolic acid) (PLGA) microspheres and embedded within chitosan guidance channels. Secondly, neural stem/progenitor cells (NSPCs) were seeded in fibrin scaffolds within the channels (Figure 12). The idea behind the study was to determine whether dbcAMP, which can influence the in vitro differentiation of NSPCs into neurons, will be able to enhance survival of transplanted NSPCs through prolonged exposure either in vitro or in vivo through its controlled release from microspheres in

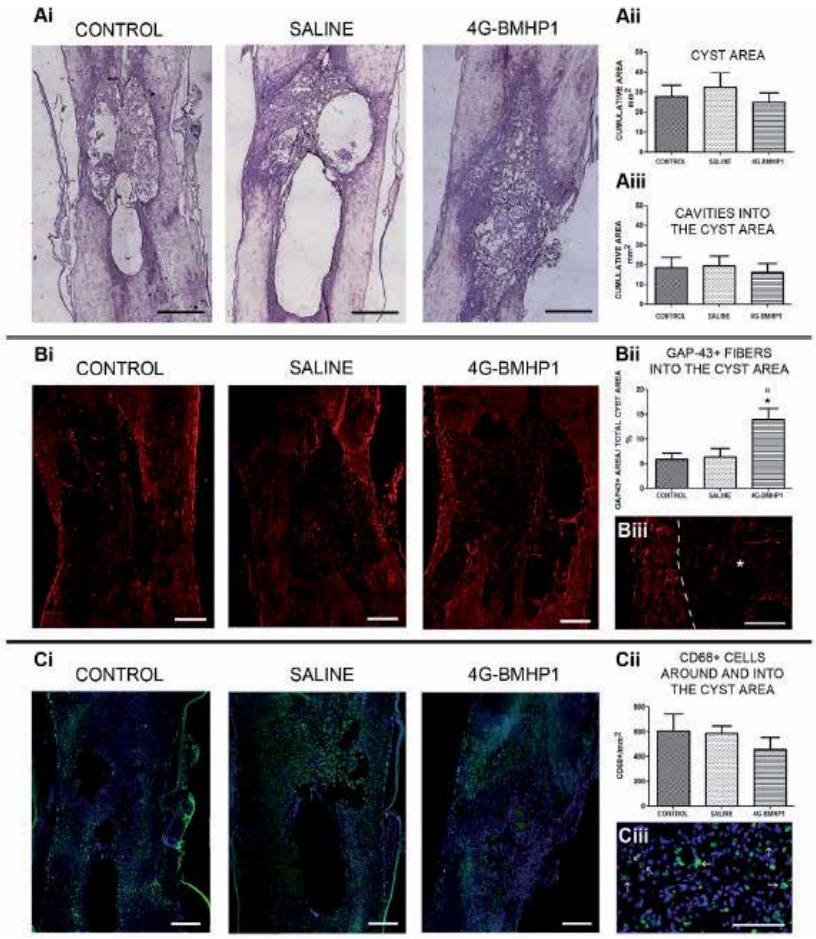

Figure 11. Quantitative histological analysis in the chronic phase of SCI. (A): lesion size was quantified on spinal cord longitudinal sections stained with hematoxylin/eosin (Ai) and it was reported as cumulative area (mm2). No significant differences among groups were found when measuring both the whole cyst area (Aii) and the cavities into the cyst area (Aiii). (B): GAP-43 positive fibers (Bi and Biii, red) were measured on six longitudinal sections after immunofluorescence staining and the values were expressed as percentage of the total area of the cyst. The GAP-43 immunopositive area was significantly higher in biomaterial-treated group (4G-BMHP1) than both control groups (saline and SCI control) (Bii). In Biii the positive GAP-43 signal is showed at higher magnification (asterisk and dotted line indicate the cyst and its border, respectively). (C): CD68 positive cells (Ci and Ciii, green) were counted on three longitudinal sections after immunofluorescence staining and reported as cumulative number per mm2. Nuclei were counterstained with DAPI. Macrophage infiltration was observed in the tissue surrounding the cyst and into the cavities of all groups (Cii). In Ciii, at higher magnification, an image representative of the CD68 positive cells (arrows) was observed in all groups [Adapted from Ref. 30 © PLoS ONE].

vicinity of NSPCs. The in vitro differentiation if NSPCs to betaIII-tubulin positive neurons was evaluated by immunostaining and mRNA expression. The in vivo studies were conducted by transplantation in spinal cord injured rats wherein the survival and differentiation of NSPCs was evaluated. For the greatest neuronal differentiation in the

presence of constant dbcAMP, the dbcAMP release from PLGA microspheres was optimized to release the bioactive for a period of 1 week based on the preliminary in vitro data. As depicted in Figure 12, drug release from native microspheres was linear over 11 days while the release of dbcAMP occurred over approximately 5 days after incorporation into channels. The mass balance results cleared this anomaly in release profiles which showed that the there was less drug content in dbcAMP microsphere loaded channels than expected microsphere quantity which was further due to drug losses during the process of embedding microspheres into the channel. Two major results were reported for the NSPC survival and differentiation as follows:

a. NSPC survival was highest in the dbcAMP pre-treated group, having approximately 80% survival at both time points as compared to that of stem cell transplantation results of less than 1% survival at similar times.
b. The dbcAMP pre-treatment resulted in the greatest number of in vivo NSPCs differentiated into neurons (3764%), followed by dbcAMP-microsphere treated NSPCs (27614%) and untreated NSPCs (1567%).
c. The reverse trend was observed for NSPC-derived oligodendrocytes and astrocytes, with these populations being highest in untreated NSPCs.
d. Chitosan channels implanted in a fully transected spinal cord resulted in extensive axonal regeneration into the injury site, with improved functional recovery after 6 weeks in animals implanted with pre-differentiated stem cells in chitosan channels [31].

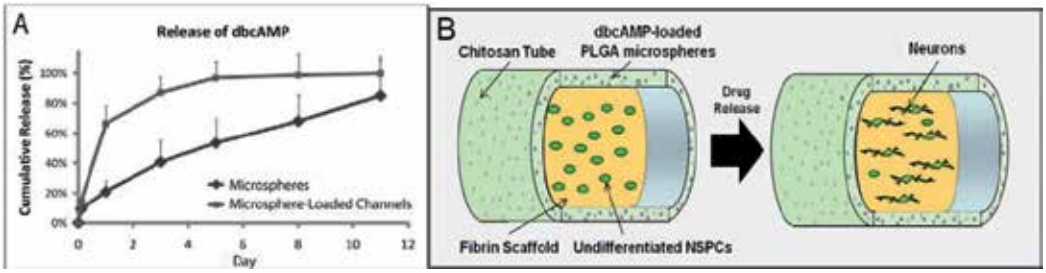

Figure 12. Microsphere-loaded channels effectively release dbcAMP in vitro. A) Cumulative release profiles of dbcAMP from free-floating microspheres and microsphere-loaded channels. The process of embedding microspheres into channel walls is likely responsible for early degradation of PLGA and faster drug release from channels. B) Schematic of the entubulation strategy. NSPCs are seeded on fibrin scaffold within a chitosan channel. Drug-loaded PLGA microspheres release the differentiation factor dibutyryl cyclic-AMP in a local and sustained manner, influencing NSPCs to preferentially differentiate into neurons [Adapted from Ref. 31 © PLoS ONE].

2.20. Local gene delivery from ECM-coated poly(lactide-co-glycolide) multiple channel bridges

In order to promote transgene expression in the injured spinal cord, Laporte et al., 2009, investigated the potential of surface immobilization to deliver complexed DNA (lipoplexes) from a multiple channel bridge in order to address the barriers related to cell survival, scar

tissue formation, and axonal elongation and guidance by targeting a range of cellular processes. The report was based on the inherent low expression levels of lipoplexes due to the lipoplex instability upon injection in vivo. The authors postulated that the "local delivery of lipoplexes from a biomaterial may have the ability to maintain lipoplex stability, and therefore increase the number of transfected cells and transgene expression". Based on their previous study, multiple linear guidance channels were able to support cell infiltration and integrate effectively into the spinal cord wherein the channels induced cell orientation along its major axis and supported and directed axon elongation across the channels [32]. Strategically, lipoplexes were immobilized to the surface of the bridges as follows:

i. incubation of DNA with extra cellular matrix (ECM)-coated PLGA surfaces (incubation),
ii. drying of ECM onto PLGA and then drying of DNA onto ECM (2-step drying), and
iii. drying a mixture of DNA and ECM proteins onto PLGA surfaces (1-step drying).

A series of in vitro studies were carried out to investigate the surface properties of the polymer, three ECM proteins (collagen, laminin, and fibronectin), and the immobilization strategies for their ability to bind and stabilize the vector, and to transfect cells. In vivo studies were performed with a rat spinal cord lateral hemisection model using conditions identified from in vitro studies. In conclusion, the combination of the multiple channel bridge and gene delivery provided the required physical and chemical guidance cues for spinal cord regeneration [33].

3. Conclusion

The current drug delivery and tissue engineering techniques discussed in this chapter demonstrate that a only a combinatorial approach can provide the desired characteristics required for an efficient repair, regeneration, restoration, reconstruction, and reorganisation of the neural tissue after traumatic CNS injuries. A synergistic therapeutic approach can be designed by incorporating scaffolds with directional cues, bioactives to promote regeneration and repair, neural/progenitor cells for release of growth factors, and functionalized polymers for better neurocompatibility. Most importantly, the design of polymers capable of releasing the drugs, cells and biofactors at a predetermined and controlled rate is essential for the success of these tissue engineering approaches (Table 4). The chapter provides a comprehensive account of recent as well as long established approaches for the delivery of bioactive of neural interest. For further reading, the readers are encouraged to read various enlightening reviews in this area as tabulated in Table 5.

S. No.	Architecture	Biomaterials employed	Bioactive incorporated	Reference
1.	Injectable gel	Hyaluronic Acid-Methyl cellulose (blend)	Nimodipine; Erythropoietin	2-4
2.	Nanoparticles-incorporated multiple channel bridges	PLGA (scaffold); Hydroxyapatite (nanoparticles)	lentiviral vectors encoding NT-3 and BDNF	5

S. No.	Architecture	Biomaterials employed	Bioactive incorporated	Reference
3.	Lipid microtubules-incorporated In situ gelling hydrogels	1,2-bis-(triscosa-10,12-diynoyl)-sn-glycero-3-phosphocholine; Agarose	brain derived neurotrophic factor (BDNF)	6
4.	Photocrosslinked 3D scaffold	Agarose; Sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate	Laminin-1	7
5.	Nanoparticles-incorporated injectable gel	PLGA (nanoparticles); agarose gel	Methylprednisolone	9,10
6.	hydrogel-microtube delivery system	Agarose; 1,2-bis-(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine microtube	Chondroitinase ABC; Rho GTPase, CA-Rac1 and CA-Cdc42; and BDNF	11,12
7.	3D Scaffolds	PLGA	Schwann cells	13
8.	micro-cylinders embedded hydrogels	1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine; Agarose	nerve growth factor (NGF)	14
9.	injectable scaffold	Agarose; methylcellulose	-	15
10.	Injectable hydrogel	acrylated poly(lactic acid)-b–poly(ethylene glycol)-b–(poly lactic acid)	NT-3	16
11.	crosslinked hydrogels containing embedded microspheres	hyaluronic acid (HA); poly(lactic-co-glycolic acid) (PLGA)	BDNF and VEGF-loaded	17
12.	scaffolds	Fibrin; heparin; bi-domain peptide (ATIII peptide)	neurotrophin-3 (NT-3)	20
13.	Nerve guidance channels combined with microspheres	poly(hydroxylethylmethacrylate-co-methylmethacrylate); PLGA	Nerve growth factor	24
14.	nanofiber scaffold	Self assembling peptides	RhoA inhibitor	25
15.	Injectable hydrogel	Agarose; carbomer	Chondroitinase ABC (chABC)	29
16.	Self assembling peptide gel	RADA16-1	bone marrow homing motif (BMHP1)	30
17.	microspheres embedded within guidance channels	poly(lactic-co-glycolic acid) (PLGA); Chitosan; fibrin	dibutyryl cyclic-AMP (dbcAMP)	31
18.	disks and multiple channel bridges	poly(lactic-co-glycolic acid) (PLGA)	DNA complexes	33

Table 4. Summary of various bioactive-loaded platforms for spinal cord injury intervention

S. No.	View point discussed in the review	Reference
1.	The current approaches to drug delivery from scaffolds for neural tissue engineering applications and the challenges presented by attempting to replicate the brain, spinal cord, and peripheral nerve tissues were summarized	34
2.	The complex processes of cell guidance occurring within native ECM; strategies to design biomimetic scaffolds able to recapitulate these processes; approaches in controlling the release of the relevant factors; challenges to design novel scaffolds; time and space orchestrated exposure of biomacromolecular moieties	35
3.	Tissue engineering and novel therapeutic approaches to axonal regeneration following spinal cord injury. Axonal growth is supported by inherent properties of the selected polymer, the architecture of the scaffold, permissive microstructures such as pores, grooves or polymer fibres, and surface modifications to provide improved adherence and growth directionality.	36
4.	The state of the art work in electrospinning and its uses in tissue engineering and drug delivery	37
5.	Electrospun fibers mimic the nanoscale properties of native extracellular matrix; the fiber morphology is affected by changing the process parameters	38
6.	Tissue-engineered implant is a biologic-biomaterial combination in which some component of tissue has been combined with a biomaterial to create a device for the restoration or modification of tissue or organ function; Specific growth factors, released from a delivery device or from co-transplanted cells aid in the induction of host paraenchymal cell infiltration and improve engraftment of co-delivered cells for more efficient tissue regeneration; polymeric device development (micropaticles, scaffolds, and encapsulated cells) for therapeutic growth factor delivery in the context of tissue engineering was outlined.	39
7.	The tissue engineering paradigm includes a matrix or scaffold to facilitate tissue growth and provide structural support, cells, and the delivery of bioactive molecules; injectable materials are designed using processing techniques inherent to both tissue engineering and drug delivery.	40
8.	Basic technology of controlled protein delivery using polymeric materials; techniques under investigation for the efficient administration of proteins in tissue engineering	41
9.	An overview of strategies using natural and artificial substrates to present active biomolecules in the development of vascular structures; the replacement and augmentation of arteries using vascular grafts or stents; the recruitment of microvasculature secondary to an ischemic event or for the purpose of developing perfused, large volume tissue-engineered constructs	42
10.	Polyblend nanofibers and nanostructures can act as proxies of the native tissue, while providing topographical and biochemical cues that promote healing; they are prepared with mixtures of synthetically and naturally derived polymers that can behave cooperatively to demonstrate unique combinations of mechanical, biochemical and structural properties.	43
11.	Synthetic polymers used for tissue growth scaffold fabrication and their applications in both cell and extracellular matrix support and controlling the release of cell growth and differentiation supporting drugs.	44

S. No.	View point discussed in the review	Reference
12.	In the future, engineered tissues could reduce the need for organ replacement, and could greatly accelerate the development of new drugs that may cure patients, eliminating the need for organ transplants altogether.	45
13.	Biodegradable and biocompatible scaffolds have a highly open porous structure and good mechanical strength to provide an optimal microenvironment for cell proliferation, migration, and differentiation, and guidance for cellular in-growth from host tissue; natural and synthetic polymeric scaffolds can be fabricated in the form of a solid foam, nanofibrous matrix, microsphere, or hydrogel; scaffolds can be surface engineered to provide an extracellular matrix mimicking environment for better cell adhesion and tissue in-growth and can be designed to release bioactive molecules, such as growth factors, DNA, or drugs, in a sustained manner	46
14.	To develop electrospun nanofibers as useful nanobiomaterials, surfaces of electrospun nanofibers can be chemically functionalized by plasma treatment, wet chemical method, surface graft polymerization, and co-electrospinning of surface active agents and polymers for achieving sustained delivery through physical adsorption of diverse bioactive molecules such as anti-cancer drugs, enzymes, cytokines, and polysaccharides; surfaces can be chemically modified with immobilizing cell specific bioactive ligands to enhance cell adhesion, proliferation, and differentiation by mimicking morphology and biological functions of ECM.	47
15.	Biomaterials can enable and augment the targeted delivery of drugs or therapeutic proteins to the brain, allow cell or tissue transplants to be effectively delivered to the brain; help to rebuild damaged circuits; promote regeneration; repair damaged neuronal pathways in combination with stem cell therapies; nanotechnology allows greater control over material–cell interactions that induce specific developmental processes and cellular responses including differentiation, migration and outgrowth	48
16.	Application of biomaterials in (i) shunting systems for hydrocephalus, (ii) cortical neural prosthetics, (iii) drug delivery in the CNS, (iv) hydrogel scaffolds for CNS repair, and (v) neural stem cell encapsulation for neurotrauma	49
17.	Recent in vivo studies for the regeneration of injured spinal cord, including stem-cell transplantation, application of neurotrophic factors and suppressor of inhibiting factors, development of biomaterial scaffolds and delivery systems, rehabilitation, and the combinations of these therapies	50
18.	Applications of polymer-based delivery of small molecule drugs, proteins, and DNA specifically relevant to neuroscience research; the fabrication procedures for the polymeric systems and their utility in various experimental models; unique experimental tools with downstream clinical application for the study and treatment of neurologic disease offered by biomaterials	51
19.	Approaches adopted for management of SCI including pharmacologic and gene therapy, cell therapy, and use of different cell-free or cell-seeded bioscaffolds; developments for therapeutic delivery of stem and nonstem cells to the site of injury; application of cell-free/cell-seeded natural and synthetic scaffolds	52

S. No.	View point discussed in the review	Reference
20.	bioengineered strategies for spinal cord repair using tissue engineered scaffolds and drug delivery systems; degradable and non-degradable biomaterials; device design; combination strategies with scaffolds.	53
21.	The merger between the two powerful disciplines—biomaterials engineering and stem cell biology	54
22.	Inclusive survey of biopolymers seeded with Schwann cells (SCs) to be used for axonal regeneration in the nervous system	55
23.	Recent studies that utilize electrospun nanofibers to manipulate biological processes relevant to nervous tissue regeneration, including stem cell differentiation, guidance of neurite extension, and peripheral nerve injury treatments	56
24.	Smart biomaterials are capable to carry and deliver cells and/or drugs in the damaged spinal cord; an overview of a wide range of natural, synthetic, and composite hydrogels	57
25.	Self-assembled materials provide the ability to tailor specific bulk material properties, such as release profiles, at the molecular level via monomer design; an overview of self-assembling molecules, their resultant structures, and their use in therapeutic delivery; current progress in the design of polymer- and peptide-based self-assembled materials.	58

Table 5. Leading review reports incorporating the inherent mechanistic and fabrication details of various neural architectures employed for neural tissue engineering and drug delivery

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Chapter 14

Novel Drug Delivery Systems for Modulation of Gastrointestinal Transit Time

Yousef Javadzadeh and Sanaz Hamedeyazdan

Additional information is available at the end of the chapter

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1. Introduction

In view of from recent advancements, in a time of augmented remarks to the efficacy and safety of the medicines embedded in dosage forms, it seems as important as ever to study the pharmaceutical concept of the latter in different aspects, prerequisite for a successful pharmacotherapy.

Despite tremendous advancements in drug delivery, oral administration of therapeutic agents still remains the favored rout for majority of clinical applications, due to the excellent accessibility, and patient compliance as well as the preferred alternative route of drug administration for non-invasive drug delivery among the other various routes [Motlekar & Youan, 2006]. Although there have been several limitations in oral administration of drugs like non-uniform drug absorption profiles, incomplete drug release, shorter residence time of dosage forms in absorption window and etc. to systemic circulation it has come a long way. Now, it has turned towards modifying and manipulating oral dosage forms to exploit from different conditions of the gastrointestinal (GI) tract for drug delivery in various ways [Hirtz, 1985].

In view of the fact that the correlation between drug intake and a clinical response is complex enough, the choice and design of the ideal pharmaceutical dosage form of a drug delivery system would be critically important to reach a progress in superior drug development. Variations between drug response within individuals could be attributed either to product bioavailability (like the rate and extent of drug absorption), drug pharmacokinetics (which includes the metabolism, distribution, and elimination of a drug), or the particular concentration-effect relationship, which in turn could potentially be influenced through a mixture of intrinsic and extrinsic variables [Hirtz, 1985; Martinez & Amidon, 2002; Javadzadeh et al., 2010].

With reference to the Biopharmaceutical Classification System, introduced by the Food and Drug Administration (FDA) in 1995, drugs have been categorized in terms of their solubility and intestinal permeability in four primary groups (Figure 1).

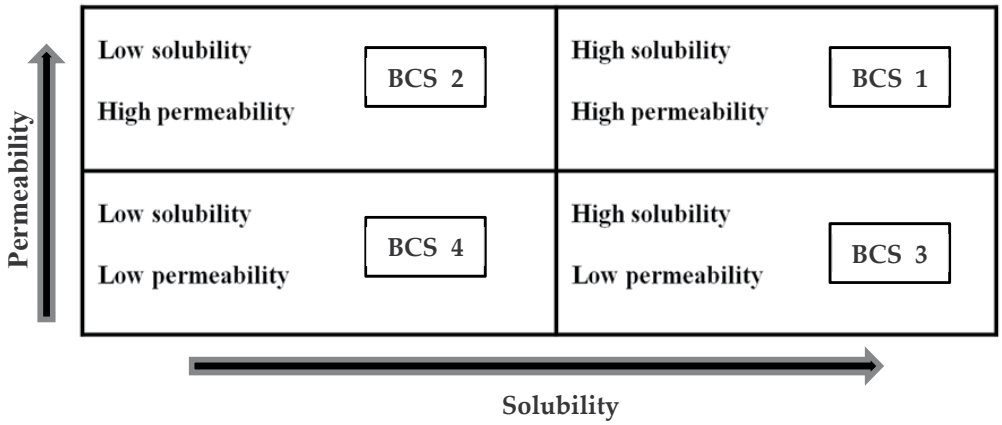

Figure 1. The Biopharmaceutical Classification System (BCS) as defined by the FDA

Drugs that are categorized in class one (BCS 1) are supposed to be well absorbed when taken orally, due to their high solubility and high permeability characteristics. Whereas, all other drugs in BSCs 2 to 4 suffer from low solubility, low permeability or both, and would present challenges to the drug developments. Drugs that are classified in low solubility groups keep drug absorption via the GI tract as the limiting factor for reaching in suitable blood-levels of drugs [Martinez & Amidon, 2002; Javadzadeh et al., 2007).

As far as we know, absorption of drugs from intake (e.g., oral delivery systems) to its site of action is a complex process, or more accurately, a combination or succession of complicated processes which is schematically presented in Figure 2.

After oral administration, drug leaves the formulation and dissolves in the aqueous digestive fluids. It reacthes and crosses the GI mucosal membrane before passing into blood, moving along the GI tract with the luminal content at a variable speed. Once the drug is reached into the blood circulation, processes of drug metabolism and elimination starts, whilst, it is also possible for some drug degradation or metabolism to occur sooner than the absorption is completed leading for a decrease in drug efficiency [Riley et al., 1992].

In general, absorption of drugs through GI tract varies on account of many factors like the nature and surface area of the GI mucosal membrane varying from the stomach to the rectum, as well as the physicochemical properties of the pharmaceutical dosage form along with the luminal content. Hence, a balance among these factors would have a notable effect on how much of the administered drug reaches to the bloodstream that is summed up by the term bioavailability. Considering the pharmacokinetics and disciplines of drug discovery and drug delivery, it has become obvious that a deep understanding of the biology and anatomy of the GI tract is of substantial value for optimizing the bioavailability of orally administered drugs.

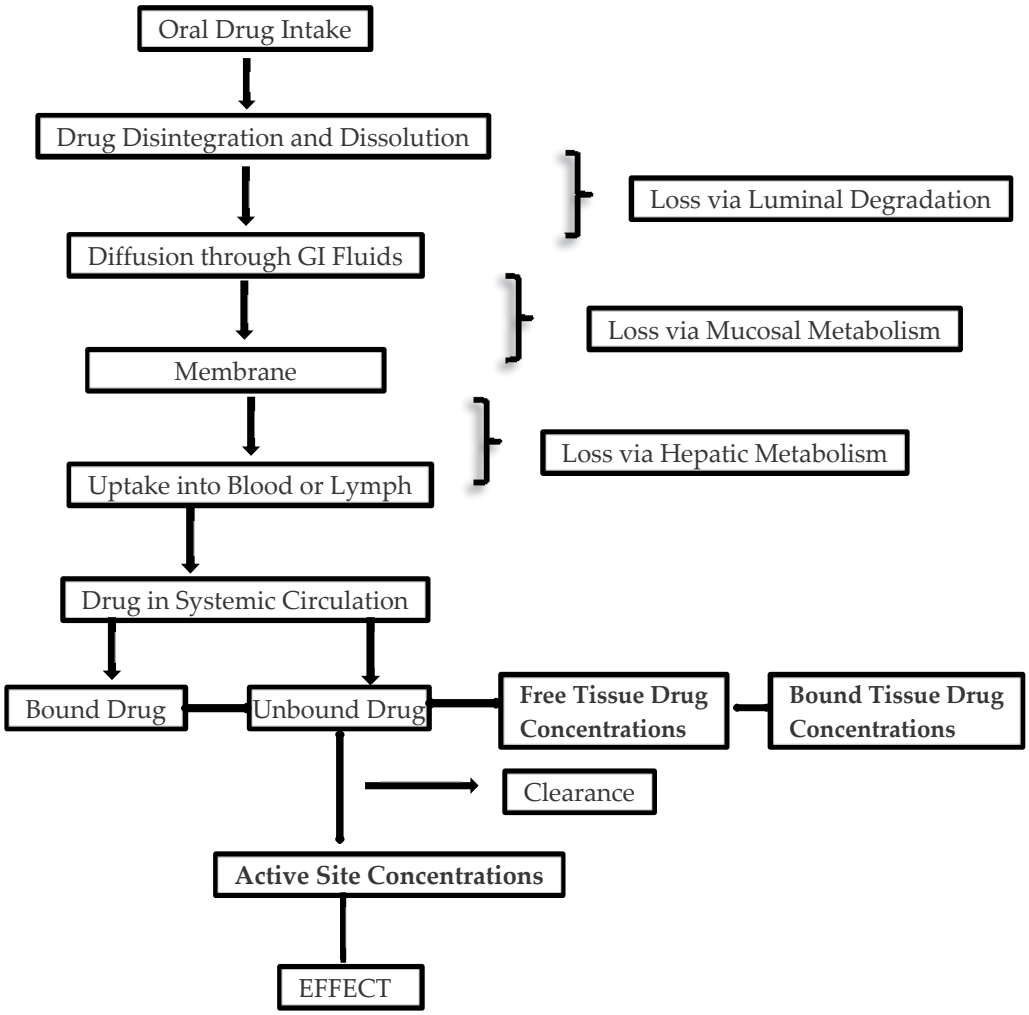

Figure 2. Schematic representation of the relationship between an oral dosage form of a drug and its ultimate effect.

2. GI anatomy and dynamics

GI tract is a group of organs joined in a long tube which is divided into several sections, each of that fulfills a specific function. The tract begins with oral cavity, follows pharynx, esophagus, stomach, small intestine and large intestine ending with rectum to anus part (Figure 3).

Each segment has certain morphological and physiological features, but there is almost a common structure for all parts of GI with muscular walls comprising four different layers: inner mucosa, submucosa, muscularis externa, and the serosa.

Stomach serves the most primarily mixing area and a reservoir that secretes pepsinogen, gastric lipase, hydrochloric acid, and the intrinsic factor. This section of the GI tract is

normally impermeable for absorption of most materials into the blood except water, ions, alcohol, and certain drugs such as aspirin. It follows small intestine, the major digestive and absorptive site of the GI tract, providing an extended surface area by the circular folds of about 10 mm height through the microvilli on the absorptive cells of the mucosa. The last part of the GI tract is the large intestine extending from the ileum to the anus with 1.5 m long and 6.35 cm in diameter serving mainly the fecal storage and water absorptive roles. It is significant that no enzymes are secreted in the mucosa, and the last stage of digestion is primarily conducted by the resident bacteria [McConnell et al., 2008].

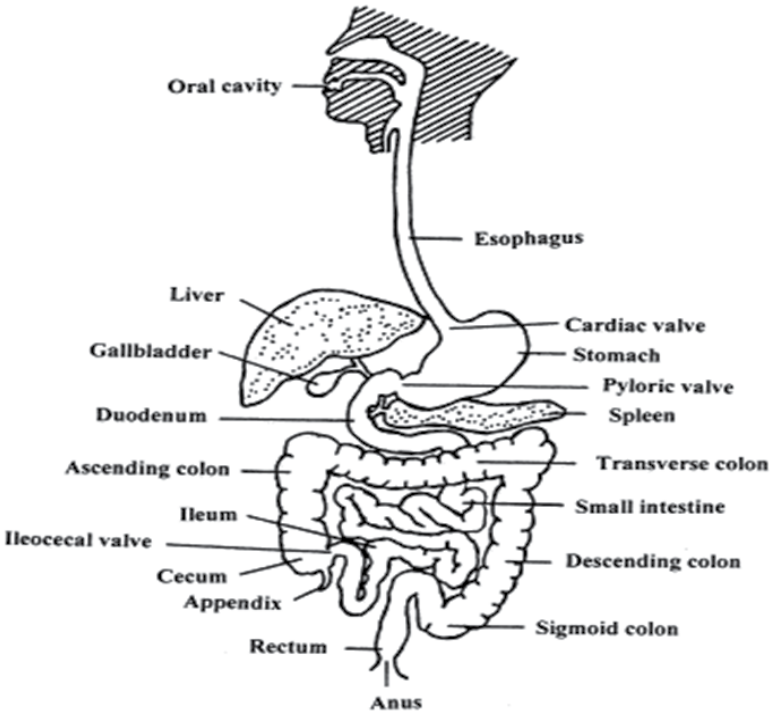

Figure 3. Anatomy of the human gastrointestinal tract [Friend & Tozer, 1992).

Not surprisingly, these anatomical and physiological parameters of the GI tract remarkably affect the rate and level of drug absorptions and should be assessed. There are several difficulties and rules in designing controlled release systems to be considered for better absorption and enhanced bioavailability of drugs embedded in the orally administered dosage forms. One principal requisite for the successful performance of orally administered drugs is that the drug should have good absorption throughout the GI tract, to certify continuous absorption of the released drug [Davis, 2005]. Regarding the BCS classification of drugs, this could be a major constraint in oral controlled drug delivery systems that not all drugs are evenly absorbed throughout the GI tract. Seeing as, most of the orally administered drugs display region-specific absorption that could be related to differential drug solubility and stability in different regions of the GI tract as a result of changes in environmental pH, degradation by enzymes present in the lumen of the intestine or interaction with endogenous compounds (Table 1). Furthermore, drugs that are substrates

of certain enzymes in particular regions of the GI tract might have regional variability in their absorption along the GI tract. One should consider this region specific drug absorption concept for designing the ideal drug formulation. It has been widely accepted that about 90% of all absorption of nutrients takes place in the small intestine, and the rest occurs in the stomach and large intestine, depending on the defining characteristics of GI fluids and membrane surface area at different locations [Davis, 2005]. Consequently, transit time of a pharmaceutical dosage form through the GI tract would determine how long a drug will be in contact with its preferred absorptive region.

Section	Length (cm)	Diameter (cm)	pH	Major constituents	Transit time (h)
Oral cavity	15- 20	10	5.2- 6.8	Amylase, Maltase, Ptyalin, Mucins	Short
Esophagus	25	2.5	5- 6	-	Very short
Stomach	20	15	1.2– 3.5	Hydrochloric acid, Pepsin, Rennin, Lipase, Intrinsic factor	0.25- 3
Duodenum	25	5	4.6– 6.0	Bile, Trypsin, Chymotrypsin, Amylase, Maltase, Lipase, Nuclease, CYP3A4	1- 2
Jejunum	300	5	6.3– 7.3	Amylase, Maltase, Lactase, Sucrase, CYP3A5	-
Ileum	300	2.5- 5	7.6	Lipase, Nuclease, Nucleotidase, Enterokinase	1- 10
Cecum	10- 30	7	7.5 - 8	-	Short
Colon	150	5	7.9 - 8	-	4- 20
Rectum	15- 19	2.5	7.5 - 8	-	Variable

Table 1. Anatomical and physiological features of the human GI tract

The usual transit time for a drug formulation or for a nutrient to pass from the stomach to the ileocecal valve (through the small intestine) is around three hours and transit time for colon is much longer up to twenty hours or more. Subsequently, the time a drug will have in its absorption site would be fairly short, more so if the drug is preferentially absorbed in the proximal small intestine rather than throughout the small intestine. Accordingly, a drug that is largely or solely absorbed from the upper GI tract would have variable bioavailability distressed by factors that change GI transit. Moreover, GI transit time of a drug formulation as the most limiting physiological factor in the development of oral drug delivery systems might be altered by the physiological properties of the GI tract too. The patterns of GI transit and gastric emptying depend on whether the person is in a fasted or fed state, and the physical state of the drug delivery system, either a solid or a liquid form [Bode et al., 2004].

Gastric emptying of liquids in the fasted state is a function of the volume administered, whereas, indigestible solids are emptied from the stomach as a function of their physical size [Bardonnet et al., 2006; Khobragade et al., 2009].

There also exist several issues affecting motility of the GI tract such as systemic disease, drug therapy and intrinsic GI disorders. Diabetes, idiopathic intestinal muscle disease, Chaga's disease, muscular dystrophy, gastroparesis and etc. are characterized by delayed gastric emptying and a reduction in GI motions. On the contrary, in the case of diarrhea, infectious diseases, thyrotoxicosis and irritable bowel syndrome an increased GI motion is usually detected [McConnell, Fadda et al., 2008]. Even though, increasing the rate of gastric emptying and GI motility increases the rate of drug absorption, for digoxin and riboflavin, increased GI motility is associated with a decrease in the rate of drug absorption. It is of note to be reminiscent of the fact that, a failure in therapeutic efficiency of drugs is inevitable due to the delayed drug absorption issued from altered gastric emptying if the drug has a short biological half-life.

Anticholinergic medications, tricyclic antidepressants, levodopa, and β-adrenergic agonists are famous groups of medicine that suppress GI motility. Alternatively, drugs that enhance GI motility by increasing contractile force and accelerating intraluminal transit are called prokinetics like metoclopramide, cisapride, tegaserod, and erythromycin (macrolide antibiotic). Other groups of medical preparations (proton pump inhibitors, antacids, histamine receptors type 2 antagonists, etc.) that are used to treat a wide variety of stomach and duodenal diseases associated with hyper-acidity by raising the intragastric pH would alter the pH-dependent solubility and stability level of a certain kind of drugs administered orally. Most drugs are absorbed via passive diffusion of the nonionized form, so the amount of ionization at the different physiological pH values encountered in the GI tract would bring about non-uniform drug absorption and bioavailability [McConnell, Fadda et al., 2008].

Although it is not possible to predict accurately the magnitude and clinical relevance of all drug absorption interactions through the GI tract, designing a suitable dosage form that would function independent of the digestive state, clinical condition, or GI motility of the individuals is an imperative fundamental of the drug delivery system dominating these constraints. So the important requisite for the successful performance of oral drug delivery systems is that the drug should have good absorption throughout the GI tract. Since, all the orally controlled-release drug delivery systems would show limited utilization in the GI controlled administration of drugs if the systems cannot remain in the absorption site for the lifetime of the dosage form, establishing a prolonged GI transit time of orally administered drugs appears to be logical.

3. Prolongation of GI retention

It is clear from the recent scientific literatures that an increased interest among the academic and industrial research groups still exists in formulating novel dosage forms that are retained in the stomach for a prolonged and predictable period of time.

The most feasible method for achieving a prolonged and predictable drug delivery in the GI tract is to control the gastric residence time by gastro retentive and sustained release dosage forms that have some beneficial in safety and efficacy over normal release systems. This method of application is especially helpful in delivery of sparingly soluble and insoluble drugs. It is acknowledged that, as the solubility of a drug decreases, the time available for drug dissolution becomes less adequate and so the transit time becomes an important factor affecting drug absorption in drugs with lower solubility. Other drug candidates suitable for gastroretentive drug delivery systems include those drugs that are locally active in the stomach, drugs with narrow absorption window in GI tract, drugs that are unstable in the intestinal or colonic environment, drugs that act locally in the proximal part of GI tract or disturb normal colonic microbes like antibiotics and also drugs that exhibit low solubility at high pH values. Concerning the pharmacotherapy of the stomach through local drug release of gastroretentive dosage forms, bringing about high levels of drug concentrations at the gastric mucosa (eradication of *Helicobacter pylori* from the submucosal tissue of the stomach), and treating stomach and duodenal ulcers, gastritis and oesophagitis, the risk of gastric carcinoma would be drastically reduced. In contrast, there are drugs that do not fit in gastroretentive drug delivery systems; Drugs that have very limited acid solubility, drugs that suffer instability in the gastric environment, and drugs intended for selective release in the colon should follow other techniques of drug delivery to reach for their intended site of action. Hence, gastroretentive dosage forms despite providing rather constant drug concentrations in the bloodstream for longer periods of time do not fulfill this benefit with several groups of drugs.

One of the advantages of the sustained release dosage forms is that medication are administered less often than other dosage forms reducing fluctuations of drug concentration in the bloodstream. As a result, patients are not repeatedly subjected to different levels of drug which are less or more than adequate. Nor does the blood chemistry undergo frequent chemical imbalances, which might be risky to the patient's health. Additionally, through gastroretentive dosage forms not only the bioavailability and therapeutic efficacy of drugs are improved but also it may allow for a possible reduction in the dose because of the steady therapeutic levels of drugs. Drugs that have poor solubility in higher pH, absorption windows in stomach, and the ones requiring locall delivery in stomach could be delivered ideally to the site of action by these gastroretentive formulations. On the other hand, drugs that cause irritation to gastric mucosa and the ones meet first-pass metabolism or have stability problems in gastric fluids are not appropriate for these kinds of drug delivery systems.

In brief, gastric retention is a means to enable a delivery strategy that will function irrespective of the digestive state, clinical condition, or GI motility of the individuals with longer drug residence time in the stomach being advantageous in superior drug bioavailabilities and also in certifying local action of some drugs in the upper part of the GI tract. Various approaches that have currently become leading methodologies for increasing the resistance time of a dosage form in the stomach would be highlighted in this section forward. Nevertheless, the early reports on gastroretentive systems have been well-reviewed in pervious literatures, only the more recent development and strategies will be discussed here.

3.1. Intragastric floating drug delivery systems

3.1.1. Introduction

Design of floating pharmaceutical dosage forms with a bulk density less than gastric fluid is one of the thriving trends for enhancing drug residence in the stomach. These systems would act independent of the highly variable nature of the gastric emptying process resulting in reduced fluctuations of drug bioavailabilities.

Apart from the commented benefits established by these floating systems, they could also have their limitations given that they require a satisfactory level of fluid in the stomach to float. Likewise, in the fed state a change in body position to supine could have a direct effect on the floating system. In order to design a more systematic and intellectual floating system it is as important as ever to study different aspects of these systems to have a deeper insight to the buoyancy, drug release mechanisms, effectiveness and reliability of these floating systems as an strategy for establishing an efficient gastroretentive drug delivery system [Blanquet et al., 2004].

In this regard, different approaches have been followed to support the buoyancy of dosage forms in the stomach. Based on the mechanism of floatation, two obviously different technologies are employed in development of floating dosage forms, effervescent and non-effervescent systems

3.1.2. Effervescent systems

These buoyant systems are usually matrices of swellable polymers along with effervescent components that generate carbon dioxide (CO_2) entrapped in swollen hydrocolloids of the dosage forms. In these systems floatation is achieved through the incorporating of a gas within the formulation lessoning density of the system and making it float on the gastric fluids [Krogel & Bodmeier, 1999; Sungthongjeen et al., 2008].

Acid - base reactions have been utilized for decades to produce various pharmaceutical preparations which effervesce as contacting with water. Sources affording the acidity in effervescent reactions are essentially provided by acids, acid anhydrides, and acid salts where organic acids, citric and acetic acids are the most commonly used materials. In contrast, carbonates and bicarbonates in contact with acids are used typically as CO_2 generation sources. Carbonates not only play critical role in making the dosage form buoyant but also provide the initial alkaline micro environment for the polymers to gel [Sungthongjeen, Sriamornsak et al., 2008; Patel et al., 2009; Rajab et al., 2010]. These carbon dioxide generating components could be intimately mixed within the matrix to produce a single-layered dosage form or a multi-layered formulation which might contain the gas generating mechanism in one hydrocolloid containing layer and the drug in the other layer formulated for the sustained release effect (Figure 4). The matrices are fabricated so that upon contact with gastric fluid, carbon dioxide is liberated with an upward motion of the dosage form maintaining its buoyancy in the gellified hydrocolloid. Totally, the reaction is due to the CO_2 generated by neutralization in the effervescent layer with the diffusion of

water through the swellable membrane layer. However, there may be a lag time relying on the duration of gas generation reaction that could end in gastric emptying of the dosage form before floatation. More reliable gastric emptying patterns are attained for multi particulate formulations consisting loads of small discrete units as compared with single unit formulations containing one unit suffering from "all or none concept" [Sungthongjeen et al., 2006]. These multi particulate formulations are also better distributed in the GI tract and are less likely to cause local irritation. Recently, much more emphasis is being laid on the development of multi particulate dosage forms rather than single unit systems to their potential benefits like increased bioavailability, reduced risk of systemic toxicity, reduced risk of local irritation and predictable gastric emptying manners.

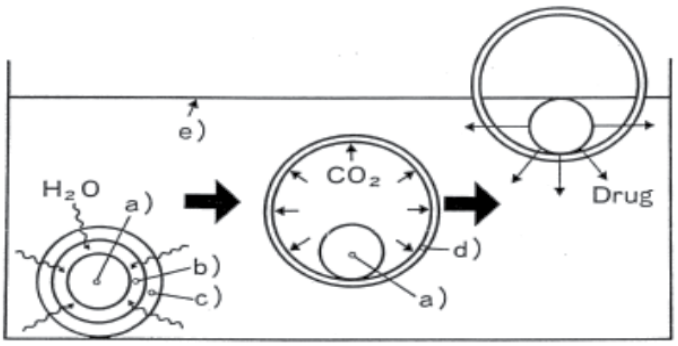

Figure 4. Mechanism of action in effervescent floating drug delivery system.

This view is supported by a variety of papers developing effervescent systems using different polymers and drugs, all agreed in the practicality and feasibility of the approach. In this regard, Pahwa et al. formulated effervescent floating tablets of famotidine with an effective and safe therapy for stomach ulcers in a reduced dose manner [Pahwa et al., 2012]. Hydroxypropyl methylcellulose K15M and hydroxypropyl methylcellulose K100M as gel forming agents along with sodium bicarbonate and citric acid as gas generating agents were used in the research. Concerning the results of the study, it was concluded that the addition of gel-forming polymers, methocel and gas generating agent, sodium bicarbonate along with citric acid were essential to achieve in vitro buoyancy profile. Faster and higher CO_2 generation caused by increasing the concentration of effervescent agents resulted in higher swelling of polymeric membrane according to a higher gas pressure [Pahwa, Chhabra et al., 2012]. Additionally, expansion of the hydrated matrices increased the surface area available for dissolution and presence of gas bubbles hindered the diffusion path, decreasing the release constant values. Concisely, in these systems generation of the CO_2, entrapped in the matrix is the constant principle rule that has been widely established in studies of this category.

3.1.3. Non-effervescent systems

Non-effervescent systems incorporate a high level of one or more gel forming, highly swellable, cellulosic hydrocolloids, poly saccharides or matrics forming polymers into the

formulations employing no gas forming agents during the procedures. These gel formers, polysaccharides and polymers hydrate in contact with gastric fluids and form a colloidal gel barrier resulting in the floatation of the dosage form. Practical approaches in formulating non-effervescent floating dosage forms are listed below.

3.1.3.1. Hydrodynamically Balanced Systems (HBS)

The HBS systems are developed with gel-forming hydrocolloids forming a hydrated gel layer in the outer exposed surface of the dosage form on contact with gastric fluids imparting buoyancy for a long period of time. Gel-forming or highly swellable cellulose type hydrocolloids such as hydroxypropyl cellulose, hydoxyethyl cellulose, hydroxypropyl methyl cellulose, polysaccharides, matrix forming polymers and the drug that is with gel-forming hydrocolloids are homogeneously thoroughly mixed within the HBS formulations [Khattar et al., 1990; Dorozynski et al., 2004]. Hydration of the inner layers brings about surface hydration and a soft gelatinous barrier around the dosage form. This soft gelatinous mass on the surface of the formulation provides a water-impermeable colloid gel barrier on the surface of the tablets. Consequently, the hydrated gel controls the rate of water penetration into the dosage form and the rate of drug release from the HBS. Different varieties of polymers could be utilized in the floating drug delivery systems as well in the HBS. Kumar et al. studied effect of different excipients and polymers on floating behavior and drug release from floating HBS matrices. It was found that the selection of suitable excipients depending on polarity of drug modulates the floatability and drug release profiles where water uptake in the floating matrix increased with the increase in the loading of polar drugs and decreased with non-polar drugs [Kumar et al., 2004]. Elsewhere, a hydrodynamically balanced delivery system of clarithromycin was developed by Nama et al. to prolong gastric residence time with a desired in vitro release profile for the localized action in the stomach, with the intention of *Helicobacter pylori* mediated peptic ulcer treatment. Different kinds of polymers with various possible concentrations were assessed to establish the optimum formulation with 66.2% clarithromycin, 12% HPMC K4M polymer, 8% sodium bicarbonate offered improved floating lag time less than 3 min and 12 h duration of floating [Nama et al., 2008].

In general, use of HBS is desirable which swell to create a gel-like structure after administration and attain a density less than that of gastric fluids where a prolonged drug delivery is required. Not many practical reports were found on the non-effervescent HBS drug formulations as compared to the effervescent systems. As a matter of fact, considering the all or none gastric emptying of HBS dosage forms, development of floating drug delivery system using multiple-unit devices to distribute uniformly within the gastric content and gradually pass through the GI tract still is one of the challenges in this field.

3.1.3.2. Porous systems

Porous materials are emerging day by day as a new category of drug delivery systems due to the several alternative features such as stable uniform porous structure, high surface area,

and tunable pore sizes with narrow distribution and well defined surface properties. Theses porous carriers have been playing one of the key roles in the pharmaceutical industries including development of novel drug delivery systems such as floating and sustained drug delivery systems in improving the solubility of poorly water soluble drugs [Ahuja & Pathak, 2009; Sher et al., 2009]. Porous structures such as silica, porous ceramic, ethylene vinyl acetate, polypropylene foam powder and titanium dioxide allow the inclusion of drugs inside a porous compartment possessing a relatively lower density than the gastric juice thus remaining buoyant in the stomach [Streubel et al., 2002]. This approach of flotation is based on the principle of the encapsulation of a drug reservoir inside a microporous compartment with pores along its top and bottom walls. Any other polymer that may be added to the dosage form would moderately cover the pores and entrap air within the system. However, the peripheral walls might get completely sealed to prevent any direct contact of the gastric surface with the drug bringing about delayed release of drugs. Besides, the entrapped air would gradually removed from the formularion in exposure to gastric medium, leading to an extended floating times with a more reproducible and predictable drug release manner. After all, interests and notable surveys have been directed in recent years towards the porous carriers as controlled drug delivery matrices.

A multi particulate floating delivery system, consisting of highly porous carrier material calcium silicate, glipizide as the drug, and eudragit S as the polymer, was developed by Pandya et al. Glipizide as a second-generation sulfonylurea with short biological half-life (3-4 h) and the site of the absorption in the stomach was an excellent candidate for development of controlled-release dosage forms that are retained in the stomach increasing the absorption, drug efficiency, along with decrease in dose requirements. In this regard, they evaluated effects of various formulation variables on the internal and external particle morphology, micromeritic properties, in vitro floating behavior, and in vitro drug release. The designed system, showed excellent buoyancy and suitable drug release pattern, possibly being advantageous in terms of increased bioavailability of glipizide over an extended period of time [Pandya et al., 2011].

More recently, Yao et al. developed a simple and rapid method of floatation to prepare a novel kind of inner-porous floating beads using foam solution using poloxamer 188 as foaming agents and alginate as foaming stabilizer. Riboflavin, a water-soluble vitamin was used as a model drug which has a narrow absorption window in the upper part of the intestine and a saturable absorption mechanism. In this method poloxamer 188 was used as an effective amphiphilic surfactant which could lower the water surface tension significantly. In addition, foam solution was formed by stirring in the presence of poloxamer 188, winding microbubbles in the alginate and stabilizing the foam solution. Findings of the study revealed that addition of the non-ionic surfactants poloxamer 188 lead to the formation of a surfactant–polymer complex through interactions between polymer and surfactant contributing in the foamability and foam stability of the formulations. Moreover, higher concentration of the poloxamer 188 increased the foamability and foam stability of the mixed solution. These results were verified through scanning electron microscopy cross-

section pictures of the beads showing inner-porous with fully bubbled nature of the beads with very thin wall bubbles stacked together. Additionally, gamma scintigraphic images and pharmacokinetic studies in vivo showed that the beads could retain in the stomach for over 6 h and improved bioavailability of the drug [Yao et al., 2012].

3.1.3.3. Alginate beads

Lately, both natural and synthetic hydrophilic polyionic systems like alginates have been a center of attention for preparation of floating systems. The interest in alginates is chiefly due to their high biocompatibility and nontoxic nature in oral administration that also demonstrate protective effect on the mucous membranes of the upper GI tract. Alginate beads with the structure of spherical gels are taken shaped through dropwise addition of aqueous alginate solution to the aqueous solution containing calcium ions and/or other di and polyvalent cations [Javadzadeh et al., 2010]. The pH dependent reswelling property of dried alginate beads let them to be administrated as a unique vehicle as the multi unit floating dosage forms in GI tract as early as 1980s [Murata et al., 2000].

In recent years, the amount of literature published on development of these alginate beads providing satisfactory data for a variety of applications has been grown remarkably. In a study, cinnarizine, a piperazine derivative as a Ca channel blocking and anti- histaminic drug suffering from incomplete and variable oral absorption which occurs mainly in the proximal small intestine was formulated as a floating multiple unit dosage form of alginate beads. Ghareeb et al. formulated floating cinnarizine olive oil-entrapped emulsion gel beads by the emulsion–gelation method. They came into conclusion that formulation variables greatly influenced the mean particle size and in vitro drug release characteristics of the prepared beads. Ultimately, the experimental observations suggested cinnarizine loaded alginate beads could potentially be useful in drug delivery systems for making controlled release gastroretentive floating beads of drugs via the ionotropic gelation technique [Ghareeb et al., 2012]. Furthermore, in our previously published paper, we prepared floating beads of metronidazole via the ionotropic gelation method employing gas forming (sodium bicarbonate) and porous (calcium silicate) agents followed by a physicochemical evaluations. It was found that the kind and amount of agents used to make the beads floated, i.e., sodium bicarbonate and calcium silicate, had a profound effect on the beads size, morphology, and floating ability as well as drug release profile [Javadzadeh, Hamedeyazdan et al., 2010]. In general, high compatibility of the alginate beads in achieving a suitable floating dosage form controlling the drug release from the beads with a definite kinetic of release was clear enough to be supported.

Novel prospects for application of floating alginate beads were accomplished by emphasizes on advantages and future potential of probiotic loaded beads in the treatment of GI disorders. As we know, probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance that may undergo antagonistic interactions with pathogenic bacteria. However, significance of probiotics survival in the GI tract, their translocation, colonization and the fate of probiotic derived active components

indicate a need and scope of packaging them into a suitable delivery system to increase the viability of the probiotics, both outside and inside the body. Singh et al. developed a floating drug delivery system of *Lactobacillus acidophilus* via orifice ionic gelation method in alginate beads. They evaluated the efficacy of the approach in experimental model of cold restraint stress induced gastric ulcer in terms of ulcer index; hemorrhagic streak length; mucus content and histopathological examinations. According to the authors, the developed formulations not only efficiently protected the entrapped probiotic cells, but also effectively delivered and retained viable bacteria in the stomach [Singh et al., 2012]. The prolonged and continuous release of the probiotic in the gastric mucosa allowed them for an ensured therapeutic efficacy of the developed floating system against ulcers post-induction, thus suggesting an efficient line of treatment.

3.1.3.4. Hollow microspheres / microballoons

One of the renowned approaches in devising multiple unit floating systems are the hollow microspheres (microballoons), spherical empty particles without core that are designed to float on gastric juice [Gholap et al., 2010]. Hollow microspheres incorporating a drug dispersed or dissolved throughout particle matrix have the potential for controlled release of drugs. These microspheres are characteristically prepared by solvent diffusion and evaporation methods where the relative polymer is dissolved in an organic solvent and the drug dissolved or dispersed in the polymer solution is emulsified into an aqueous phase containing suitable additive (surfactants / polymer) to form oil in water emulsion (Figure 5). After removal of the organic solvent, polymer precipitates at the oil/water interface of droplets, forming cavity and thus making them hollow to impart the floating properties. Frequently used polymers in developing hollow microspheres are polycarbonate, chitosan, methocil, polyvinyl acetate, carbopol cellulose acetate, calcium alginate, eudragit, agar and also pectin [Soppimath et al., 2006].

In general, hollow microspheres are believed to be prominent buoyant systems as they provide a multi-unit system with an improved floating property. This view is supported by scores of reports on development of the emulsion solvent diffusion method to form hollow microspheres employing different polymers and drugs to follow a promising drug delivery system floating on the gastric juice. Rosiglitazone hollow microspheres were prepared through modified quasi-emulsion solvent diffusion method by Gangadharappa et al. to improve the oral hypoglycemic agent's bioavailability. The formulated dosage form controlled the drug release, avoiding dose dumping, in an extended floating duration [Gangadharappa et al., 2011]. Elsewhere, Rane et al. prepared hollow microspheres of rosiglitazone were by O/W emulsion-solvent diffusion technique using biodegradable anionic acrylic resin as a polymer. They provided evidence that a modification of external aqueous phase with salt ($CaCl_2$ and NaCl) improved the drug entrapment efficiency for moderately soluble drug by 45–50% as a result of salting out effect. Predominantly, the release mechanism for formulations was diffusion controlled and followed the first order kinetics [Rane et al., 2012].

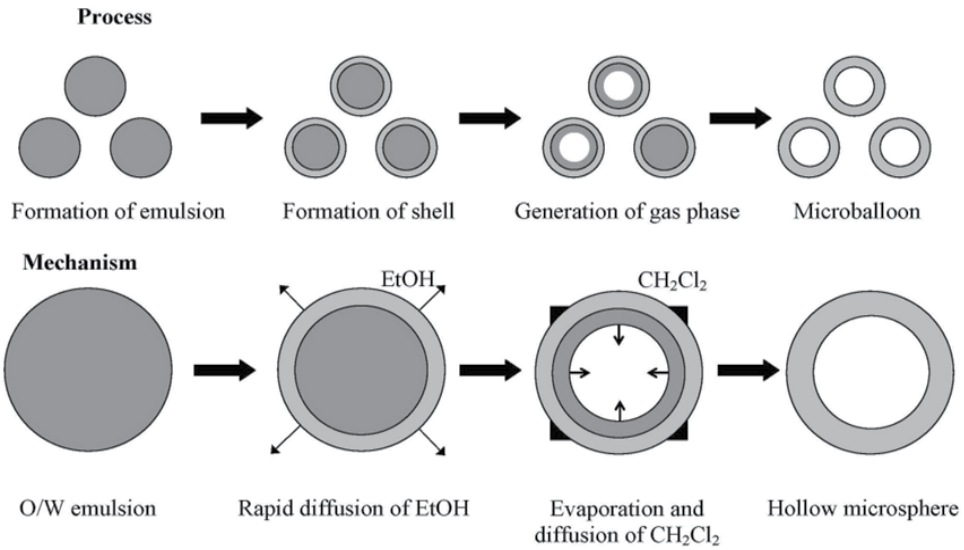

Figure 5. Solvent diffusion and evaporation technique in formation of the hollow inner core microspheres.

3.1.4. Physicochemical evaluations

In development phase for floating dosage forms, designing a selection of suitable control principles is of crucial importance. This would be beneficial in preventing the development efforts to costly late stage product failures throughout the manufacture and administration periods too. Besides, health authorities worldwide necessitate accurate adjustment of the physicochemical parameters of the formulation for all individually administered dosage forms in advance to clinical phase [Parikh & Amin, 2008].

3.1.4.1. In vitro buoyancy and drug release properties

Not surprisingly, insufficient and inconsistent formulation buoyancy, drug dissolution and the subsequent inadequate drug efficacy are some of the routine challenges to be coped with during the development of floating drug delivery systems when they are administrated orally. It is of essential for these kinds of dosage forms to have a satisfactory floating activity, for giving the drug a chance for sufficient dissolution rate, absorption, and a suitable clinical efficacy, as well. Since, different physiological situations may affect the results for buoyancy and dissolution and drug release from pharmaceutical dosage form, in vitro tests for buoyancy and drug release studies are usually carried out in close proximity to the physiological conditions of the human body to lessen the variation derive from altered dissolution medium. The apparatus and procedures of USP are preferred the best to make a better correlation between in vitro and in vivo results of the floating and drug release tests. Generally, the tests are determined in 900 mL of simulated gastric fluid (HCl/NaCl with 0.02% Tween 80, pH 1.2) maintained at 37°C using basket or paddle stirring elements of the USP dissolution apparatus [Mudie et al., 2010]. Besides, the time needed for

the dosage form to float and duration of the buoyancy are noted as the floating lag-time and flotation time are evaluated as the floating behavior of the formulations.

There have been numerous published reports on diverse challenges of the floating drug delivery systems applying a variety of floating agents to encourage buoyancy of the pharmaceutical dosage forms with a controlled drug release. Gupta at al. prepared floating microspheres of clopidogrel bisulfate using different viscosities of ethyl cellulose and triethyl citrate as a plasticizer using emulsion solvent diffusion technique. It was detected that the drug release from the floating microspheres matrix was completely controlled by the polymer as the polymer proportion in the formulation was increased, a decrease in drug loading, drug release was observed. The floating ability of different formulations was found to be altered according to polymer ratio. Overall, the prepared formulations showed appropriate balance between buoyancy and drug release rate dependent on the amount of formulation variables [Gupta & Rajpoot, 2012]. Most of the relative papers dealing with the development and optimization of the buoyancy lag time and duration of drug release employed both swellable polymers and gas generating systems to increase the buoyancy and lag times. Several studies in this filed have shown that different factors influence the buoyancy and drug release from a pharmaceutical dosage form and contributing in different patterns of drug release in similar floatation preparation methods.

Accordingly, different factors influence the buoyancy and drug release from a pharmaceutical dosage form. Solubility of the drug in dissolution medium as well as the formulation variables in the dosage form are the dynamic items, contributing distinctively in drug release differences of the floating dosage forms with similar basic floatation preparation methods. It has always been a great challenge to modify the drug release in an expected manner and assess the efficiency of the dosage form in an in vivo environment. Even if the more or less standardized in vitro methods described in the pharmacopeial monographs are used in different studies, the correlation between in vivo and in vitro results must be confirmed from case to case in individuals prior to the clinical application.

3.1.4.2. In vivo assessments

Very early on, in the development phase of drugs a reliable relationship has to be established between in vitro and in vivo test, in order to expose therapeutically relevant discrepancies first between different formulations and later also between drug production batches. This correlation is most likely if the test conditions are in close proximity to the physiological circumstances. Hardly ever, do the in vitro assessments fully counterpart the in vivo results, since the standard USP methods have not been a reliable predictors of in vivo performance of the floating pharmaceutical dosage forms [Kaus et al., 1999]. Apart from the drug release pattern of the dosage form, it is the absorption, distribution, metabolization and excretion influence the in vivo drug concentration at the site of action. In view of the fact that interplay of the pharmacokinetic and pharmacodynamic parameters for a particular drug also has a noteworthy effect on the efficiency of the floating dosage form, in vivo evaluations appear to be requisite. Tracking the in vivo floating behavior and location of dosage form through GI tract providing a broad insight for formulating the

optimal floating drug delivery system could be established via radiography, gamma scintigraphy, gastroscopy, ultrasonography and magnetic resonance imaging. Considering the majority of the surveys in this case, not only the relevant correlation between in vivo and invitro assessments was confirmed but also effectiveness and reliability of these floating systems as an excellent strategy for controlled delivery of drugs was ascertained [Jain et al., 2006; Ali et al., 2007; Wei & Zhao, 2008; Guguloth et al., 2011].

3.1.4.3. Drug release kinetic for the floating drug delivery systems

Over the past few decades, the large variety of formulations devoted to floating drug delivery systems with varied physicochemical properties have become manifested which influence the drug release patterns of these formulations. In the same way as the ideal purpose of any drug delivery system is to maintain drug concentration in the blood or in target tissues at a desired value as long as possible, focusing on the drug release profiles to design a more systematic and intellectual floating system is has become even more rational. Accordingly, study of the drug release kinetics is often useful in obtaining one or two physically meaningful parameters which are employed for comparative purposes and relating the release parameter with important parameters such as bioavailability [Wagner, 1969; Barzegar-Jalali et al., 2008; Dash et al., 2010].

There are number of kinetic models, which described the overall release of drug from the dosage forms. Seeing as both qualitative and quantitative changes in a formulation could alter drug release and in vivo performance of the dosage form, developing tools that facilitate drug development by reducing the necessity of bio-studies is always desirable [Arifin et al., 2006; Javadzadeh, Hamedeyazdan et al., 2010]. Referring our former studies on drug release mechanisms from floating drug delivery systems, no a single kinetic model is customary for the floating dosage forms, even if the flotation strategies were the same [Adibkia et al., 2011]. Formulation variables, approaches in establishing formulation buoyancy and the kinetic models in analyzing the drug release data should have chosen parallel to present a suitable model fitting for these systems. In general, evaluating the mechanism behind drug release profile from floating dosage forms is complicated, and requires careful observance of the physicochemical properties of the dosage form.

3.2. Mucoadhesive gastrointestinal drug delivery systems

3.2.1. Introduction

For years pharmaceutical scientists have been fascinated by the concept of bioadhesion. The term bioadhesion implies attachment of a drug carrier system to a specific biological location like epithelial tissue. This bioadhesion phenomenon is referred to as mucoadhesion if the adhesive attachment is to a mucus coat [Davidovich-Pinhas & Bianco-Peled, 2010]. Mucoadhesive drug delivery systems are used to localize a delivery device within the lumen to enhance the drug absorption in a site-specific manner since the luminal surface of the GI tract is a richly glycosylated tissue that presents considerable advantages in the use of formulations with mucoadhesive properties.

3.2.2. Factors affecting mucoadhesion

Mucoadhesive characteristics are affected by a factor of both the bioadhesive polymer and the medium in which the polymer will exist in. The mucoadhesive properties of polymers themselves are concerned by diversity of factors such as molecular weight, flexibility, hydrogen bonding capacity, cross-linking density, charge, concentration, and hydration (swelling) of a polymer, which is briefly discussed below [Lahoti et al., 2011].

3.2.2.1. Polymer-related factors

Molecular weight: In general, it has been revealed that the bioadhesive strength of a polymer increases with molecular weights above 100,000.

Flexibility: Bioadhesion could be result of the diffusion of the polymer chains in the interfacial region. Then, containing of a substantial degree of flexibility is essential in order to get the desired entanglement with the mucus. In general, mobility and flexibility of polymers can be related to their viscosities and diffusion coefficients, where in the case of higher flexibility, greater diffusion into the mucus network was seen.

Hydrogen bonding ability: It was found that for occurring of mucoadhesion, polymers must contain functional groups that are able to form hydrogen bonds. It was also showed that flexibility of the polymer is imperative to advance this hydrogen bonding potential.

Cross-linking density: It gives the impressions that with increasing density of cross-linking, diffusion of water into the polymer network take place at a lower rate resulting an inadequate swelling of the polymer and a decreased rate of interpenetration between polymer and mucin.

Charge: Nonionic polymers show a smaller degree of adhesion compared to anionic polymers. It was also shown that some cationic polymers display superior mucoadhesive properties, especially in a neutral or slightly alkaline medium. Furthermore, some cationic polymers such as chitosan that has high molecular weight have shown good adhesive properties.

Concentration: In the lower concentration of the polymer, the number of penetrating polymer chains per unit volume of the mucus is small, and then the interaction between polymer and mucus is unstable. In general, the more concentrated polymer would result in a longer penetrating chain length and better adhesion. However, there is a critical concentration, above which the polymer generates an "unperturbed" situation due to a significantly coiled structure. As a result, the accessibility of the solvent to the polymer decreases, and chain penetration of the polymer is considerably reduced. Therefore, higher concentrations of polymers do not necessarily improve mucoadhesive properties and, in some cases, diminished effect was seen.

Hydration (swelling): For any polymer in order to develop the interpenetration process between polymer and mucin, hydration is required to expand and create a proper "macromolecular mesh" of sufficient size, and also to induce mobility in the polymer chains.

3.2.2.2. Environmental factors

The mucoadhesion of a polymer not only depends on its molecular properties, but also on the environmental factors adjacent to the polymer (pH, feed condition, and movement of the GI tissues while eating, drinking, or talking). Therefore, an optimum time period for the administration of the dosage form is necessary in order to avoid many of these interfering factors [Lahoti, Shep et al., 2011].

3.2.3. Polymers used for mucoadhesive drug delivery

Polymers have played an important role in designing mucoadhesive drug delivery systems. Customarily, in these systems mucoadhesive polymers adhere to the mucin layer on the mucosal epithelium followed by a decrease in drug clearance rate from the GI absorption site, thereby increasing the time available for drug absorption [Rajput et al., 2010]. Polymers used in such system may have natural or synthetic origin. These polymers are classified as:

3.2.3.1. Hydrophilic polymers

These polymers are soluble in water. When put into an aqueous media, start to swell and subsequent dissolution of the matrix is occurred. The polyelectrolytes show superior mucoadhesive property in comparison with neutral polymers. Anionic polyelectrolytes, e.g. poly (acrylic acid) and carboxymethyl cellulose, have been broadly used for preparation of mucoadhesive formulations for their ability to exhibit strong hydrogen bonding with the mucin. Chitosan has been widely used as a cationic polyelectrolyte mucoadhesive polymer developing mucoadhesive property of these systems via electrostatic interactions with the negatively charged mucin chains. Moreover, these ionic polymers might have a drug delivery matrix exhibiting mucoadhesive property by developing ionic complex with the counter-ionic drug molecules. Alternatively, non-ionic polymers, such as poloxamer, hydroxypropyl methyl cellulose, methyl cellulose, poly (vinyl alcohol) and poly (vinyl pyrrolidone), have been used for mucoadhesive properties, as well. The hydrophilic polymers would be used as viscosity modifying/enhancing agents in the development of liquid ocular delivery systems for increasing the bioavailability of active agents by reducing the drainage of the administered formulations since they form viscous solutions when dissolved in water. Accordingly, a mucoadhesive drug delivery system would be established by these polymers when they are directly compressed in the presence of drugs. Polymers that are used frequently in ocular mucoadhesive delivery systems are usual polysaccharides and its derivatives like chitosan, methyl cellulose, hyaluronic acid, hydroxypropyl methylcellulose, hydroxypropyl cellulose, xanthan gum, gellan gum, guar gum, and carrageenan [Roy et al., 2009]. Besides, sustained release of drugs through these hydrophilic mucoadhesive polymers is accomplished in combination of the cationic cellulose derivatives (e.g. cationic hydroxyethyl celluloses) with various anionic polymers.

3.2.3.2. Hydrogels

Three-dimensionally crosslinked polymer chains have the ability to hold water within its porous structure forming hydrogels that are usually characterized with the presence of

hydrophilic functional groups like hydroxyl, amino and carboxyl groups bringing about the water holding capacity of these hydrogels. However, it is of value to note that with increase in the crosslinking density of hydrogels a decrease in the mucoadhesion would be inevitable. Preparation of the hydrogels by the condensation reaction of poly (acrylic acid) and sucrose pointed out an increase in the mucoadhesive property with the increase in the crosslinking density that was attributed to the rise in the poly (acrylic acid) chain density per unit area. In this regard, Wood and Peppas developed a system in which ethylene glycol chains were grafted on methacrylic acid hydrogels and were functionalized with wheat germ agglutinin. In this system the intestinal residence time of the delivery system was improved by binding of the wheat germ agglutinin with the specific carbohydrate moieties present in the intestinal mucosa. Mucoadhesive hydrogel based formulations not only have beneficial in drug targeting but also they are of use in improving the bioavailability of the poorly water soluble drug. Typically, Muller and Jacobs prepared a nanosuspension of buparvaquone, a poorly water soluble drug, by incorporating it within carbopol and chitosan based hydrogels [Muller & Jacobs, 2002]. Improved bioavailability of the drug in mucoadhesive delivery systems was determined when compared over the nanosuspension indicating the increased retention time of the delivery system within the GI tract.

3.2.3.3. Thiolated polymers

The mucoadhesive properties of the polymers (e.g. poly (acrylic acid) and chitosan) along with the paracellular uptake of the bioactive agents would be significantly improved through the presence of free thiol groups in polymeric skeleton which could help in the formation of disulphide bonds with that of the cysteine-rich sub-domains present in mucin. Some of the polymers including thiol groups are chitosan–iminothiolane, poly (acrylic acid)–cysteine, poly(acrylic acid)–homocysteine, chitosan–thioglycolic acid, chitosan–thioethylamidine, alginate–cysteine, poly(methacrylic acid)–cysteine and sodium carboxymethylcellulose–cysteine [Roy, Pal et al., 2009].

3.2.3.4. Lectin-based polymers

Lectins that are found in both animal and plant kingdom in addition to various microorganisms, are the proteins which have ability to reversibly bind with specific sugar or carbohydrate residues, providing with specific cyto-adhesive property for targeted delivery systems. Lectins extracted from *Ulex europaeus I*, soybean, peanut and *Lens culinarius* have demonstrated specific binding to the mucosa. Additionally, wheat germ agglutinin amongst other available lectins has shown limited immunogenic reactions exhibiting potential capacity to bind to the intestinal and alveolar epithelium as the favorable muccoadhesive polymer [Roy, Pal et al., 2009].

3.2.4. Methods of evaluation

Mucoadhesive polymers and drug delivery systems can be evaluated by testing their adhesion strength by both in vitro and in vivo tests (Table 2).

In vitro / ex vivo methods	In vivo methods
• Methods determining tensile strength	• Use of radioisotopes
• Methods determining shear stress	• Use of gamma scintigraphy
• Adhesion weight method	• Use of pharmacoscintigraphy
• Fluorescent probe method	• Use of electron paramagnetic resonance (EPR) oximetry
• Flow channel method	• X ray studies
• Mechanical spectroscopic method	• Isolated loop technique
• Falling liquid film method	
• Colloidal gold staining method	
• Viscometer method	
• Thumb method	
• Adhesion number	
• Electrical conductance	
• Swelling properties	
• In vitro drug release studies	
• Mucoretentability studies	

Table 2. Variety of in vitro / in vivo tests for evaluation of the adhesion strength in mucoadhesive sustems

The GI transit time of these dosage forms could be measured by using one of the many radio opaque markers like barium sulphate which is coated to the bioadhesive dosage form so as to assess the GI transit by means of X-ray inspection. Both the distribution and retention can be studied by using of gamma scintigraphy. Using a non invasive technique to determine the transit time of mucoadhesive polymers is also popular and this could be imaged via labeling of the polymer with a gamma emitting nucleotide determining with the help of gamma scintigraphy. Another recent technique is to use magnetic resonance imaging to localize the point of release of thiolated polymers from dosage forms via the use of gadolinium determining by ascertaining the residence time of the fluorescently tagged thiomer on intestinal mucosa of rats after 3 hours [Tangri et al., 2011]). Although, considering the high cost, time consuming and ethical factors, these techniques are less usual common, evaluating the true mucoadhesive potential of the pharmaceutical dosage form is of essential [Shaikh et al., 2011].

Accordingly, there have been several successful attempts in identifying these putative bioadhesive or mucoadhesive materials using in vitro and in vivo tests but success usually has failed in translating to human studies in the stomach and small intestine [Sohrabi et al., 2009; Movassaghian et al., 2011]. It may be due to the lack of correlation between mucosal conditions in the GI tract, and test models that are not in close proximity to the physiological circumstances. This phenomenon reinforces the note that appropriate testing methods are

required to be developed, with suitable compositions and mechanical forces. Moreover, the continuous production of mucous by the gastric mucosa to replace the mucous that is lost through peristaltic contractions seems to limit the potential of mucoadhesion as a gastroretentive force. On the contrary, colonic mucoadhesion due to the lower mucus turnover and sensitivity to mucus secretory stimulus is much more successful than small intestinal or gastric approaches, due to a thicker mucus layer and lower disruptive colonic motility.

On the whole, it is suggested that this system normalizes the overall variations in drug GI transit time and allows for a more consistent performance of the formulations within and between individuals, improving the overall efficacy of drugs. However, the high turnover rate of gastric mucus and the probable local irritation by these drug formulations has remained challenging in this approach.

3.3. Conclusion

Ever since, the pharmaceutical concept of a dosage form seriously manipulate the stability, solubility, bioavailability, and other features of a medicine identifying these potential liabilities allows us to predict, control and avoid any complexities that may arise during pharmacotherapy. Hence, acquiring a clear notion of the assorted characteristics of drug delivery systems will not only allow proficient design of drug formulation, but also an improved in vitro and pre-clinical in vivo behavior of the drug, better in vitro - in vivo correlations, as well as opening new features in administrating drugs via novel drug delivery systems.

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Chapter 15

Advanced Drug Carriers Targeting Bone Marrow

Keitaro Sou

Additional information is available at the end of the chapter

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1. Introduction

The progression of nanotechnology has produced engineered fine nanoparticles for use in biomedical applications. Drug delivery systems are in a particularly promising field for the use of the unique properties of nanoparticles in biomedical applications. The pharmacokinetics of nanoparticles differs considerably from that of small drug molecules. Therefore, a drug delivery system based on nanoparticles offers a novel direction of drug discovery as well as an improved delivery system for use with conventional drugs. It has been demonstrated that drug delivery systems using nanoparticles are advantageous for stable solubilization of lipophilic drugs, reduced toxicity, inhibition of enzymatic degradation of the drugs, and so on (Moghimi et al., 2001; Papahadjopoulos et al., 1991; Torchilin, 2005). Nanoparticulate drugs show longer circulation time by avoiding renal excretion compared to drugs with small molecules. Moreover, one important technology termed "PEGylation", surface modification of nanoparticles by polyethylene glycol (PEG) chains, prolonged the circulation time (Klibanov et al., 1990; Owens & Peppas, 2006). These long-circulating nanoparticles are effective to increase the passive delivery of anticancer drugs into tumor tissues having leaky blood capillaries with wide fenestrations (Matsumura & Maeda, 1986; Gabizon et al., 1994).

Practical nanotechnology and methodologies to target a specific organ or cell actively are of current interest in the development of further advanced drug delivery systems. For this purpose, the specific interaction which is typically mediated by the receptors on a cell surface must be ascertained. Furthermore, non-specific interactions must be minimized to achieve high selectivity to the target. These specific and selective mechanisms are working constantly in communication, transport, and metabolism processes in living system. Drug carriers offer a platform to target these biological mechanisms. Several results of studies have shown that the bone marrow is the principal organ for the specific uptake of bioparticles such as senescent cells, lipoproteins, and nuclei from erythroid precursor cells through the mononuclear phagocyte system (Hussain et al., 1989a; Qiu et al., 1995; Rankin, 2010). This importance suggests that the phagocytic activity of bone marrow is a potent target of nanoparticle-based

drug delivery. In fact, it has been demonstrated that nanoparticles modified with a specific molecule on their surface are distributed selectively into bone marrow tissues (Harris et al. 2010; Mann et al., 2011; Moghimi, 1995; Porter et al., 1992; Schettini et al., 2006; Sou et al. 2007, 2010, 2011a). These bone marrow-specific drug carrier systems are expected to improve diagnostic and therapeutic systems to treat hematopoietic disorders. This chapter presents a review of the specific targeting of bone marrow using nanoparticles as carriers. Furthermore, future aspects of their medical applications are discussed.

2. Functions of bone marrow for blood cell turnover

Bone marrow, a soft and spongy tissue found in the hollow spaces in the interior of bones, constitutes about 4% of the total body weight of adult humans. Progenitor cell (stem cell) lines in the bone marrow produce new blood cells and stromal cells. It has been estimated that as many as 4.9×10^{11} senescent blood cells are eliminated from blood circulation per day in adult humans (Fliedner et al., 1976, 2002). Figure 1 shows that almost as many blood cells are released from bone marrow to maintain a constant number of circulating blood cells. Bone marrow possesses an efficient system that takes nutrients from blood circulation selectively for blood cell production.

Mononuclear phagocyte systems in liver and spleen have been regarded as the main pathway to eliminate senescent blood cells. However, a recent study has shown that mouse bone marrow is an important organ for eliminating white blood cells, especially neutrophils, where neutrophils are ultimately phagocytosed by bone marrow stromal macrophages (Dalli et al., 2012; Furze & Rankin, 2008; Rankin, 2010). The expression of the anti-inflammatory molecule annexin A1 by resident macrophages is necessary for clearance of senescent neutrophils, which are determined by their higher levels of CXCR4 expression and annexin V binding in the mouse bone marrow (Dalli et al., 2012). Consequently, it can be speculated that annexin A1 on bone marrow resident macrophages specifically interacts with senescent neutrophils. High uptake of white blood cells in bone marrow can also be observed in humans following administration of ^{111}In-radiolabeled white blood cells that are administered routinely to humans for the detection of occult infection which is first recognized by secondary manifestations such as increased neutrophils in the circulation or fever of unknown origin. Whole body region-of-interest analysis frequently reveals that 60–70% of the administered white blood cells localize to bone marrow, whereas 30–40% localize to liver and spleen (Sou et al., 2011a).

Chylomicrons are large lipoprotein particles that consist of triglycerides, phospholipids, cholesterol, and proteins. Hussain and co-workers reported that rabbit and marmoset bone marrow had significant uptake of chylomicrons labeled with [^{14}C] cholesterol and [^{3}H] retinol (Hussain et al., 1989b). Perisinusoidal macrophages protruding through the endothelial cells into the marrow sinuses were responsible for accumulation of the chylomicrons in the marmoset bone marrow. In contrast to marmosets, chylomicron clearance from the bone marrow of rats, guinea pigs, and dogs was much less, and the spleen in rats and guinea pigs took up a large fraction of chylomicrons. Consequently, Hussain et al. concluded that the observed differences in chylomicron metabolism result

from the presence of perisinusoidal macrophages in bone marrow. It was also believed that the differences between bone marrow and spleen uptake of chylomicrons might provide insights into the role of chylomicron catabolism in these organs, both of which are involved in hematopoiesis. It was speculated that the chylomicrons play a role in the delivery of lipids to the bone marrow and spleen as a source of energy and for membrane biosynthesis or in the delivery of fat soluble vitamins. In addition, bone marrow macrophages, which are associated with erythroblasts in a hematopoietic environment, participate in erythropoiesis control, and engulfment of nuclei from erythroid precursor cells (Chasis & Mohandas, 2008; Qiu et al., 1995; Sadahira & Mori, 1999; Winkler et al., 2010; Yoshida et al., 2005). These features of bone marrow for uptake of senescent blood cells, lipoproteins, and nuclei can be original models of the specific bone marrow targeting system.

The molecular mechanism of the uptake of lipid particles such as lipoproteins and apoptotic cells plays an important role in the transport and metabolism of fats such as cholesterol, triglycerides, and phospholipids. The attractive matter on the molecular mechanism is the ligand-receptor system, which permits the specific interaction of the lipid particles with specific cells in organs. For example, low-density lipoprotein (LDL), a native lipid particles consisting with cholesterol, ApoB-100, phospholipids, and lipophilic vitamins functions to transport cholesterol and vitamins from liver to peripheral tissues. LDL receptors on cells are responsible to the binding and following endocytosis of the LDL where ApoB-100 and ApoE on the LDL act as ligands to the LDL receptors. However, once the LDL is oxidized, scavenger receptors, instead of LDL receptors, act to recognize the oxidized LDL (OxLDL) where ApoB-100 and ApoE are not ligands to the scavenger receptors. Scavenger receptors are typically expressing on the mononuclear phagocyte cells including macrophages, monocytes, and endothelial cells.

It is believed that OxLDL specifically expresses carboxyl groups on its surface and that scavenger receptors bind to the OxLDL via the carboxyl groups as ligands. Phosphatidylserine (PS), a characteristic phospholipid with a carboxyl group on its hydrophilic head group, is a known component of bilayer membrane and is asymmetrically localized in the intracellular membrane of living cells. However, PS is detected specifically on the surface of apoptotic cells that are detectable by the specific binding of annexin V to PS (Koopman et al., 1994; Martinet al., 1995). Consequently, it can be assumed that the asymmetry of the bilayer membrane disappears on the apoptosis of cells and that PS is exposed on the cell surface by the flip-flop. In addition, previous reports describe that the PS is detected on the activated platelets (Thiagarajan & Tait, 1990), senescent erythrocytes (Schroit & Zwaal, 1991), and nuclei from erythroblasts (Yoshida et al., 2005). A specific receptor to PS has been identified on the phagocytes, enabling phagocytes to recognize the biomembrane exposing PS and to eliminate it (Fadok et al., 1992, 2000).

In selective elimination of lipid particles, oxidation of the unsaturated phospholipids plays a critical role. Natural phospholipids have polyunsaturated fatty acids such as linoleic acid and arachidonic acid. These polyunsaturated fatty acids are susceptible to free radical-initiated oxidation. The general process of lipid peroxidation consists of three stages:

initiation, propagation, and termination (Catala, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction by which radicals such as hydroxyl (•OH) react with polyunsaturated fatty acids to produce a lipid radical (L•), which in turn reacts with molecular oxygen to form a lipid peroxyl radical (LOO•). The LOO• can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical (Catala, 2006). The LOOH thus formed can suffer reductive cleavage by reduced metals such as Fe^{2+}, producing a lipid alkoxyl radical (LO•). Both alkoxyl and peroxyl radicals stimulate the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms (Buettner, 1993). Finally, the decomposition of the alkoxyl radical produces a terminal carboxyl group. Several kinds of oxidized phospholipids having the terminal carboxyl group have been identified. These oxidized phospholipids are known as ligands or strong agonists to the scavenger receptors, especially CD36 (Gao et al., 2010; Podrez et al., 2002). Further study has indicated that the carboxyl group of oxidized phospholipid is not located in the bilayer membrane but that it extends from the cellular surface like whiskers, in the "Whisker model", to interact with the receptors (Greenberg et al., 2008; Hazen, 2008). This whisker model is expected to be important to design of surface of the drug delivery carrier, which is expected to interact with the receptors.

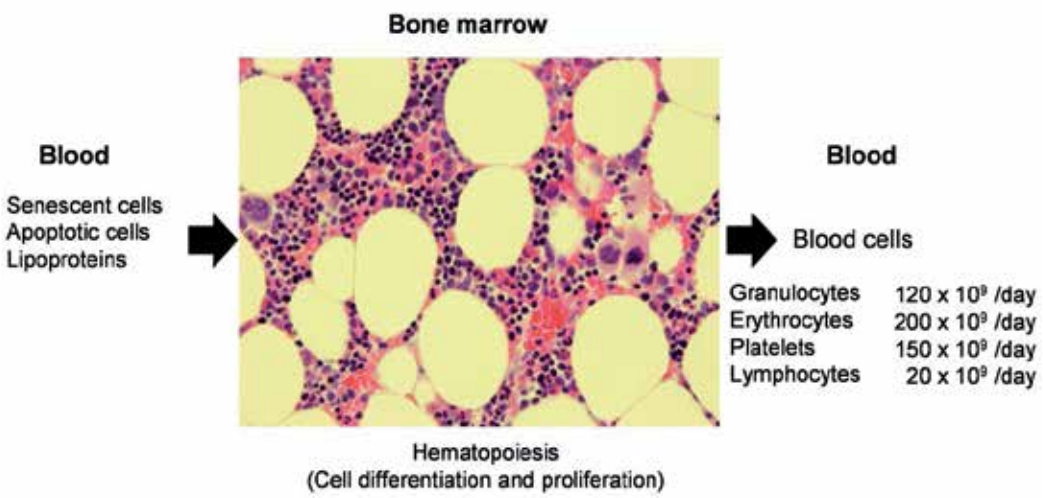

Figure 1. Bone marrow in blood cell turnover. Bone marrow can be regarded as a cell culture system in which nutrients for hematopoiesis can be supplied from circulating blood as senescent cells, apoptotic cells, lipoproteins, and so on. The blood cell turnover in an adult human is $20–200\times10^9$/day which is estimated from the life time and total number of cells of each type. The image shows a hematoxylin and eosin stained rabbit bone marrow tissue section, where nuclei of hematopoietic cells are stained darkly and the circular blanks are adipocytes.

3. Engineered carriers for bone marrow targeting

Intravenous injected nano-sized materials are typically eliminated from blood circulation through a mononuclear phagocyte system (MPS). The main organs for uptake of the nano-

sized materials are the liver and spleen in general. Little attention has been given to the bone marrow though the bone marrow is a part of MPS because the contribution of the bone marrow for uptake of nano-sized materials is believed to be small compared with those of the liver and spleen. However, results of several studies have shown that bone marrow is the most important organ for the uptake of nanoparticles that have been modified with specific molecules on their surface as presented in Table 1.

Carrier type	Targeting	Target cells	Species	Ref
Liposome	Passive (Reduced size)	Macrophages	Dog	Schettini et al., 2006
Liposome	Active (Succinic acid-lipid) and passive (PEG-DSPE)	Macrophages	Monkey, Rabbit, Hamster	Sou et al., 2007; 2010; 2011a
Polystyrene microspheres	Active (Poloxamer 407)	Endothelial cells	Rabbit	Porter et al., 1992; Moghimi, 1995
Polymer complex	Active (Cationic peptide)	Monocytes, T-cell lineage cells	Mouse	Harris et al., 2010
Porous silicon particles	Active (E-selectin thioaptamer ligand)	Endothelium	Mouse	Mann et al., 2011
Nanoparticlesof dendritic molecule	Active (Guanidinium group)	Osteoclast precursors	in vitro	Chi et al., 2010
Branched polypeptide	Active (Succinyl group)	Macrophages	in vitro	Szabó et al., 2005

Table 1. Proposed drug delivery carriers targeting bone marrow. These carriers based on surface-modified nanoparticles or polymers target bone marrow phagocytes such as macrophages and endothelial cells.

3.1. Liposomes

Liposomes are the most studied nanoparticles for drug and gene delivery to date. Great interest has been devoted to interaction between liposomes and cellular components *in vitro* and *in vivo*. Allen et al. studied the uptake of liposomes by cultured mouse bone marrow macrophages as a function of liposome composition (Allen et al., 1991). In this study, surface modification with monosialoganglioside (G_{M1}) and polyethylene glycol-lipid (PEG-PE) greatly decreased DPPC liposome uptake by bone marrow macrophages in a concentration-dependent manner. However, incorporation of PS increased liposome uptake by macrophages substantially in a concentration-dependent manner. These observations were correlated with the *in vivo* behavior of liposomes. Consequently, results of this study showed that liposome uptake by bone marrow macrophages is sensitive to the liposome composition, and that PS, which is a biological marker of apoptotic cells, accelerates the

uptake of the liposomes. Several other studies have also shown the accelerated uptake of liposomes containing PS or oxidized PS by macrophages, suggesting a similar molecular mechanism on apoptotic cell clearance (Fadok et al., 1992; Greenberg et al., 2006; Ishimoto et al., 2000).

Schettini and co-workers prepared a novel liposomal formulation of meglumine antimoniate, a drug used for treating leishmaniasis, to deliver the drug to the bone marrow (Schettini et al., 2006). The liposomes were produced from distearoylphosphatidylcholine (DSPC), cholesterol, and dicetylphosphate (molar ratio of 5:4:1). The targeting of antimony to the bone marrow was improved approximately three-fold with the small liposomal formulation compared to the large liposome formulation used in dogs with visceral leishmaniasis. These liposomes had no active targeting factor to bone marrow, but the passive targeting of the liposomes to the bone marrow of dogs was improved by the reduction of vesicle size from 1200 nm to 400 nm.

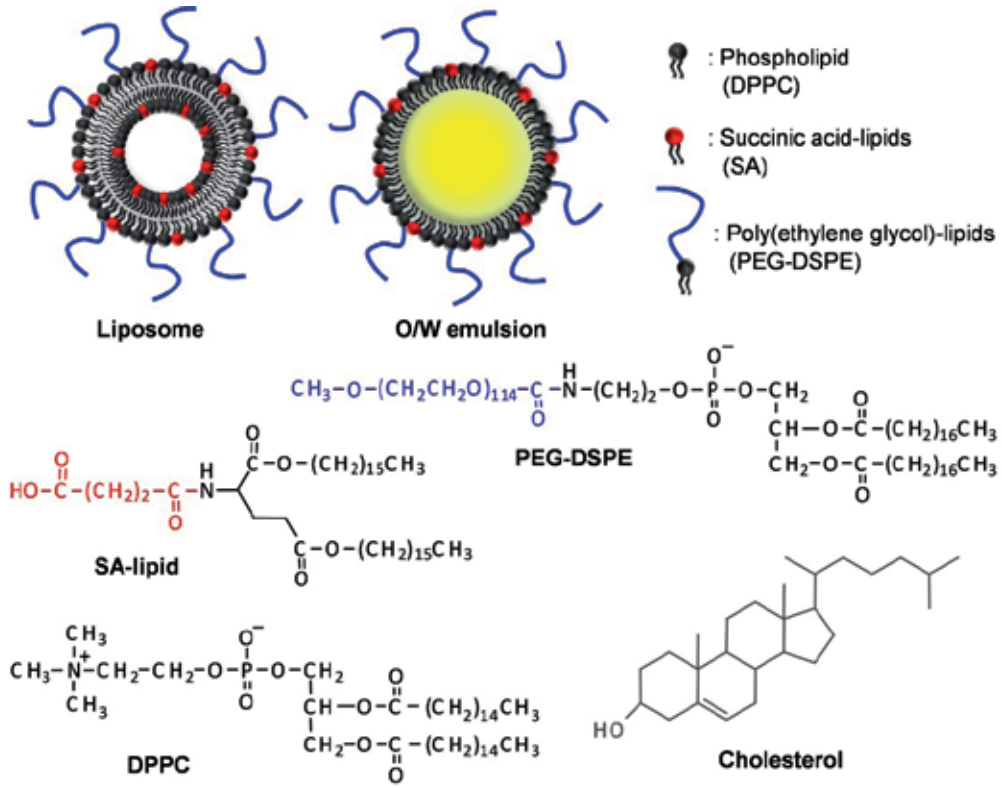

Figure 2. Lipid components of bone marrow-targeted lipid-based nanoparticles. Liposomes composed by DPPC, cholesterol and SA-lipid have high encapsulation capacity for water-soluble materials (Sou 2011b). Oil-in-water (O/W) emulsions have advantage in embedding lipophilic drugs in their oil core. The uptake of lipid-based nanoparticles by bone marrow phagocytes in rhesus monkeys, rabbits, and hamsters is induced by the incorporation of SA-lipid. PEG-DSPE enhances the uptake of the nanoparticles by bone marrow phagocytes passively by preventing the uptake by hepatic and splenic phagocytes (Sou et al. 2007; 2010; 2011a).

Sou et al. found a liposome formulation that is specifically distributed to bone marrow in rabbits and rhesus monkeys (Sou et al., 2007, 2010, 2011a). Figure 2 shows that the bone marrow-targeted liposomes comprise lipids of four kinds: 1,2-dipalmitoyl-*sn*-glycero-3- phosphocholine (DPPC), cholesterol, L-glutamic acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA-lipid), and poly(ethylene glycol) (PEG). The SA-lipid component has been identified as the active factor leading to their phagocytosis by bone marrow phagocytes, presumably macrophages in rabbits (Sou et al., 2007). Furthermore, as little as 0.6 mol% of PEG-DSPE depressed hepatic uptake but did not depress the bone marrow uptake. PEG-DSPE can be incorporated into the outer surface of preformed liposomes using the post incorporation method (Sou et al., 2000; Uster et al., 1996). Otherwise PEG-DSPE is mixed with other lipid components before preparation of liposomes. The active targeting factor of SA-lipid and passive targeting factor of PEG-DSPE appear to increase the distribution of the liposomes to bone marrow cooperatively.

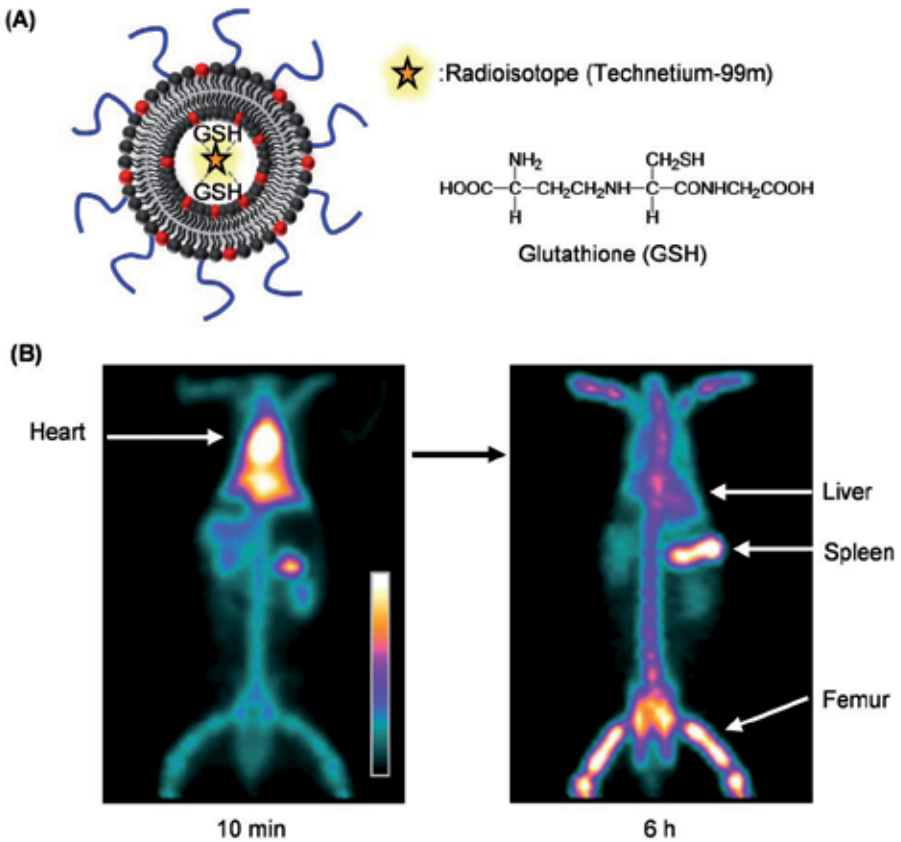

Figure 3. Imaging analysis of animal receiving technetium-99m (^{99m}Tc)-labeled bone marrow-targeted liposomes. (A) Preformed liposomes encapsulated glutathione (GSH) can be radiolabeled with ^{99m}Tc by a remote loading method (Phillips et al., 1992). (B) Gamma camera images of rabbits acquired at 10 minutes and 6 hours after injection of ^{99m}Tc-labeled bone marrow-targeted liposomes (Sou et al., 2007).

The size of the liposomes between 200–270 nm is not a significant factor for uptake by bone marrow. The liposomes were designed to have high entrapment capacity with the interfacial

electrostatic interaction to form a unilamellar membrane (Sato et al., 2009; Sou et al., 2003; Sou 2011b). These characteristics are expected to facilitate the application of the bone marrow-targeted liposomes as pharmaceutical carriers to bone marrow.

To evaluate the bone marrow-targeting capability quantitatively, whole body scintigraphic imaging in living animals is a particularly powerful tool (Goins and Phillips 2001, Phillips et al., 2009, 2011). Technetium-99m (^{99m}Tc), which is a good tracer for imaging, is used widely for single photon emission computed tomography with a gamma camera. Figure 3A shows that the ^{99m}Tc labeling of the liposomes encapsulating glutathione (GSH) can be accomplished using a complex of the ^{99m}Tc and GSH in inner aqueous phase of the liposomes (Phillips et al., 1992). Stoichiometric analysis has shown a 2:1 molar ratio of GSH and ^{99m}Tc for stable complex formation (Baba et al., 1999). Images presented in Figure 3B show the distribution of ^{99m}Tc-labeled bone marrow-targeted liposomes in a rabbit at 10 min and 6 hr after intravenous injection (lipid dose: 15 mg/kg b.w.). The image at 10 min is a typical blood pool image representing large blood pool at heart and liver, where the liposomes exist in blood circulation. At 6 hr, the radioactivity at the bone including marrow is increased, although it is decreased at the heart and liver. At this point, 69.7±0.3%ID of bone marrow-targeted liposomes accumulated in bone marrow. At the same time point, the liver and spleen respectively contained much smaller amounts of 11.5±2.88 and 5.0±1.19%ID (Sou et al., 2007).

In addition to the macroscopic and quantitative observation of the distribution of the liposomes by scintigraphic imaging, histological observation by microscopic techniques enables further microscopic localization of the liposomes in bone marrow tissues. Transmission electron microscopy (TEM) is the frequent method used to observe the nanoparticle sample to determine their size and size distribution. Observations of liposomes in cells and tissues can be made using TEM (Sakai et al., 2001). Figure 4 shows that microscopic localization studies demonstrate that bone marrow macrophages are the cellular components responsible for clearance of bone marrow-targeted liposomes from circulation and that they are also responsible for their uptake by the bone marrow. The liposomes are located in the phagosomes and lysosomes of bone marrow macrophages, indicating that the bone marrow macrophages capture the liposomes through phagocytosis. Therefore, it can be speculated that bone marrow-targeted liposomes interact with a receptor on the bone marrow macrophages, which stimulates the phagocytic activity of bone marrow macrophages specifically.

Also flow cytometry analysis is a useful method to determine the targeted cell population quantitatively (Harris et al., 2010). In this method, bone marrow cells will be labeled with markers to identify specific cell subtypes. Flow cytometry analysis might be particularly useful for animal experiment in mice and rats because the markers to identify specific cell subtypes are extensive for these species. Other possible method to identify the cell type is the immunofluorescent staining. The cells type emitting fluorescence from nanoparticle carriers or cargos in tissue section could be determined from the comparative analysis with images of immunofluorescent staining (Longmuir et al., 2009).

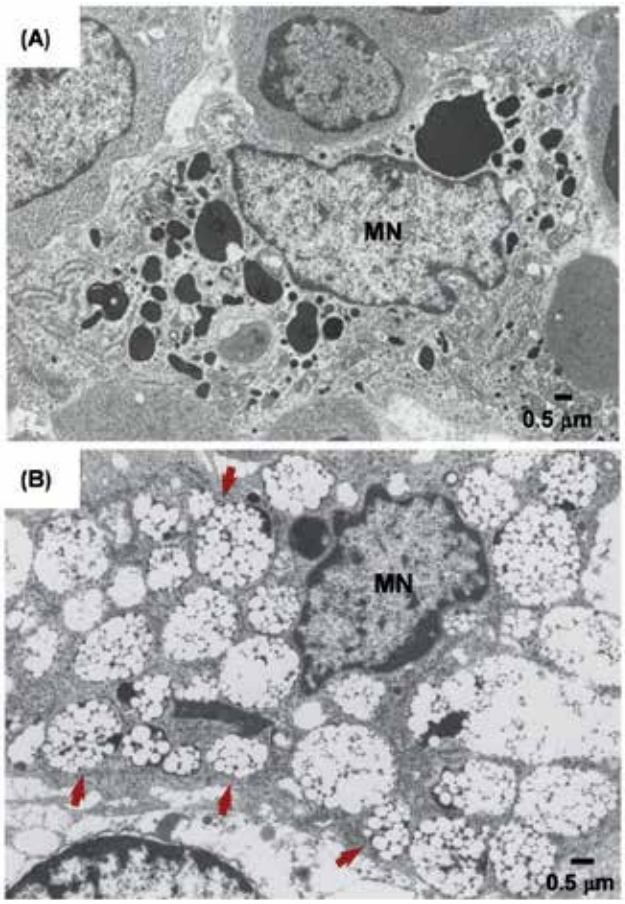

Figure 4. Transmission electron microscopic observations of bone marrow-targeted liposomes. (A) Bone marrow macrophages in a femoral bone marrow tissue section, taken from rabbit. Many phagosomes and lysosomes (electron-dense vacuoles) are visible. (B) Bone marrow macrophages in a femoral bone marrow tissue section, taken from a rabbit at 6 hr after intravenous injection of bone marrow-targeted liposomes (lipids: 15 mg/kg b.w.). Arrows indicate phagosome maturations trapping liposomes with the original diameter (average 270 nm) (Sou et al., 2007). MN: macrophage nucleus

Fluorescent techniques enable detection of the distribution of particular components labeled with fluorescent probes in live cells and tissues microscopically, with exquisite sensitivity and selectivity. For liposomes, both the lipid bilayer membrane (green fluorescent C1-BODIPY C12) and inner aqueous phase (red fluorescent Texas Red- superoxide dismutase, TR-SOD) were labeled with fluorescence probes as shown in Figure 5A. At 6 hr after injection of the dual-labeled bone marrow-targeted liposomes in rabbits (lipids: 15 mg/kg b.w.), the femoral bone marrow section was observed using confocal scanning microscopy. Figure 5B shows that the bone marrow sections have fluorescence from both the TR-SOD and C1-BODIPY C12, where the fluorescence from membrane probes and encapsulated probes are co-localized in bone marrow. This observation revealed that the bone marrow-targeted liposomes and encapsulated agents are distributed at the same locations into bone

marrow tissues, clearly indicating that the encapsulated agents were delivered to bone marrow tissues by the liposomes.

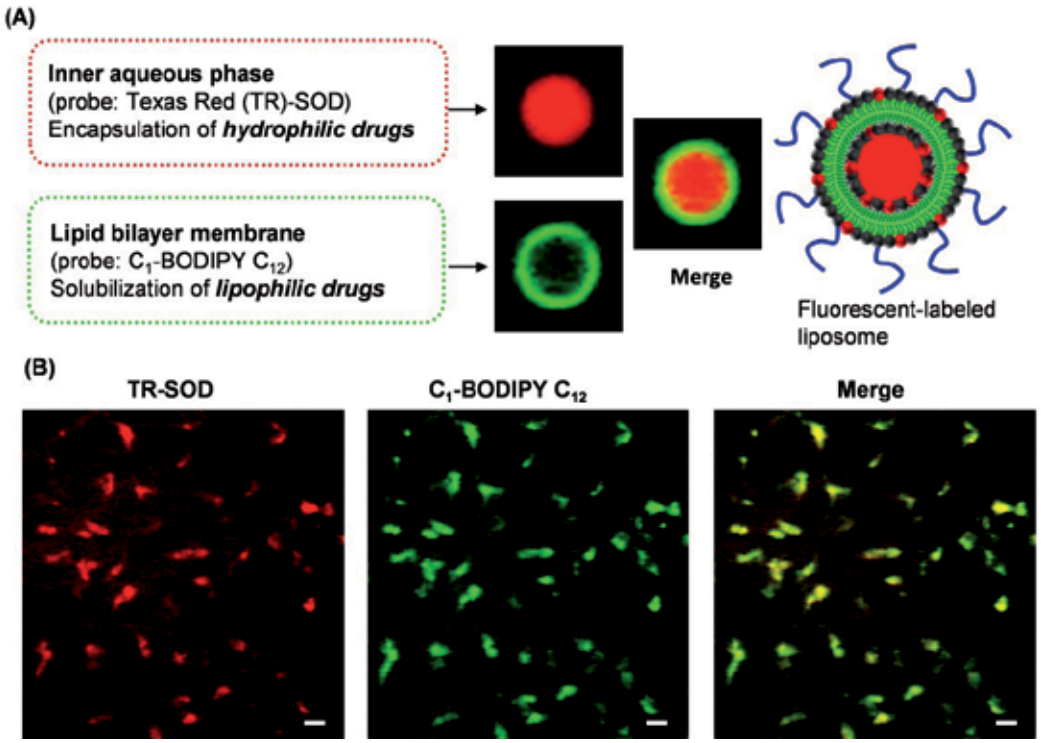

Figure 5. Histological examination of fluorescence delivered into bone marrow tissues using bone marrow-targeted liposomes as carriers. (A) Fluorescence localization in double fluorescence-labeled large multilamellar bone marrow-targeted liposomes with ca. 10 mm diameter. (B) Confocal scanning images of femoral bone marrow taken from a rabbit at 6 hr after i.v. injection of double fluorescence-labeled bone marrow-targeted liposomes (lipids: 15 mg/kg b.w.) (Sou et al., 2007). Scale bars show 20 μm.

3.2. Polymeric nanoparticles

Regarding engineered colloidal particles, Porter and co-workers observed remarkable accumulation of small colloidal particulates (150 nm and smaller diameter) that were coated by the block co-polymer poloxamer-407, a non-ionic surfactant, in the bone marrow after intravenous administration in rabbits (Porter et al., 1992). In this case, the coated colloids were sequestered by the sinusoidal endothelial cells of the bone marrow instead of macrophages. Importantly, no marked uptake was achievable with other block co-polymers having a similar structure to that of poloxamer-407, suggesting the participation of a specific interaction mechanism between the particle and the sinusoidal endothelial cell surface.

Chi and co-workers prepared dendritic amine and guanidinium group-modified nanoparticles for the delivery of model peptide drug into primary osteoclast precursor cells

(bone marrow macrophages) (Chi et al., 2010). It can be speculated that positively charged guanidinium groups have favorable interactions with negatively charged functional groups in the cell membrane of osteoclast precursors (Rothbard et al., 2005). Physicochemical interaction between a positively charged drug carrier and a negatively charged cell surface can enhance the cellular uptake in cells of various kinds with a negatively charged surface. However, the selectivity to specific bone marrow cells might be low in practical applications for drug delivery carriers.

Harris and co-workers studied tissue-specific gene delivery via nanoparticle coating (Harris et al., 2010). They prepared cationic nanoparticles with plasmid DNA and then coated their cationic surface with poly anionic poly(glutamic acid)-based peptides with and without cationic insert. Particles coated with a low 2.5:1 peptide:DNA weight ratio (w/w) form two large micro-sized particles that can facilitate specific gene delivery to the liver in mice. However, the same particles coated at a higher 20:1 peptide with cationic insert:DNA (w/w) form small 200 nm particles that can facilitate specific gene delivery to the spleen and bone marrow. They have confirmed that the terminal sequence insert, cationic amino acid sequence G-dP-dL-G-dV-dR-G, to the poly(glutamic acid)-based peptides is a critical factor enhancing bone marrow and spleen-specificity of gene delivery *in vivo*. Regions of luminescence selected around the femur bones showed nearly 40-fold enhancement, and regions around the spleen showed nearly 30-fold enhancement by the cationic insert. Flow cytometry analysis of bone marrow cells from a mouse tail-vein injected with green fluorescent protein-encoding nanoparticles coated with 20:1 w/w peptide with a cationic insert revealed that green fluorescent protein expression relative to the whole population of bone marrow cells is enriched in monocyte and T-cell lineage cells. This system might be available for bone marrow-specific drug delivery and for gene delivery. The molecular mechanism at work in this system is not obvious.

E-selectin is an attractive molecular target for active targeting of a drug carrier to bone marrow because E-selectin is expressed selectively on endothelial cells of adult and fetal hematopoietic organs (Schweitzer et al., 1996). It has been suggested that the E-selectin plays a role in the homing of hematopoietic progenitor cells and that its constitutive expression on endothelial cells of hematopoietic organs is necessary in the initial step of the homing process. Mann and co-workers identified a thiophosphate-modified aptamer (thioaptamer) against E-selectin (Mann et al., 2010). They confirmed that the thioaptamer ligand selectively binds to E-selectin with nanomolar binding affinity (K_D=47 nM) while exhibiting minimal cross reactivity to P-selectin and L-selectin. Recently, they developed porous silicon particles modified with E-selectin thioaptamer ligands to target bone marrow endothelium (Mann et al., 2011). A mice study demonstrated that the accumulation of the porous silicon particles modified with E-selectin thioaptamer ligands in the bone marrow was eight times higher than control porous silicon particles, which were accumulated primarily in the liver and spleen instead of bone marrow. Histological analysis supported the presence of porous silicon particles modified with E-selectin thioaptamer ligands on the endothelial wall of the bone marrow tissue. The molecular target of this ligand-receptor reaction might be readily apparent in this system and be promising for delivering drugs to bone marrow endothelial cells specifically.

Moghimi reviewed the clearance mechanism of particulate materials from the circulation by bone marrow (Moghimi, 1995). The endothelium of bone marrow sinusoids can remove particles from circulation by both transcellular and intercellular routes. The intercellular route occurs through the fenestrate in the endothelial wall. Therefore this mechanism is strongly dependent on the particle size. The intercellular distance is less than 2 nm for the tight junctions in capillaries and less than 6 nm for post-capillary venules under normal conditions (Simionescu et al., 1978; Bundgaard, 1980). In contrast, the size of the fenestrate in the endothelial wall is reportedly 85–150 nm (Huang, 1971). These fenestrated capillaries exist in bone marrow as well as in the liver and spleen. Therefore, the intercellular route is a possible pathway to target bone marrow through circulation. Liposomes consisting of DSPC, cholesterol, PEG(5000)-DSPE, and α-tocopherol prepared in various sizes (136–318 nm diameter) have been tested for organ distribution in rabbits. None of these liposomes show a significant accumulation in bone marrow (Awasthi et al., 2003). Therefore, the passive diffusion of nanoparticles from blood circulation to extravascular space through an intercellular route is not an important factor in bone marrow targeting when no active targeting factor exists. Indeed, based on the proposed drug delivery carrier described above, the surface modification of nanoparticles with specific molecules is a critical factor to achieve bone marrow targeting with nanoparticles, although further study is necessary to ascertain details of its molecular mechanism. Consequently, discovery of receptors specifically expressing in bone marrow tissues facilitates the development of drug carriers targeting bone marrow.

4. Future prospects for bone-marrow-targeted drug carrier systems

The balance between blood cell production and removal in blood cells of each type is important to maintain the number of circulating blood cells. When blood cell production is suppressed, erythrocytopenia, leukopenia, and thrombocytopenia are induced. The blood cell production suppression results from the direct bone marrow dysfunction or indirect factor such as deficiency of the hematopoietic growth factors such as erythropoietin (EPO), granulocyte colony-stimulation factor (G-CSF), and thrombopoietin (TPO). These hematopoietic growth factors, EPO and G-CSF, have been established as recombinant products. Recombinant EPO products are used clinically for the renal anemia patients or autologous blood. These growth factors function with hematopoietic cells in bone marrow, so that a drug delivery system to target bone marrow can be expected to offer an improved therapeutic system.

Recently, Winkler and co-workers reported that the bone marrow macrophages are pivotal to maintain an endosteal hematopoietic stem cell niche and that the loss of such macrophages engenders the egress of hematopoietic stem cells into the blood (Winkler et al., 2010). They administered clodronate-loaded liposomes intravenously to deplete the bone marrow macrophages. After the macrophage depletion, hematopoietic stem cells were found in the blood. These findings provide evidence supporting the critical role that macrophages play in the support of hematopoietic cells in bone marrow. Such specific biology of bone marrow macrophages can present a therapeutic target for the treatment of hematopoietic disorders.

Leishmaniasis is an infectious disease caused by a protozoan parasite that is parasitic on resident macrophages. Promastigotes, which are injected into the skin by a sandfly, are phagocytized by macrophages. Thereafter, promastigotes transform into amastigotes inside macrophages. The amastigotes multiply in cells, including in macrophages, at various tissues. The typical symptoms in leishmaniasis are damage to the spleen and liver, and anemia by damage to bone marrow. Therefore, macrophages in bone marrow as well as in the liver and spleen are target cells in leishmaniasis treatment. Thus several nanoparticles loaded with therapeutic agents such as nanoparticles loaded with amphotericin B (Gupta & Vyas 2007; Nahar et al., 2010), PLGA nanoparticles loaded with saponin (Van de Ven et al., 2012), lipid nanoparticles loaded with oryzalin (Lopes et al., 2012), PLGA nanoparticles loaded with kinetoplastid membrane protein-11 (Santos et al., 2012) have been developed to deliver the drugs in leishmania-infected macrophages. Drug carriers targeting bone marrow, especially bone marrow macrophages, would have a great potential to deliver these therapeutic agents in leishmania-infected macrophages.

The abnormal increase of cancerous cells such as leukemia cells, which are immature white blood cells in bone marrow, suppress normal hematopoiesis. Different from a solid cancer, surgical resection is ineffective as a treatment for leukemia. Most cases of leukemia are treated using chemotherapy. Therapies are typically combined into multi-drug chemotherapy. Drug delivery systems targeting bone marrow can increase the efficacy of such methods. Moreover, bone marrow is sensitive to chemotherapy and radiation therapy. Several pharmaceuticals are available to protect soft tissues from chemotherapy and irradiation. Drug carriers targeting bone marrow offer a promising platform to deliver such pharmaceuticals to bone marrow effectively.

5. Conclusion

Conventional drug delivery to bone marrow is uncontrolled passive diffusion of drugs into bone marrow through blood circulation. The fraction of drugs reaching the target site through such passive diffusion is negligibly small. Bone marrow-targeted drug delivery carriers provide a platform to develop more efficient and safer diagnostic and therapeutic systems. These therapeutic systems can target bone and bone marrow diseases such as rheumatoid arthritis, bone regeneration and repair, bone metastases, osteoporosis, infectious diseases, multiple myeloma, and hematopoietic dysfunction.

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Chapter 16

Gene Delivery Systems: Recent Progress in Viral and Non-Viral Therapy

Erdal Cevher, Ali Demir Sezer and Emre Şefik Çağlar

Additional information is available at the end of the chapter

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1. Introduction

Thanks to the changes in medicine, pharmacological treatment rapidly progresses into new fields. There is an emphasis on the development of treatment methods to eliminate underlying factors rather than to treat the symptoms of a disease. Therefore, research is increasingly utilizing knowledge from the field of genetics. Genetic mutation and deletion lead to many genetic disorders. Genetic disorders in metabolic pathways, regulation of cell cycle, ligand/receptor function, cell skeleton and extracellular proteins cause serious diseases (Yaron et al., 1997).

Both genetic and acquired diseases may be treated using gene therapy. Genetic diseases are generally caused by deletion or mutation of a single cell. Conversely, in acquired diseases, a single gene cannot be defined as the sole cause of a disease. Gene therapy has increased in prominence, and has shown great potential in treating acquired and genetic diseases (Conwell and Huang, 2005).

Gene delivery systems are categorized as: viral-based, non-viral-based and combined hybrid systems. Viral-mediated gene delivery systems consist of viruses that are modified to be replication-deficient, but which can deliver DNA for expression. Adenoviruses, retroviruses, and lentiviruses are used as viral gene-delivery vectors (Escors and Breckpot, 2010).

Non-viral gene delivery systems were introduced as an alternative to viral-based systems. One of the most important advantages of these systems is improved transfection. Non-viral systems are categorized according to preparation, as physical or chemical types. The most common physical methods are microinjection, electroporation, ultrasound, gene gun, and hydrodynamic applications. In general terms, physical methods refer to delivery of the gene *via* the application of physical force to increase permeability of the cell membrane. In contrast, chemical methods utilize natural or synthetic carriers to deliver genes into cells. In

this method, polymers, liposomes, dendrimers, and cationic lipid systems are used as gene delivery systems (Miyazaki et al., 2006; Prokop and Davidson, 2007).

2. Gene therapy

This chapter provides general information on gene delivery systems, how they are used, their relative merits, and selection of the most appropriate methods for new studies on gene therapy.

The drug sector is entering a new era that will enable treatment of the underlying cause rather than the symptoms of a disease. Many human diseases result from mutations or deletions, in genes, which lead to disorders in metabolic pathways, ligand/receptor function, cell cycle regulation, cell skeleton or extra-cellular protein structure and function (Sullivan, 2003). With gene therapy, the disease can be treated by the injection of exogenous nucleic acid sequences designed to target the diseased tissues of the body (Yaron et al., 1997).

Diseases that can be treated by gene therapy are categorized as either genetic or acquired. Genetic diseases are those which are typically caused by the mutation or deletion of a single cell. Conversely, a single gene is not defined as the sole cause of acquired diseases. Although gene therapy was initially used to treat genetic disorders only, it is now used to treat a wide range of diseases such as cancer, peripheral vascular diseases, arthritis, neurodegenerative disorders and AIDS (Mhashilkar et al., 2001). The expression of a single cell, directly delivered to the cells by a gene delivery system can potentially eliminate a disease. Prior to gene therapy studies, there was no alternative treatment for genetic disorders. Today, it is possible to correct genetic mutation with gene therapy studies (Sullivan, 2003).

2.1. Chronology

The term gene therapy was first introduced at the International Congress of Genetics (Yaron et al., 1997). The techniques utilized by Gregor Mendel in the 1850s, which were then developed by Ronald Fischer at the beginning of the twentieth century, formed the turning points in genetics. The work of both Mendel and Fischer laid the foundations of genealogy. The material they studied was later termed as gene by the Danish botanist Wilhelm Johannsen (Yaron et al., 1997). Towards the end of the 1970s, the background of the majority of genetic disorders was understood, and gene therapy came to the fore. The first gene therapy trial in humans was conducted at the beginning of the 1970s, and it was observed that naturally occurring DNA and RNA tumor viruses successfully delivered new genetic information to the genomes of mammal cells (Escors and Breckpot, 2010).

Due to developments in the science of genetics, at the beginning of the twentieth century, it was understood that diseases such as hemophilia were genetic diseases. Similarly, it was found that diseases such as colon cancer, diabetes, and retinoblastoma were also genetic-based diseases. In the 1980s, gene transfer to mammalian cells came to the fore after the

development of retroviral vectors (Yaron et al., 1997). In the mid-1980s, gene transfer to mammalian cells became a routine procedure. Retrovirus-based gene therapies brought significant advantages, as they can stably integrate their genomes to host-cell chromosomes. After the 1980s, DNA was defined as a genetic material. Later, it was structurally analyzed and further advances allowed the modification of genetic code. These discoveries on genetic material made cloning possible (Escors and Breckpot, 2010).

In 2006, melanoma was successfully treated for the first time. The treatment used a retroviral formulation of melanoma antigen (MART-1)-specific T-cell receptor (TCR), which encodes α- and β- chains. In recent years, great advances have been made in gene therapy, ranging from somatic cloning and completion of human genome project to the discovery of microRNA-based gene-regulating systems (Escors and Breckpot, 2010).

The use of non-viral gene delivery systems in gene therapy also significantly increased in recent years. Naked plasmid DNA, coated with gold particles was effectively introduced to cells using the non-viral gene gun technique. This technique was first used in plant cells in 1987, and is today commonly used in mammal cells and tissues. One of the most successful current gene therapy techniques is hydrodynamic injection (Willemejane and Mir, 2009).

2.2. Gene delivery systems

Gene delivery systems use various methods to allow uptake of the gene that has been selected to target the cell (Conwell and Huang, 2005). The successful design of a gene delivery system requires complete understanding of the interaction mechanism between the target cell and delivery system. Understanding intercellular traffic and targeting mechanism is the most important factor in designing a more effective gene delivery system. Cell targeting refers to delivery of the therapeutic agent to a specific compartment or organelle of the cell. It is the most commonly used mechanism in endocytosis gene therapy, particularly in cellular uptake of non-viral gene delivery systems (Prokop and Davidson, 2007). After the cellular uptake of the delivery system by endocytosis, cellular release takes place to initiate DNA transcription and translation, and to produce the related protein. A successful gene delivery procedure involves minimizing potential inhibitory inflammatory response while also overcoming certain barriers at each step of the gene delivery procedure, in order to optimize gene activity (Conwell and Huang, 2005).

Viral gene delivery systems consist of viruses that are modified to be replication-deficient which were made unable to replicate by redesigning which can deliver the genes to the cells to provide expression. Adenoviruses, retroviruses, and lentiviruses are used for viral gene delivery. Viral systems have advantages such as constant expression and expression of therapeutic genes (Sullivan, 2003). However, there are some limitations that restrict the use of these systems, which particularly include the use of viruses in production, immunogenicity, toxicity and lack of optimization in large-scale production (Witlox et al., 2007) (Figure 1).

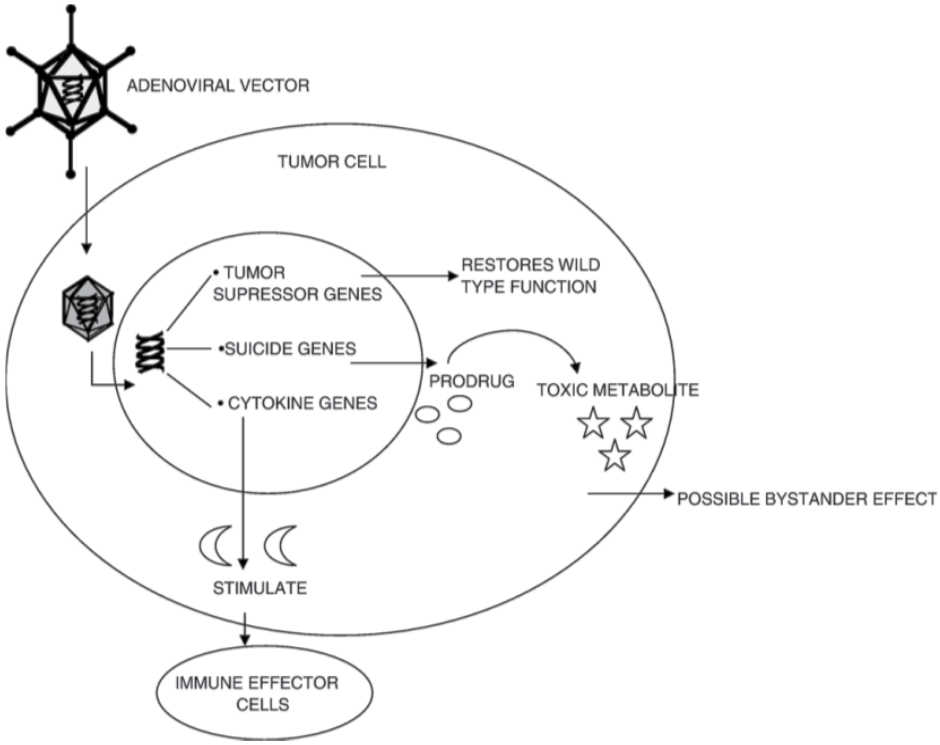

Figure 1. Gene therapy strategies; mutation compensation, suicide gene therapy and immuno-potentiation (Witlox et al., 2007).

Non-viral gene delivery systems were developed as an alternative to viral-based systems. One of the most important advantages of these systems is that they develop transfection. Non-viral gene delivery systems are divided into two categories: physical and chemical. Microinjection, electroporation, gene gun, ultrasound-mediated methods, and hydrodynamic systems are the most widely used physical methods. Physical methods involve the use of physical force to increase the permeability of the cell membrane and allow the gene to enter the cell. The primary advantage of physical methods is that they are easy to use and reliable. However, they also have the disadvantage of causing tissue damage in some applications.

Chemical methods involve the use of carriers prepared from synthetic or natural compounds for gene delivery into the cell, including synthetic and natural polymers, liposomes, dendrimers, synthetic proteins, and cationic lipids. The biggest advantages of these systems are that they are non-immunogenic and generally have low toxicity.

2.2.1. Viral gene delivery systems

Since a large number of viruses have appropriate mechanisms for transfer of genetic material to the target cell, current gene technologies concentrated on the use of viral vectors that provide high transduction effectiveness and advanced level of gene expression. The

optimal design of a viral vector depends on the types of virus to be used (Wunderbaldinger et al., 2000).

For safe application of *in vivo* gene therapy, firstly some problems should be eliminated. Therapeutic factors should only be expressed in related cell types, and should not cause any undesired effect in healthy cells (Yaron et al., 1997).

2.2.1.1. Adenoviral systems

Adenoviruses (Ad) were first discovered in 1953 by isolation from human adenoid tissue cultures (Campos and Barry, 2007; Majhen and Ambriovic-Ristov, 2006). They are commonly used as gene vectors (Dinh et al., 2005). C group adenoviruses Ad2 and Ad5 are the most widely studied adenoviruses. The capsid of an adenovirus determines virus tropism. Groups A and C–F first bind to highly-expressed coxsackie virus B-adenovirus receptor, and thus realize their high infectivity in many tissues. In contrast, group B binds to complement-regulatory protein CD46. Adenoviruses are replicated and produce virions, which contain the nucleus of the infected cell (Dinh et al., 2005). These vectors have the ability to be replicated, and purification of the vectors generally involves easier and shorter processes (Armendariz-Borunda et al., 2011).

Adenoviruses are well-characterized, non-integrated, ~26–40 kb in length, relatively large, non-enveloped, linear dsDNA viruses coated with icosahedral particle, with a diameter of ~ 950 Å (excluding elongated fiber proteins) and a molecular weight of approximately 150MDa. They are unreplicated, and infect cells quickly. Adenoviral particles do not contain lipid or membrane; therefore they are stable in solvents such as ether or ethanol. The use of adenoviruses, which are one of the first developed vector systems for gene expression, is a great discovery (Campos and Barry, 2007).

Adenoviruses have important characteristics, which make them indispensable for gene transfer. The most important ones are their well-known molecular biology; delivery capacity of foreign DNA fragments up to 36kb; and ability to transfect DNA into many cell types (Sullivan, 2003).

Adenoviruses are one of the largest and most complex viruses, whose Ad structure was analyzed with cryo-electron microscopy and X-ray diffractometry. An Ad genome of approximately 36kb codes more than forty genomes; however, only 12 of these were demonstrated as the component of the virus particle. As seen in Figure 2 it was reported that crystal structures of single Ad proteins contained fiber knob, shaft, domains, penton base, hexon, and cysteine protease. Ad capsid consists of 252 sub-units called capsomeres, which contain 240 hexonproteins and 12 of the penton base. In addition, the capsid contains pIIIa, pVI, PVIII, and pIX proteins. Each of the 12 capsid corners contains penton bases wrapped by 5 hexons. The penton base serves as a hook for the fiber protein, which projects from the virus like an antenna. Fiber is a homotrimer, where 3 identical polypeptides bind in the same direction, and which contains 3 structurally and functionally different domains: N-terminus, which binds the fiber non-covalently to the penton base; C-terminus, which gives the disc-like knob form, which is responsible for binding to the receptor; and the rod-like

shaft, which varies in length according to the serotype of the virus (Medina-Kauwe LK. 2003; Majhen and Ambriovic-Ristov, 2006).

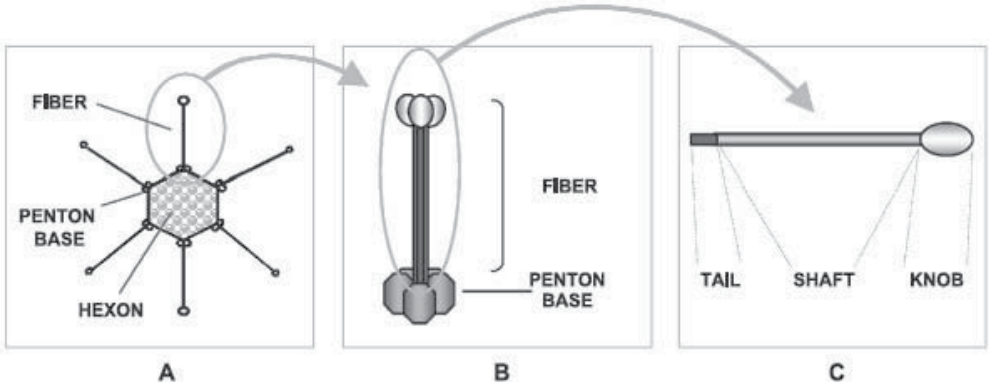

Figure 2. Schematic of the adenovirus capsid. (A) Whole capsid identifying fiber, penton base, and hexon. (B) Enlargement of circled region in (A), showing homotrimeric fiber bound to homopentameric penton base. (C) Fiber monomer, identifying tail, shaft, and knob domains. Pictures are not drawn to scale (Medina-Kauwe LK. 2003).

A viral genome is in a condensed state inside cell nucleus, together with protein V, VII and X, and the 5′ terminus of the AdDNA is covalently linked to a terminal protein (TP). The terminal protein (TP) functions as the initiation primer in viral DNA replication. An Ad genome consists of 5 early (E1A, E1B, E2, E3 and E4), 4 interim (IVa2, IX, VAI, VAII) and one late transcriptional unit. E1A is the first viral transcriptional unit, which will be expressed after entering the cell. E1A proteins activate other *trans*early transcription units, which will create an optimum medium for virus replication by causing the products to enter in S phase of the cell E1B proteins bind to p53, Bak, and Bax proteins, and allows the infected cell survive by inhibiting p53 dependent apoptosis. The E2A unit encodes the proteins that function in viral genome replication, including DNA polymerase, prethermal protein, and single-stranded DNA-binding protein. While the E4 transcription unit encodes the proteins that affect cell cycle control and transformation, E3 protein disrupts the immune response of the host and ensures the continuation of the infected cell (Majhen and Ambriovic-Ristov, 2006).

The nucleus is surrounded by an icosahedral capsid with a symmetrical structure, formed by the non-covalent interactions between 7 proteins (II, III, IIIa, IV, VI, VIII, and IX) (Smith et al., 2010). Adenoviral structural proteins are responsible for the stabilization of genome and encapsidation of the nucleoprotein nucleus. The icosahedral capsid consists of seven peptides:trimerichexons, which are found in complex form with three minor capsid polyproteins (VI, VIII and IX), which provides stabilization (II); pentonbase (III); receptor binding fiber (IV) and penton-bound protein which serves as a bridge between protein and the hexon base (III). The fiber consists of 3 domains which are tail in N-terminus, rod-like shaft and globular knob in C-terminus (Couglan et al., 2010). Adenoviruses enter target cells *via* receptor-mediated endocytosis. Instead of combining its own DNA with the genomes of

the host cell, the adenovirus, remains as an episome within the infected cell. This provides high efficiency (10); however, it should not be ignored, since it will restrict the longevity of expressed trans genes; in other words, protein expression will quickly cease (Yaron et al., 1997; Muzzonigro et al., 1999).

Penton and fiber proteins of virus capsid interact with the coxsackievirus-adenovirus receptor cell surface protein to provide cell binding. These proteins are then localized in clathrin-coated pits through NPXY motive of β_3 and β_5 integrin sub units of the cell. Dinamine, which is systolic GTPase, mediates cellular uptake of the virus into the cell via endocytosis by providing construction and development of clathrin-coated pits. Viral capsid proteins dissociate prior to endocytosis, and the pH value of the viral endosome decreases due to proton pumps. At pH 6.0, the virus has the ability to detach from the vesicle and enter the cytosol. Capsid proteins of the virus move towards the nucleus through microtubules and fragment in the following stages. Although a considerable part of the capsid proteins remain in the periphery of the nucleus, viral DNA crosses *via* nuclear pores (Ziello et al., 2010).

For successful delivery of DNA to the nucleus, viruses must facilitate cell-specific binding, endocytosis internalization, propagation from endocytic vesicles to cytosol, delivery into cytoplasm, translocation from one terminus of the nuclear envelope to other, and finally expression of the delivered gene (Dinh et al., 2005).

Direct injection or bolus delivery through inhalation is the easiest form of viral delivery. However, since the virus will spread from the injection area, a high dose is required to achieve therapeutic effectiveness. The spread of the virus from the injection site limits the regional effectiveness and immune response. Adenoviral vectors can be targeted *via* selective modification of coat proteins. There is a relatively high level of protein expression following the transduction, equal to approximately 35% of total cellular protein (Zhang et al., 2009).

Adenoviruses have many natural properties that enable them to be used as a vector for on colitic, vaccine, or gene therapy. Non-enveloped viruses can be kept in lyophilized form in a stable state inside a flacon tube or capsule; they can be transported without cold chain; by mediating in high transduction effectiveness in dividing and non-dividing cells, they can form 104 virus particles per infected cell (Khare et al., 2011). Adenoviruses achieve suitable transduction through a high level of expression and become beneficial in *in vivo* conditions (Muzzonigro et al., 1999).

Adenoviruses have been one of the most promising methods for high effectiveness in *in vivo* gene therapy. For example, following systemic vector delivery, transduction levels of hepatocytes were observed by adenoviral systems. These are some of the most effective vectors for gene delivery to various organs; however, they have very significant disadvantages. Some target cells have low ratios of appropriate primary and/or secondary adenoviral receptors, and this requires a high dose of vector application to cause target-cell cytotoxicity. Additionally, non-discriminating tropism of the virus can also lead to transduction to untargeted cells (Reynolds et al., 1999). The most serious problem in the use of Ad vectors is their tendency to cause strong immune and inflammatory responses at high doses (Navarro et al., 2008).

2.2.1.2. Retroviral systems

Retroviruses are diploid, single-stranded, circular-enveloped RNA viruses of the family Retroviridae, with a genome of 7–11 kb, and a diameter of approximately 80–120 nm (Osten et al., 2007; Escors and Brecpot, 2010). The Retroviridae family is divided into two sub-families: Orthoretrovirinae and Spumaretrovirinae species (Osten et al., 2007). Retroviruses cause diseases such as AIDS, leukemia, and cancer; however, their use as a vector in gene therapy brought new developments in treatment (Pages and Danos, 2003).

The ability of retrovirus-based gene delivery vectors to carry foreign genetic material was first realized in the early 1980s (El-Aneed, 2004; Escors and Brecpot, 2010). Retroviruses are viruses that integrate with host genome to produce viral proteins (gag, pol, env) that are extracted during gene delivery (El-Aneed, 2004). Retroviral vectors have the capacity to delivery DNA up to 8 kb (Navarro et al., 2008). The most commonly used retroviruses are the Moloney murine leukemia virus species, which have the capacity to deliver exogenous genetic material up to approximately 9 kb (Muzzonigro et al., 1999).

A virion nucleus consists of circular or triangular gag-encoded capsid proteins. They are coated with gag-encoded nucleocapsid protein. The nucleus contains pol-encoded enzymes, reverse transcriptase, and integrase. Retrovirus genomes have various characteristics. For example, these viruses are diploid in the real sense, and their genomes are produced by cellular transcriptional mechanism. In addition, these viruses require cellular (tRNA) for replication (Luban, 2000). The genome of these (+) sense RNA viruses is not directly processed as mRNA immediately after infection Considering the structure of the genome, retroviruses are categorized as either simple or complex forms. Simple and complex retroviruses encode gag (group-specific antigen), pro (protease), and pol (polymerase) genes; complex retroviruses also encode a large number of additional genes. Retroviruses were the first viruses to be modified for gene delivery, and have also been used in the majority of clinical trials of gene therapy (Hu and Pathak, 2000).

Most of retroviral vectors used in clinical studies are based on the Moloney murine leukemia virus (MMLV). MMLV is a well-studied and characterized virus; its genome encodes three polyproteins that are required in trans form for replication and packaging: gag, pol and env (Escors and Brecpot, 2010).

An ideal retroviral vector for gene delivery should be cell-specific, regulated, and safe. Effectiveness of delivery is important, as it will also determine the effectiveness of therapy (Hu and Pathak, 2000). Retroviruses have a lipid envelope. In order to enter a host cell, they use the interactions between cellular receptors and virally encoded proteins, which are embedded in the membrane (Hu and Pathak, 2000). Combination of chimeric envelope protein with viral particles allows the retrovirus to bind receptor-positive target cells (Yi et al., 2011). SU in the virion surface binds to CD, which is a cell-surface protein expressed by some T-cells and is found in macrophages. Binding to CD4 induces some changes in the envelope protein (SU) of the domain of epidermal growth factor (EGF), which allows for interaction with chemokine receptors (CKR). CKRs are a family of cell-surface-G-proteins functioning as receptors to stain molecules called chemokines.

Infusion of viral and cellular membranes starts the internalization of the viral nucleus (Yi et al., 2011); the viral envelope combines with the cell membrane. Envelope glycoprotein (Env) of retroviruses is responsible for determining tropism. Binding of Env to cellular receptor and fusion of viral and cellular membranes are the first stages of infection. Trials that targeted retroviruses by adding single-stranded antibodies or Env cell-binding ligands showed limited success. The addition of such large ligands to Env protein prevents Env from binding to the virion (Bupp and Roth, 2002). Env protein takes a particle-coated structure and releases the viral nucleus into the cytoplasm. Single-stranded DNA gets reversely transcripted to double-stranded DNA in the nucleus (Osten et al., 2007; Karlsson et al., 1987). Viral RNA is reverse transcribed to a DNA copy using virally-encoded reverse-transcriptase enzyme in the virion (Hu and Pathak, 2000). Retroviruses follow a different replication cycle through transformation of single-stranded RNAs into double-stranded DNA in the infected cell. Viral DNA synthesis takes place in cytoplasm by virion-dependent DNA polymerase, which is termed reverse transcriptase. Viral DNA enters the nucleus, where takes the ring form for the first time, and serves as a draft for RNA synthesis (Olsen and Swanstorm, 1985). The first DNA product is a linear double layer molecule, which forms during DNA synthesis. Within the nucleus, some linear DNA is turned into many different forms of ring DNA; these mainly contain two copies of LTR units in tandem, or a single copy of LTR unit (Olsen and Swanstorm, 1985). The end of each LTR unit creates short, deficient repetitions; this represents the strand domain required for this integration (Olsen and Swanstorm, 1985), and is delivered to the nucleus. This delivery takes place through cell division for oncoretroviruses, and through active transport for lentiviruses. Here, lentiviruses are observed to be more advantageous than oncoretroviruses, as they have the ability to deliver genes to non-dividing cells. When viral DNA enters the nucleus, it integrates with the DNA of the host cell (provirus). Retroviruses introduce their genetic material to the host cell genome in a stabile manner during mitotic division. Since these types of retroviruses only transfer genes to dividing cells, many procedures that utilize retroviruses are applied *ex vivo*. However, more recent lentiviral-based retroviral vectors reduce these restrictions (Muzzonigro et al., 1999). Most retroviruses infect cells that can be actively divided during mitotic division. This property protects normal tissue, and although it naturally targets the tumor, all tumors contain non-dividing cells in G0, which is the resting phase of the tumor cell cycle (Hu and Pathak, 2000; Kitamura et al., 2003; El-Aneed, 2004) (Figure 3).

Infected cells are then transcribed and spliced. Full-length viral RNA (which is the RNA that encodes all proteins) is delivered to cytoplasm and translated; other cellular procedures are modification and delivery of RNA from the nucleus (Hu and Pathak, 2000). Spliced full-length viral RNA is packaged into viral particles. Retroviruses are RNA viruses that are replicated over integrated DNA intermediate products. Retroviral particles surround two copies of full-length viral RNA, containing all of the genetic information required for virus replication, with capsid (Hu and Pathak, 2000). Virologic and genetic studies indicated that specific packaging of retroviral genomic RNA was carried out through the interaction with the nucleocapsid (NC) domain of Gag polyprotein. Retroviral NC domains are generally very simple, and contain one or two zinc knob motifs consisting of C-C-H-C sequences. Zinc knobs form metal-coordinated reverse turn, stabilized by NH-S hydrogen bonds. Most retroviral zinc knobs contain a hydrophobic cut on the surface of the mini-globular domain,

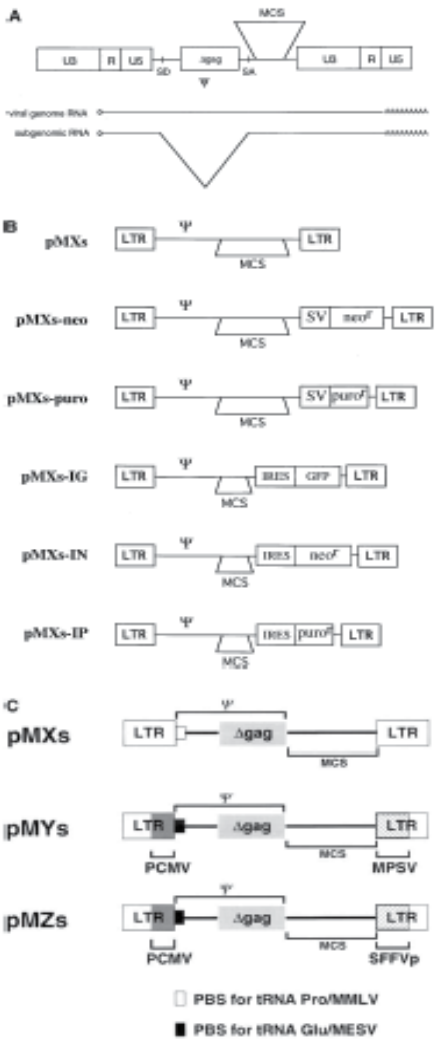

Figure 3. Structures of retrovirus vectors. **(A)** Basic structure of retrovirus vectors and two transcripts from the vector. In the replication competent MMLV, the Gag-Pol and the Env proteins are translated from the genomic RNA and the subgenomic RNA, respectively. The subgenomic RNA is a spliced form RNA, and the splicing occurs from the splice donor site (SD) to the splice acceptor site (SA). Both 5´ and 3´ LTRs consist of U3, R, and U5 regions. Ψ = packaging signal; Δgag = truncated *gag* sequence. **(B)** Structures of versatile pMXs-derived vectors. LTR = long terminal repeat; Ψ = packaging signal; MCS = multi-cloning site; IRES = internal ribosomal entry site; GFP = green fluorescent protein' neor = neomycin-resistant gene; puror = puromycin-resistant gene. (C) Structures of pMXs, pMYs, and pMZs vectors. The 5´ LTR, primer binding site (PBS), and the extended packaging signal of pMXs are derived from the MFG vector. PBS used in pMYs and pMZs are derived from MESV (murine embryonic stem cell virus) and binds tRNA-glu instead of tRNA-pro. MCS is designed for cDNA library construction and is preceded by triple stop codons (not shown). White box = MMLV LTR; gray box = PCMV LTR; hatched box = MPSV LTR or SFFVp LTR. MCS = multi-cloning site (Kitamura et al., 2003).

recognizing the specific structure of RNA or DNA. Base N- and C- terminal tails of nucleocapsid domains are conformationally indurable (Miyazaki et al., 2011). Retroviral genome packaging is generally localized between the binding donor (SD) region and the gag start codon in the 5′ head region. It should be noted that the packaging sign generally coincides with the dimerization region, which indicates that packaging combines with genome dimerization (Miyazaki et al., 2011). Inhibition of genome dimerization through addition or deletion of mutations at dimer initiation sites (DIS) causes a significant decrease in genome packaging. Studies of mutant viruses containing two retroviral genomes showed that these were non-covalently dimerized in progen viruses. 5′ untranslated region (UTR) packages monomeric genome, which indicates that the genome packaging procedure occurs *via* the interaction of two 5′ UTR (Miyazaki et al., 2011). Virion maturation occurs by the budding of the particle from the cell (Escors and Brecpot, 2010). The translational mechanism of the host synthesizes and modifies viral proteins. Newly synthesized viral proteins and full-length RNAs combine to produce a new virus form, which will be budding from the host cell (Hu and Pathak, 2000).

A retrovirus infects the target cell by providing interaction between viral envelope protein and cell surface receptor on the target cell. The virus then internalizes to the place where its single-stranded RNA turns into double-stranded DNA. Double-stranded DNA is delivered to the nucleus and integrated to the host cell genome there. Many types of retrovirus types require degradation of mitosis and then the nuclear envelope for the arrival of a viral genome within the nucleus (Robbins and Ghivizzani, 1998).

Stable binding of viral DNA to the host genome is advantageous because it will provide long-term expression of transgenes required for therapeutic effect. However, one of the disadvantages of current retroviral transfer systems is that they are not specific to types of target cells (Yi et al., 2011).

One of the most important properties of retroviruses is that they can integrate a reverse transcribed genome to the host cell chromosome (Miyazaki et al., 2011).

Unlike adenoviral vectors, retroviral vectors realize transfection through transgene integration to the target cell genome. However, transgene expression ceases within a few days or weeks. Retroviral vectors reveal gene transfer that is unsuitable for many cells in *in vivo* conditions, and this partly stems from rapid inactivation of the human complement system. This silencing is not well characterized; however, it appears as a result of methylation in DNA or promoter regions, or participation of splice region to condensed chromatin (Reynolds et al., 1999; Navarro et al., 2008).

The ability of retroviruses to enter their genomes to host DNA enables them to make stable modifications to the life cycle of the host cell. This stability is greater than viruses such as adenovirus, herpes simplex virus (HSV), and papilloma virus, which remain episomal (Robbins and Ghivizzani, 1998).

2.2.1.3. Lentiviral systems

In recent years, studies mainly concentrated on the use lentiviral vectors. Lentivirals are viral systems without small, retrovirus-like viral proteins and no capacity for replication;

they provide gene delivery to non-dividing cells. This characteristic property is an advantage for various gene therapy applications used in targeting in post-mitotic and highly differentiated cells. The most important advantage of lentiviruses compared with other retroviruses is their ability for gene transfer to non-dividing cells (Escors and Brecpot, 2010; Yi et al., 2011; Matrai et al., 2009; Freed, 1997). This characteristic is believed to be related to the pathogenic characteristics of lentiviruses, which infect terminally differentiated cells of monocyte/macrophage lineage (Freed, 1997). For this reason, lentiviral vectors can be used for transgene expression to neuron cells (Yi et al., 2011).

Genome of lentiviruses have a more complicated structure; they contain accessory genes which regulate viral gene expression, control combination of infectious particles, modulate viral replication in infected cells, and are associated with the continuance of infection (Howarth et al., 2010). In other words, the fact that it contains regulatory and accessory genes apart from gag, pol and env, requires lentiviruses to adopt a more complex form (Osten et al., 2007). For example, HIV-1 is one of the most widely used lentiviral vectors, and contains six accessory genes (tat, rev, vif, vpr, nef, vpu) (Osten et al., 2007; Yi et al., 2011). These proteins are involved in all steps of cell cycles, which are termed: budding, maturation, and integration. In addition to helping combination of virions, these proteins ensure nuclear export and transcription ratio of RNA (Yi et al., 2011).

HIV infects CD4-positive T lymphocytes and macrophages (CD4 antigen acts as a primer surface receptor for HIV-1), and causes chronic immune deficiency, known as acquired immune disorder syndrome (AIDS). HIV-1 genome additionally contains 2 regulatory genes (*tat* and *rev*), and 4 accessory genes (*vpr, vpu, vif, nef*). The lenti viral genome is 9.2 kb in length. *Tat* protein increases transcriptional activity in 5′ LTR of integrated provirus from promoter regions. Rev protein transports non-spliced HUV mRNA from the nucleus. In general, accessory proteins increase virulence against the host organism. Development of HIV-1-based vectors was first achieved by utilizing the abilities of lentiviral vectors of infecting post-mitotic cells. The design of other retrovirus-based vectors, such as self-inactivating MoMLV vectors, can be directly transferred to lentiviral vectors. In the most recent generations of HIV-1-based vectors, all accessory genes and the regulatory tat gene are deleted (Osten et al., 2007). In the self-inactivating (SIN) expression vector, the U3 promoter region is deleted from the 3′LTR. This region was copied to the 5′ end of dsDNA during reverse-transcription. Therefore, SIN modification results in transcriptional inactivation of the integrated provirus, which allows for the restriction of the likelihood of recombination with latent retroviral sequences and mobilization of latent retroviral sequences in the host cell genome (Osten et al., 2007).

Tropism of lentiviral vectors depends on the type of envelope protein used for viral production (Escors and Brecpot, 2010). The use of heterologous Env proteins is called pseudo typing. The most widely used Env protein is "vesicular stomatitis virus glycoprotein (VSV-G)". This protein permits the virus high titration values *via* ultracentrifugation and a wide tropism (VSV-G binds to suitable phospholipid component of plasma membrane) (Osten et al., 2007).

Lentiviral vectors do not require degradation of the nuclear membrane for integration. Lentiviruses that are encoded with the Gag matrix protein integrase enzyme and vpr protein

interact with the nuclear import mechanism of the target cell and manage active transport of pre-integration complex *via* nucleopores. This characteristic enables transfection to non-dividing cells and, in conclusion, "complex retroviruses" become suitable for use as gene transfer vectors, particularly for post-mitotic cells like neurons (Howarth et al., 2010). Receptors have been defined for many retroviruses. The best-characterized example is CD4 molecule, which serves as a receptor for lentiviruses including HIV (Freed, 1997).

Frequently, pseudotype lentiviral vectors are formed by "vesicular stomatitis virus" envelope (VSV-G). VSV-Gis a glycoprotein that interacts with the phospholipid component of a number of receptors or cell membrane. VSV-G offers large host-cell range and high vector particle stability, which are attractive characteristics for *ex vivo* gene modification. However, restriction of infection to specific cells, which is called transduction altargeting, is effective and reliable and critical in the case of *in vivo* gene delivery. In addition, it is the key to increase therapeutic effectiveness, reduce adverse effects, and reducing the required amount of the vector. Two methods can be used to achieve this: (a) benefiting from natural characteristics of existing viral proteins, (b) using gene engineering to continue, stop or increase the original tropism of the vector (Escors and Brecpot, 2010).

Recently-developed lentiviral gene transfer systems have many characteristics of retroviral systems. A viral genome integrates with host chromosomes, and genes that are desired to remain permanently are placed (Yi et al., 2011).

2.2.2. Non-viral gene delivery systems

The basic concept underlying gene therapy is that it develops gene expression to specific cells in order to treat human diseases or for transfer of genetic material to inhibit the production of a target protein (He et al., 2010).

In theory, viral carriers can achieve rapid transfection of foreign material spliced to viral genome with high transfection ratio. However, many studies that used viral vectors reported unsatisfactory results, due to the immunologic and oncogenic adverse effects of these vectors. On the other hand, non-viral vectors have many advantages, such as easy of fabrication, cell/tissue targeting, and low immune response. The biggest disadvantage of non-viral vectors in clinical use is low transfection efficiency (He et al., 2010). Therefore, the most important points that should be taken into account in gene therapy are the introduction of the gene into the cell and increasing transfection efficiency. Using transfection, cells are induced for the production of chemicals used in pharmacological arrangements like hormone replacement or specific protein production (Godbey and Mikos, 2001). Naked DNA molecules cannot effectively enter to the cell due to their hydrophilic structures and large size resulting from negatively-charged phosphate groups. In addition, they are easily fragmented by nuclease enzymes. Therefore, the biggest difficulty in gene therapy is the development of physical methods to ensure gene transfer to target cells of the gene delivery vectors and delivered gene (Al-Dosari and Gao, 2009).

As seen in Figure 4, natural and synthetic polymers are used to prepare non-viral gene delivery systems. Compared to viral delivery systems, non-viral carriers are less toxic and

immunogenic. Another advantage of non-viral vectors is ease-of-production (DeLaporte et al., 2006). Much of the previous research found that non-viral systems were less effective than viral systems. However, in gene therapy conducted with physical methods, the effectiveness of gene transfection and therapy increased, and the duration of gene expression was extended significantly in clinical terms (Al-Dosari and Gao, 2009).

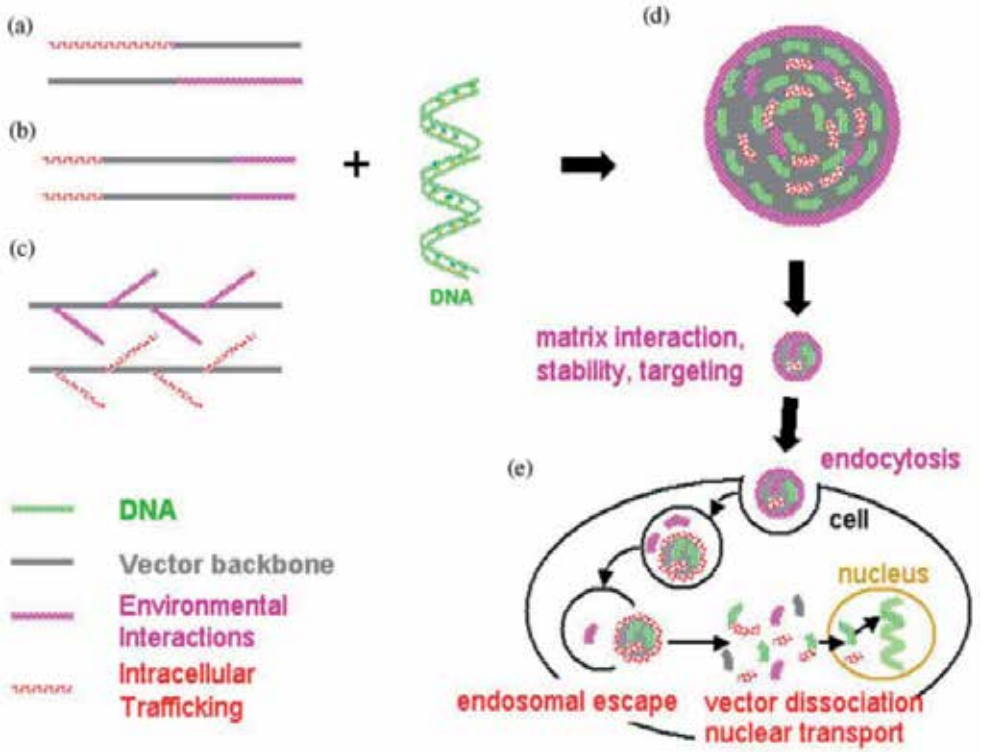

Figure 4. Modular design of non-viral vectors schematic. Modules associated with vector design are: vector backbone (grey), functional groups for regulating environmental interactions (purple), and intracellular trafficking (red). The vector backbone, typically containing polymers, lipids, or polysaccharides, is designed for DNA binding and complexation, which can protect against nuclease degradation, create a small, less negatively charged particle that can be internalized by cells, and facilitate some intracellular trafficking. The function of the vector backbone is being augmented by the attachment of groups that address the extracellular and intracellular barriers. The environmental functional groups can serve to limit interactions with serum components, promote specific cell binding or tissue targeting, or facilitate interactions with the extracellular matrix or biomaterials. The intracellular functional groups aim to enhance nuclear accumulation of the DNA either by facilitating endosomal escape, movement along the cytoskeleton, or nuclear pore trafficking. The individual modules can be assembled in different ways (a – c) for complexation with DNA (green), which may affect the structure and function of the resulting non-viral vector. (d) Schematic illustrating the distribution of the modules and DNA throughout the vector cross section, with the desired organization of functional groups regulating the environmental interactions presented primarily on the exterior and the groups for intracellular trafficking protected within the vector interior for activity following internalization. (e) Vectors are internalized by endocytosis and must subsequently escape the endosome for transport to the nucleus. Additionally, the modular components must dissociate from the DNA to allow for transcription (DeLaporte et al., 2006).

A number of barriers need to be overcome in order to increase the effectiveness of non-viral vectors in humans. These barriers are classified as production/formulation/storage; extracellular barriers; and intracellular barriers (Davis, 2002). Anatomic barriers are extracellular matrixes coating the cells, which prevent direct transport of macromolecules to target cells through epithelium and endothelial cell sequences. Phagocytes like Kupffer cells in the liver and residential macrophages in the spleen are responsible for theclearance of DNA-loaded colloidal particles in blood circulation. Similarly, nucleases found in blood and extracellular matrix cause free and unprotected nucleic acids to be rapidly inactivated following systemic application. The most critical barrier to effective DNA transfection was regarded as the transition of plasma membrane. Typically, naked nucleic acids cannot cross cell membrane by cellular uptake mechanisms such as endocytosis, pinocytosis, and phagocytosis without application of physical methods or being loaded to a carrier (Al-Dosari and Gao, 2009). There is a substantial body of research on developing effective physical, chemical, and biological systems to deliver transgenes into the cell to provide appropriate expression (Gao et al., 2007). Physical approaches, including electroporation, gene gun, ultrasound, and hydrodynamic delivery are based on the application of a force to increase the permeability of the cell membrane and promote intracellular gene transfer. In chemical applications, synthetic or natural carriers are used to transport transgenes to the cells. An ideal gene delivery system should meet 3 criteria: The carriers should protect the transgene from nuclease enzymes inside intracellular matrixes; should transport the transgene from plasma membrane to the target cell nucleus; and should not cause any toxic effect (Gao et al., 2007).

Two of the most important advantages of synthetic carriers are that they do not display immunogenicity, and large-scale production is easy. Furthermore, the size of the gene to be delivered does not reduce efficiency of these systems. Even mammalian artificial chromosomes of 60 megabase were successfully transfected by these types of carriers (Schatzlein, 2003).

Non-viral vectors can trigger an inflammatory response, since they do not provide a specific recognition; they are much less dangerous than viral vectors in terms of antigen specific immune response. However, although non-viral vectors seem to be more suitable, there are some important points to be contemplated in vector design. Non-viral vectors should be designed according to specific cell targeting; cellular uptake and cellular release should be optimized; and potential immune response should be minimized (Conwell and Huang, 2005; Mrsny, 2005).

2.2.2.1. Physical methods

Gene Gun

Delivery with gene gun method is also termed ballistic DNA delivery or DNA-coated particle bombardment, and was first used for gene transfer to plants in 1987 (Al-Dosari and Gao, 2009; Lin et al., 2000). This method is based on the principle of delivery of DNA-coated heavy metal particles by crossing them from target tissue at a certain speed. The particles achieve sufficient speed due to a pressurized inert gas (generally helium). Generally, gold, tungsten or silver microparticles were used as the gene carrier (Mhashilkar et al., 2001; Al-

Dosari and Gao, 2009; Miyazaki et al., 2006). Momentum allows penetration of these particles to a few millimeters of the tissue and then cellular DNA release (Gao et al., 2007). These particles typically have a diameter of 1μm. Due to its small size, the particle easily penetrates the cell membrane and can transport DNA into the cell. At this point, DNA separates from the carrier particle and can be expressed (Lin et al., 2000).

Gas pressure, particle size and dose frequency are critical factors in determining the degree of tissue damage and penetration effectiveness of the application (Al-Dosari and Gao, 2009).

Gene-gun-based gene transfer is a widely tested method for intramuscular, intradermal and intratumoral genetic immunization (Miyazaki et al., 2006). Numerous animal tests and clinical trials indicated that this method caused a greater immune response than microinjection, even in low doses (Al-Dosari and Gao).

The advantages of gene gun over other *in vivo* gene delivery systems are as follows: [1] It does not use toxic chemicals or complex biological systems, [2] delivery is achieved without the need for a receptor, [3] DNA fragments of various sizes, including large ones, are transported, [4] there is no need to introduce foreign DNA or protein, [5] it has high repeatability, [6] production of heavy metal particles is easy (Lin et al., 2000). However, in this method, gene expression is short-term and low (Mhashilkar et al., 2001).

Electroporation

The future of gene therapy requires the advancement of effective and non-toxic polynucleotide gene delivery mechanisms. Electroporation includes controlled electric application to increase cell permeability. Electroporation was first developed in 1960s, with studies on the degradation of cell membrane with electric induction. Neumann et al., first reported transfection of eukaryotic culture cells through electroporation in 1982. In many subsequent studies, transfection was performed on animal and plant cells *via* electroporation (Al-Dosari and Gao, 2009; Somiari et al., 2000).

Electroporation introduces foreign genes into the cell by electric pulses. In this method, pores are formed on the membrane surface to enable the DNA to enter the cell. Pore formation occurs very rapidly, in approximately 10 nanoseconds. The size of the electric pore is estimated to be smaller than 10 nm radius. If the molecule is smaller than the pore size (as in oligonucleotides and chemical compounds), it can be transferred to the cell cytosol through diffusion (Somiari et al., 2000; Miyazaki et al., 2006). In addition to passive diffusion, loaded molecules and ions can be transported from the membrane *via* electrophoretic and electro-osmotic means *via* the effect of electric regions. The pores close following cellular uptake of DNA. In recent years, *in vivo* electroporation became common in nucleic acid vaccines and improving non-viral gene therapy. There are critical steps defined for transportation *via in vivo* gene delivery with electroporation. The first step is fusing together, attachment or proximity between the cell membrane and nucleic acid. The second step is adding nucleic acid to the membrane, which is immediately followed by the third step, involving the delivery of nucleic acid from the membrane. The delivery procedure results in decreased pore size and permeability (Somiari et al., 200).

In vivo electroporation technique is a generally reliable method that is more efficient than other non-viral systems. When the parameters are optimized, this technique is equally effective as viral vectors. In addition to local injection and electroporation, it was previously shown that *in vivo* electroporation can be applied in localized form after the injection of plasmid DNA (Al-Dosari and Gao, 2009).

There are some difficulties in *in vivo* applications of electroporation. There is an effectiveness region of approximately 1 cm between the electrodes, and this makes transfection of the cells in large regions of the tissues difficult. It is difficult to use in internal organs, and surgery is required to implant electrodes. High temperature due to high voltage application can cause irreversible tissue damage. The application of high voltage to cells can affect the stability of genomic DNA (Al-Dosari and Gao, 2009; Gao et al., 2007).

Ultrasound

Ultrasound has many clinical advantages as a gene delivery system, due its easy and reliable procedure (Pitt et al., 2004; Ferrara, 2010). In recent years, it was found that microbubbles applied by ultrasound increased gene expression. Microbubbles or ultrasound contrast agents decrease cavitation threshold with ultrasound energy. In many applications, perfluoropropane-loaded albumin microbubbles (Mallinckrodt, San Diego, USA) were used. The microbubbles were modified with plasmid DNA before the injection and then ultrasound was applied (Niidome and Huang, 2002).

Ultrasound has been used for therapeutic purposes for more than a half century, even before the use of ultrasound for diagnosis and imaging purposes. In diagnostic use, low-intensity ultrasound is used to prevent energy accumulation in tissue causing biological effects. Conversely, therapeutic applications are based on the principle of intensification of ultrasound energy within the tissue (Frenkel, 2008).

After the discovery of the applicability of ultrasound to gene therapy at the cellular and tissue level, this was taken one step further with physical methods. Reporter gene expression, where naked DNA is used, advanced for10 times thanks to this discovery. The transfection efficiency of this system is based on frequency, time of ultrasound treatment, the plasmid DNA mount used, etc. In conclusion, size and local concentration of DNA plays an important role in the effectiveness of transfection (Gao et al., 2007).

Hydrodynamic therapy

Hydrodynamic gene delivery is based on the principle of understanding the characteristics and structure of capillaries, an understanding of the dynamic characteristics of the fluids passing through blood veins (Suda and Liu, 2007). Hydrodynamic gene delivery uses the hydrodynamic pressure created by the injection of the large volume of DNA solution with blood pressure inside veins (Suda and Liu, 2007), and thus the permeability of the capillary endothelium increases and pores form in the plasma membrane encircling parenchyma cells (Gao et al., 2007; Suda and Liu, 2007). DNA or other related molecules can reach the cell from these pores. Membrane pores are later closed, and these molecules are retained within the cell. In hydrodynamic gene therapy, the principle reason for targeting parenchyma cells

is that capillary endothelium and parenchyma cells are closely related, and when the endothelial barrier is breached, this allows DNA to easily reach parenchyma cells. In addition, the capillary's thin wall structure has a stretchable and easily fragmented structure (Suda and Liu, 2007). The effectiveness of hydrodynamic procedure depends on capillary structure, the structure of the cells encircling capillaries, and the hydrodynamic force applied (Gao et al., 2007; Suda and Liu, 2007).

2.2.2.2. Chemical methods

Polymers

Polymers are long-chained structures composed of small spliced molecules called monomers. Polymers that are composed of a repeated monomer are called homopolymers, while those composed of two monomers are called copolymers. Natural and synthetic polymers are used in drug delivery systems. Biodegradable and non-biodegradable polymers are used according to the type of controlled release mechanism. Biodegradable polymers are non-water soluble, and undergo chemical or physical change in biologic environments (Tuncay and Calis, 1999). Polyamides, dextran, and chitosan are examples of biodegradable polymers. On the other hand, non-biodegradable polymers are not degraded in biological environments; hydrophilic polymers are hydrogels, which are non-water soluble and swell in water, while hydrophobic polymers are non-water soluble and do not swell. Examples of hydrophilic hydrogel polymers include polyvinylalcohol, polyvinylacetate, polyethyleneglycol, polyacrylic acid, polyhydroxyethyl methacrylate, and polymethacrylic acid. Examples of hydrophobic polymers include silicones, and polyethylene vinyl acetate. Polymers such as ethyl cellulose (EC), hydroxypropyl methyl cellulose (HPMC), cellulose acetate phthalate (CAP), and eudragit derivatives are commonly used in controlled release systems (Tuncay and Calis, 1999).

Selection and design of a polymer is difficult due to structural differences; achieving the desired chemical, mechanical, and biological functions requires understanding of the characteristics and surface of a polymer. For polymer selection, in addition to its physicochemical characteristics, characterization of extensive biochemical characteristics and preclinical tests are required to demonstrate its reliability (Pillai and Panchagnula, 2001).

The physical properties of a polymer carrier, such as hydrophilicity, surface charge, permeability and diffusibility, biocompatibility with tissue and blood (Pillai and Panchagnula, 2001).

For successful delivery, polymers should package DNA in small sizes. Thus, extracellular and intracellular stability of DNA is increased; cellular uptake by endocytosisis enabled; and, by transporting it to the nucleus, the active form of DNA can be released within the nucleus (Mrsny, 2005).

Polymeric gene transporters deliver genetic material through electrostatic interactions with nano-sized polyplexes for gene therapy (Kim et al., 2011).

A synthetic gene delivery system: [1] should protect the negatively-charged phosphate DNA skeleton against anionic cell surface from load repulsion, [2] should be condensed to suitable

length intervals of DNA with a macromolecular structure for cellular internalization (condensing at nanometer size for receptor compatible endocytosis or condensing at micrometer size for phagocytosis); and [3] should protect DNA from all extracellular and intracellular nuclease degradations. To meet this need, researchers concentrated on three strategies: electrostatic interaction, encapsulation, and adsorption (Wong et al., 2007).

Electrostatic interaction

The majority of polymeric vectors presently in use form complexes with negatively charged DNA by electrostatic interaction. All polymers have amino groups. At adequate nitrogen–phosphate ratio, the polymer and the DNA form nanocomplexes, which allows both cellular DNA uptake and also protects the DNA from nuclease enzyme (Wong et al., 2007).

Encapsulation

An alternative to electrostatic condensation of DNA is encapsulation of DNA with a biodegradable polymer. Polymers that have an ester linkage in their structures (like polyesters) are hydrolytically degraded to short oligomeric and monomeric compounds, which are more easily discharged from the body. Furthermore, the degradation mechanism and DNA release can be controlled by changing the physicochemical characteristics and composition of the polymer. DNA is protected from enzymatic degradation by encapsulation (Wong et al., 2007) (Figure 5).

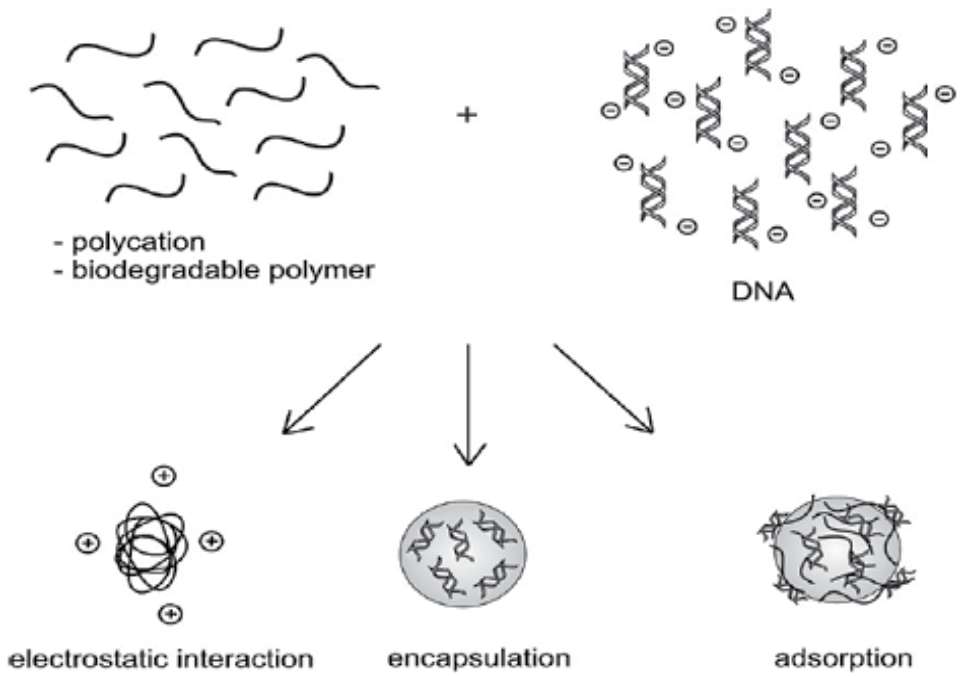

Figure 5. Gene packaging—The three main strategies employed to package DNA are *via* (1) electrostatic interaction, (2) encapsulation within or (3) adsorption onto biodegradable nano- or microspheres (Wong et al., 2007).

Adsorption

Alternative DNA packaging methods, such as adsorption techniques, were developed to overcome the limitations of encapsulation methods. DNA adsorption represents the combination of the above mention two models. In this method, DNA is conjugated to the surface of biodegradable cationic particles, or is electrostatically adsorbed due its negative charge. This method prevents the DNA from being exposed to heavy encapsulation conditions, and also increases rapid release DNA (Wong et al., 2007).

Adsorption methods attracted much research interest, as it does not cause immunogenicity, does not require the integration of exogenous genes to host chromosomes, is a simple and cheap method, and is suitable for effective gene delivery. However, low transfection efficiency, some cytotoxic effects, and *in vivo* instabilities are still significant restricting factors in gene therapy. One of the strategies to solve these problems is the synthesis of biologically degradable polymers (Kim et al., 2011).

Liposomes

Liposomes are colloidal drug delivery systems. They are biologic membrane-like sacs in sphere form, formed by one or more lipid layers, and include an aqueous phase. The phospholipid phase consists of principle components like aqueous phase and cholesterol. Liposomes are classified according to the number of layers they contain. The advantages of liposomes include effectiveness at small doses, extended dosing interval, and ideal transport for active substances with a short half-life. Cellular uptake mechanism of active substances in liposomes can be categorized as endocytosis, combination by melting, and adsorption. Liposomes are used for application of carcinogenic, antifungal, antiparasitic, antiviral, and anti-inflammatory drugs, hormones, DNAs, and cosmetics (Bogdansky, 1990).

Cationic lipids, which are used for the entrance of plasmid DNAs in the cell, have been studied for more than 20 years. During this period, many cationic liposome formulations were developed, and were tested as nucleic acid transported in animals and human with phase I and phase II studies. When compared to other gene delivery systems such as viral vectors and transfection agents, cationic liposome delivery systems are more easily formulated and cause no biological damage like viral vectors (Dass and Choong, 2006). Critical parameters that determine the behavior of liposomes in *in vitro* and *in vivo* conditions include size, number of layers, and surface charge. Unilamellar or mutilamellar vesicles can be produced depending on the preparation method; the diameter of these vesicle ranges from 25 nm to 50 μm (Bogdansky, 1990).

Liposomes are divided into three categories according to their charges: cationic, anionic, or neutral. Compared to viral vectors, liposome delivery systems are non-pathogenic and non-immunogenic, with easy of preparation. However, the most important disadvantage of liposomes is short gene expression time and low transgene expression level. Since liposomes are generally non-toxic and non-immunogenic structures, they are considered reliable carriers for gene therapy. Liposome-based gene delivery was first reported by Felgner (1987). In the 1990s, liposomes were used in numerous cationic lipid gene therapy tests.

Liposomes are not as effective as viral vectors in *in vivo* gene delivery, and it is generally difficult for them to transfect targeted gene to selected cells (Miyazaki et al., 2006).

Dendrimers

Dendrimers were first defined in the late 1970s and early 1980s. Dendrimers consist of symmetrical branches projecting from a central core (Dufes et al., 2005; Genc, 2008; Bulut and Akar, 2012). Dendrimers are 10–200 Angstrom in diameter; they are repeating, branched, large spherical molecules, and have functional groups on their surface that can be used as a building block to connect many biological materials (Ward and Baker, 2008). These functional groups determine the variability of dendrimers, whereas branching provides the growth of the structure (Dufes et al., 2005; Bulut and Akar, 2012). As seen in Figure 6, the most important advantages of dendrimers are conjugation of a large number of different molecules on the dendrimer surface, and the conjugation of molecules that will be used for diagnosis and treatment (Ward and Baker, 2008; Dufes et al., 2005).

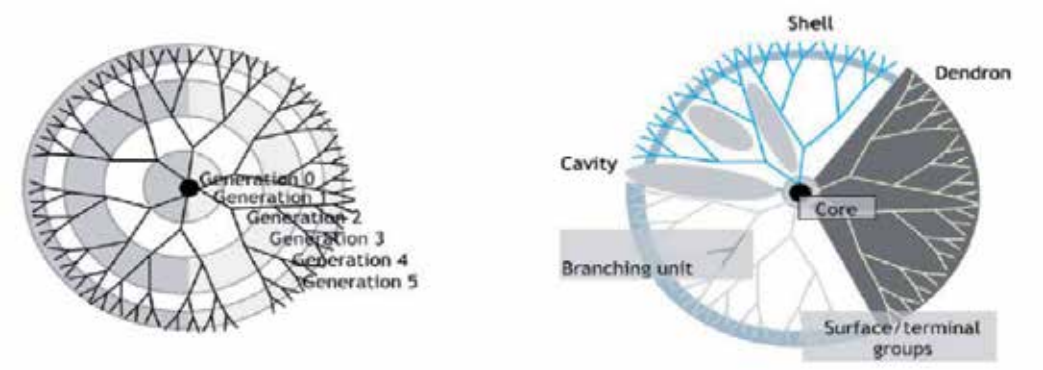

Figure 6. Dendrimer structure. The stepwise synthesis of dendrimers means that they have a well defined hierarchical structure. This hypothetical dendrimer is based on a core with three covalent root attachment points but other common cores have di- or tetracovalent cores. The valency of the core dictates the number of linked dendrons and the overall symmetry of the molecule. The dendrons are synthesised by covalent coupling of the branch units. For each additional layer or *generation* that is being added to the structure the reaction sequence is repeated. In this case the units have two new branching points at which additional units can be attached. (The generation count is not always consistent: normally *generation* 0 refers to the core while sometimes it is used to describe the dendrimer after the first reaction cycle.) The number of branching points, branching angles, and the length of the branching units determine to what extent each generation increases molecular volume vs. surface area. For the higher generations the density of the terminal groups reaches a point where for steric reasons no groups can be added (starburst effect). Dendrimers of higher generation also have a typical molecular density profile under favourable conditions; the high peripheral molecular density establishes a steric outer shell and the lower density at the centre creates cavities which can accommodate guest molecules. (Dufes et al., 2005).

The degree of polymerization of dendrimers is represented by the number of generations (G) of repeated branching cycles during synthesis. Generation number can be easily identified by calculating number of branching points from the core to the outer surface.

The number of branching points increases proportional to dendrimer growth. For example, a dendrimer with no connection points is termed zero generation (G-0) (Bulut and Akar, 2012).

Equal layer groups of dendrimers with an equal number of layers are highly suitable for use in drug delivery due to their perfect encapsulation characteristics and highly controllable chemical behaviors. Drugs can be encapsulated in a dendrimer or can electrostatically or covalently connect to functional groups on the surface (Ward and Baker, 2008; Bulut and Akar, 2012). On the other hand, targeted drug delivery can be achieved by covalent conjugation of the targeting agent to functional groups of dendrimers (Ward and Baker, 2008).

Although the toxicity of dendrimers that form complexes with DNA generally decreases, toxicity and cellular effects of free dendrimers should be taken into account (Dufes et al., 2005).

Cationic lipid compatible systems

Felgner et al. pioneered cationic lipid-based gene transfer (Felgner et al., 1987; Al-Dosari and Gao, 2009). Today, hundreds of lipids have been developed and tested for gene transfer. Lipid-based systems consist of a positively-charged hydrophilic head and hydrophobic tail structure.

Most common hydrophilic head groups are primary, secondary, tertiary amines, or quaternary amine salts. These positively charged sections connect to negatively charged phosphate groups in nucleic acid. The hydrophobic tail consists of aliphatic chain, cholesterol, or steroid rings. The connection region between hydrophilic and hydrophobic sections consists of the bonds that affect biodegradability ratio, such as ether, ester, carbamate, or amid (Al-Dosari and Gao, 2009; Uyechi-O'Brian and Szoka, 2003).

Each lipid has a different sized head group and hydrocarbon tail length, and these characteristics have a significant impact on the formation of DNA–lipid complexes and intake by the cell (Balasz and Godbey, 2010). The transfection effectiveness of cationic lipids varies according to the structure of the lipid and the charge ratio used to form the DNA–lipid complex. Compared to negatively-charged DNA, a positively-charged lipid spontaneously forms structures called lipoplexes. In lipoplex structure, the DNA molecule is encircled by positively-charged lipids, which protect it from extracellular and intracellular nucleases. In addition, due to their positive charges, lipoplexes electrostatically interact with the cell membrane and thus cellular uptake is achieved (Al-Dosari and Gao, 2009; Uyechi-O'Brian and Szoka, 2003).

Cationic lipids such as N-[1-(2,3-diloliloksi)propyl]-N,N,N-trimethlammoniumchloride (DOTMA), [1,2-bis(oliloxy)-3-(trimethlamonnium) propane] (DOTAP), 3β[N-(N_,N_-dimethlaminoethane)-carbamile] cholesterol (DCChol) and dioctadecylamidolysisspermine are used to prepare lipid carriers (Balasz and Godbey, 2010).

The aim of cationic lipid-based gene therapy is to transport plasmid DNA to the cell and thus to achieve the desired protein/peptide transcription and translation. DNA–lipid complex is internalized into the cell through the vesicular path, which is followed by the release of cell cytoplasm of DNA from the lipoplex. Some fractions of the released DNA are transported to the nucleus (Uyechi-O'Brian and Szoka, 2003). Strong DNA–cationic lipid complexes alone are insufficient for gene transfer; however, DNA release before or after transport to the nucleus is critically important (Khalid et al., 2008). Lipid–cationic lipid ratio, cationic lipid–DNA ratio and DNA mount presented to the cell are important parameters that significantly influence transfection efficiency (Hofland et al., 1996).

Although gene expression is provided by cationic lipids, for clinical benefit, the effectiveness, expression time and degree of gene transfer were not found to be adequate. The most significant disadvantages of cationic lipids are structural instability and heterogeneity, inactivation in blood, low effectiveness, and weak targetability when compared to other systems (Navarro et al., 2008).

Cationic lipid-based transfection systems are easy to prepare and reliable; they also have low immunogenicity, and there is no need to classify the size of DNA which will be transported (Mortimer et al., 1999). Many cationic lipid-DNA based systems are effectively taken up by endocytosis; however, the mechanism of DNA release from endosome and translocation to the nucleus are not well known (Mortimer et al., 1999).

2.3. The use of gene delivery systems in treatment

2.3.1. Cancer

Any change in the structure of genes that cause diseases is called mutation. In a mutated cell, variations occur in the sequence of nucleotides. As a result, chemical substances that are produced under the control of the gene have abnormal structure and function. If the gene is associated with the growth, differentiation, or division of cells, then the mutation might cause cancer (Wang et al., 2012). There are two main groups of genes responsible for formation of cancer. These are oncogenes and tumor suppressor genes. Oncogenes are mutated versions of normal genes that are responsible for controlling cell growth. After mutation, they compel the cell to become a cancer cell. There are more than 100 known oncogenes, most of which were identified in human tumors. The "ras gene" is the most widely known and most commonly studied of these genes. After mutation, the ras gene initiates division and cancer formation in cells with the "ras protein" it forms (Luo et al., 2012).

Tumor suppressor genes normally inhibit cell division and tumor development in cells. The mutated forms of these genes cannot inhibit cell division, and instead cause tumor formation. Among these genes, the RB and P53 genes are the most widely known. These genes show their effect with p-RB protein and P53 protein, which are produced under their control. Normally, these proteins inhibit DNA replication and cell division, and regulate apoptosis. If the created genes are mutated, these proteins lose their activity and

uncontrolled cell division occurs, which results in cancer development. It was indicated that RB protein was inactive in approximately 40% of human cancers. Similarly, it was found that P53 gene had an abnormal structure in 50% of all tumors (Morandell & Yaffe, 2012).

Gene therapy is based on the principle of replacing damaged genes in diseased cells with corresponding healthy genes that were prepared outside the body. This approach is the most difficult type of gene therapy. Successful treatment involves healthy genes, which are prepared out side the body, targeting specific cells in the body *via* certain transporters (carriers). Current technology has simplified the process of preparing healthy genes. However, the subsequent process of targeting cells remains imperfect (Lagisetty & Morgan, 2012).

In addition to the correction of defective genes, gene therapy includes other treatment principles. In a different method, cancer patients were injected "cytosinedeaminase" enzyme, which is not normally produced in humans, *via* viral carriers,thereby allowing this enzyme to be synthesized in tumor cells (Logue & Morrison, 2012). The drug 5-FC is injected at high doses with no adverse effects; this is converted to 5-FU in tumor cells by cytosine deaminase enzyme. Using this method, effective levels of drug activation are achieved without damaging healthy cells.

In a similar method, carrier viruses injected with "thymidinekinase enzyme gene" found in the herpes simplex viruses reach cancer cells and produce thymidine kinase enzyme in these cells. This enzyme enables the drug ganciclovir, which has no adverse effects on normal body cells, to be active in tumor cells and destroy cancer cells (Etienne-Grimaldi et al., 2012)

Another treatment approach involves inhibition of oncogene proteins that were mentioned above. Oncogenes cause cancer formation *via* the proteins they control. If the production of these proteins is inhibited, their cancer-causing effects will be reduced. One example is the farnesyltransferase enzyme, which plays a role in the formation stages of RB protein. It was reported that new tumor formation was inhibited in patients who were injected with drugs that inhibited the function of this enzyme (Logue & Morrison, 2012). Cancer treatment is the most studied application of gene therapy. A large number of genetic changes occur by the transformation of a normal cell to a neoplastic cell (El-Aneed, 2004).

Tumor suppressor genes eliminate cancer cells through apoptosis, whereas oncogenes accelerate proliferation. For this reason, apoptotic genes and anti-oncogenes are frequently used in cancer treatment. In addition to oncogenes and tumor suppressor genes, chemotherapy and gene therapy were combined *via* a 'suicide gene' strategy. A suicide gene encodes an enzyme that does not belong to mammals, and this enzyme converts non-toxic product into active cytotoxic metabolite in cancer cells. Tumor suppressor genes, anti-oncogenes, and suicide genes target cancer cells at the molecular level. Generally the genes that encode cytokine revive immune response to cancer cells (Wang et al., 2012).

Transformation of a normal cell to a neoplastic cell occurs by multi-mutationary changes of the cells at the genetic level. Due to the complicated nature of cancer, cancer gene therapy involves many therapeutic strategies. These strategies can be analyzed according to two categories: immunogenic and molecular (El-Aneed, 2004).

2.3.2. Immunologic approach

The human immune system has two pathways to respond to foreign antigens. The first pathway includes antibodies secreted by B cells following activation of membrane immunoglobulin via (B cell receptor)-antigen connection. Antibodies are water-soluble proteins that move within the circulatory system to reach the target antigens. T cells, the second immune pathway, directly interact with antibodies without secreting antibody. T cells can produce multiple immune reactions, including cytotoxic effects (Lagisetty & Morgan et al., 2012).

Cancer cells are immunogenic in nature with their cancer antigens, which are intracellular molecules. Therefore, cellular immunity (T-cell mediated), is more important than humoral immunity (B-cell mediated) (Restifo et al., 2012). Normal immune response is not sufficient to eliminate tumor cells. The ability of cancer cells evade immune-system responses is associated with secretion of immunosuppressive factors, down-regulation of antigen expression or MHC (major histocompatibility complex) molecules, and co-stimulation deficiency. One of the common genetic immunotherapy strategies includes transfer of immune-stimulating molecular genes like cytokines. Immune response is created by the activation of interleukin-12 production by tumor cells with numerous components of immune system, particularly including cytotoxic T lymphocytes and natural killer cells (McDonald et al., 2012).

Another immunologic approach involves *in vitro* manipulation of the antigen-presenting cells to produce active tumor antigens. Dendritic cells are the strongest antigen-presenting cells (El-Aneed, 2004). Application of antigen-encoding genes *via* direct vaccination might induce the desired immune response to cancer cells. When injected subcutaneously or intramuscularly, DNA enters local cells like fibroblast and myocytes, and antigen is subsequently produced and secreted. Antigen-presenting cells move towards lymphoid organs where new antigens will be caught and the desired immune response will be initiated (El-Aneed, 2004). Another method to increase the specificity of chemotherapy might be enzyme-encoding enzyme-activate prodrug therapy, which converts non-toxic products into specific and toxic metabolites (Altaner, 2008). This approach is known by many different names, including enzyme prodrug therapy in gene management; suicide gene therapy or enzyme prodrug therapy in virus management; and gene prodrug activation therapy (Scheier et al., 2012). All of these expressions define the same two-step treatment process. The first step involves the targeting and transport of the foreign enzyme to the tumor *via* various pathways. In the second step, non-toxic prodrug is applied and converted to active systolic metabolite. In *in vivo* approaches, expression of foreign genes does not occur in each cell of the target tumor. As a result, the active drug

can enter the transduced cell and cell deaths take place, including the cells which do not express enzyme. This procedure is also termed the bystander effect or neighbor cell death effect. In addition, dead cells can induce host immune response due to T cells and NK cells. This therapeutically beneficial effect is known as the slight bystander effect. To increase the effectiveness of enzyme-activated product therapy, therapeutic genes in the vector should be expressed at a sufficiently high ratio to achieve effective prodrug transformation; it should remain as active as possible in order to support the killing mechanisms of the produced systolic metabolite, and the suicide gene should be specifically targeted only to the tumor. These arrangements will also reduce adverse effects (Altaner, 2008).

2.3.3. Cardiovascular disorders

Recombinant adenovirus is one of the most practical vectors for localized gene therapy. In addition, these vectors allow for tissue-specific delivery, and are suitable for re-targeting. Bispecific antibodies were used to coat adenovirus particles by connecting the antibody ligand on the cell receptor surface. Genetic modifications also include gene modifications for some viral coat proteins to change tropisms of adenoviruses (Ji et al., 2012).

Antiviral vectors are the most recently developed vectors; they are HIV-based and generally used for HIV treatment. These vectors have the ability to transfect non-dividing and dividing cells. The effectiveness and reliability of these cells are currently being analyzed (Kozarsky, 2001).

2.3.3.1. Angiogenesis

One exciting recent development is that blood circulation can be provided to ischemic tissues. The development of new blood veins was observed in the region of endothelial growth factor (VEGF) expression (Katz et al., 2012). Gene transfer has advantages for protein therapy, and the use of permanent VEGF is preferred. This method can be fulfilled by a single injection of gene-transfer vector; however, transgene expression of VEGF should last for a limited period, because long-term unregulated VEGF expression might cause abnormal angiogenesis. Therefore, rapid gene expression obtained from adenoviral and plasmid vectors are among the ideal vectors for local delivery of VEGF gene by direct injection (Kozarsky, 2001; Katz et al., 2012).

2.3.3.2. Cardiac failure

Treatment of cardiac failure by gene therapy is still limited, due to the need to determine suitable transgenes and develop effective gene delivery methods to the myocardium. Genes can be directly transported to the heart. However, if these genes have to be transported to larger regions, as in the case of cardiac failure, this method is not regarded as successful. Another method involves transporting gene delivery vectors to coronary veins *via* a catheter; however, some catheter materials inactivate recombinant adenovirus (Kozarsky, 2001; Kairouz et al., 2012).

2.4. Pulmonary disorders

According to some authors, lungs are ideal organs for gene therapy. However, cystic fibrosis and α_1-antitripsin deficiency are the only common disorders with known genetic background for which existing treatments are not curative. Although cystic fibrosis is the first target for pulmonary gene therapy, this method is used to treat many acquired diseases, mainly cancer (Davies et al., 2003).

After the discovery of CTFR in 1989, considerable new progress was made to improve gene transfer system for somatic gene therapy of cystic fibrosis. Cystic fibrosis affects approximately1 in 2500 newborns, which makes it the most common recessive genetic lethal disorder (Davies et al., 2003). Even a copy of a single normal SF gene is sufficient for normal function (Klink et al., 2004). Therefore, somatic gene therapy is relatively straightforward. The only requirement is to provide a cell affected by a gene which makes CFTR protein expression (94). CFTR is cAMP-regulated chloride channel in epithelium cells. Dysfunction of CFTR arises from production deficiency, problems in transport of apical membrane of the cell to the effective region, or due to functional defects (Davies et al., 2003). Air tract epithelium is the most important target among lung diseases that cause mortality and morbidity. In order to address this aim, many gene delivery systems were developed and their effectiveness *in vitro* and *in vivo* was analyzed.

The use of adenoviral vectors gave rise to safety concerns due to low transduction effectiveness and increasing inflammation in human lungs. Tests with cationic liposomes showed low toxicity and effectiveness (Klink et al., 2004).

Some individuals have to be overcome to perform gene therapy. Mucociliary clearance is the primary one of these individuals. Air tract secretions and the mucociliary clearance system act as significant barriers to exogenous gene transfer. The epithelia of a healthy respiratory system create a thin mucus layer required for normal cilia function; however, this layer inhibits gene transfer by cationic liposomes (Davies et al., 2003).

Similarly, a potential gene transfer agent has to overcome the barrier formed by secretions in order to enter the cell. The glycocalyx structure and endocytosis ratio of a cell were defined as the factors that restrict the effectiveness of gene transfer. A number of intracellular process, including endosomal system, cytoplasmic delivery, and the entrance to the nucleus serve as barriers to successful gene transfer. Similarly, host immune response causes problems in the application of gene transfer agents to lungs (Davies et al., 2003).

Adenovirus/CFTR was applied to the nasal epithelia of three patients to treat cystic fibrosis. No viral replication was observed, but mild inflammation was reported. PD returned to normal baseline values in all three patients (Davies et al., 2003). However, one of the problems of this vector system is that low expression levels of viral gene wastes leads to cytotoxic T-cell response that targets vector-containing cells. The fact that adenoviruses are generally air tract pathogens is a both advantageous and disadvantageous. Many potential

receivers have circulating antibodies that reduce vector effectiveness and T-cell response. Most importantly, systemic application of adenoviral vectors causes acute and potentially life-threating inflammation responses due to activation of antigen-presenting cells (Klink et al., 2004). On the other hand, when compared to other gene delivery systems, lentiviral vectors are advantageous. Lentiviral vectors are integratable retrovirus derivatives with a high cloning capacity. When suitable regular sequences such as LCS (locus control sections) are used, stable and cell-specific expression can be achieved. When envelope proteins of pseudo-written lentiviral vectors are applied to apical surface with vesicular stomatitis virus, it is not possible to effectively transduce polarized air tract epithelia. Retroviral vectors, pseudo-written with apical membrane-connecting envelope proteins, are used to overcome this problem (Klink et al., 2004).

The first clinical trial of liposome-compatible CFTR gene transfer was reported in 1995. This method involves direct application of DC-Chol/DOPE/CFTR to the nasal epithelia of SF patients. No adverse clinical effects were observed, and nasal biopsy analyses showed no histologic change. Specific mRNA and plasmid DNA were found in five out of eight patients who received CFTR gene vector (Klink et al., 2004).

3. Conclusion

Current gene delivery systems are divided into three categories: viral-based, non-viral based, and combined hybrid systems. Viral gene delivery systems consist of viruses that are modified to be replication-deficient which can deliver genes to the target cells to provide expression. Although viral systems have many advantages, such as long-term expression, and the effectiveness and expression of therapeutic genes, they also have various disadvantages. These disadvantages include risks due to working with viruses, optimization problems when producing large batches, and problems of immunogenicity and toxicity. To date, adenoviruses, retroviruses, and lentiviruses have been used as viral gene carrier vectors. Non-viral systems include physical and chemical methods. In its broadest sense, this refers to delivery of the gene into the cell by applying a physical force in order to increase the permeability of the cell membrane. The most common physical methods are microinjection, electroporation, ultrasound, gene gun, and hydrodynamic delivery. Chemical methods use natural and synthetic compounds are to deliver the gene into the cell. The gene delivery system generally includes polymers, liposomes, dendrimers, and cationic lipids. Non-viral vectors have many advantages, such as easy of synthesis, effective targeting of cell/tissue, low immune response, and potential to use plasmid at desired molecular weight. The biggest disadvantage of non-viral vectors in clinical use is low transfection efficiency. A large number of viral and non-viral gene delivery systems have been developed. Both systems have many advantages and disadvantages. Hybrid systems were developed to combine the advantageous parts of both the individual systems, while minimizing their respective disadvantages, thereby producing a gene therapy with higher effectiveness and low toxicity. The biggest advantage of hybrid systems is that they are less toxic than viral systems, and are not as low-efficient as non-viral systems.

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Chapter 17

Is Chronic Combination Therapy of HAART and α-ZAM, Herbal Preparation for HIV Infection Safe?

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Additional information is available at the end of the chapter

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1. Introduction

Since discovery of acquired immunodeficiency syndrome (AIDS) and subsequent isolation of the virus (HIV) more than 30 million have been infected [1]. Sub-Saharan Africa was affected most with HIV infection with estimated 2/3rd of the World's HIV infected people. Nigeria is the largest populated country in Africa and it was estimated that about 3 million of the population is infected with human immunodeficiency virus [2]. Thus Nigeria is the second largest country after South Africa with largest HIV infected population in the World [1]. Nigerians like any other people in Africa are favourable to the use of herbal remedies for major illnesses, thus, that HIV infection has no cure medically serves as a catalyst to source for cure in herbal remedies [3].

Herbal remedies which are considered as herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or plant materials, or combinations thereof used to treat a multitude of ailments throughout the world[1]. Because many HIV patients denied the use of herbal products when asked by medical practitioners, the safety of combination therapy of orthodox drugs and herbal remedies had been a major concern to many people especially when the chemical constituents of the latter product are not known and would be used for a long period [4, 5].

There was no doubt about the effectiveness of herbal remedies in HIV infection. There are many classes of herbal remedies used for HIV infection based on chemical constituent: alkaloids, carbohydrates, coumarins, flavonoids, lignans, phenolic, proteins, quinones, terpenes and tannins. There are many herbal remedies that have been found to inhibit one or more steps in HIV replication [6, 7]. Alkaloids derivatives herbal remedies (e.g. *Ancistrocladus*

korupensis) from tropical liana plant inhibit reverse transcriptase and HIV induced cell fusion [8]. p14 (HIV tat regulatory protein that activates transcription of proviral DNA) had been documented to be inhibited by a carbohydrate derivative, pentosan poly-sulphate [9]. A coumarin herbal remedy in form of canolides from tropical forest tree (*Calophyllum lanigerum)* was rated as non-nucleoside reverse transcriptase inhibitor in potency [10]

However, every drug is a potential poison. Thus some herbal remedies are toxic especially when used over a long period. Despite alkaloid containing herbal remedies had been found to be very effective in HIV infection however some michellamines group of alkaloid were found to be cytotoxic thus limiting them for therapeutic use [11]. Quinones and xanthones derivatives herbal remedy were weakly effective against HIV infection and the proved effective medications were cytotoxic [10, 12].

Because HIV infection is a terminal illness, there is an increase in use of herbal remedy in Nigeria [13]. Many HIV patients resulted to herbal therapy at the on-set of HIV diagnosis before commencement of highly active antiretroviral therapy (HAART) while some use it as complementary therapy [14, 15]. The major concern is possible negative drug interaction of herbal therapy and HAART. It was documented that garlic and St John's wort negatively interact with HAART [16].

The fact that many consumers could not distinguish between the safe herbal drugs from potential harmful led to general acceptance or rejection of herbal therapy. A safe herbal therapy may become harmful if used for a long period. African potato (hypoxis) and *Sutherlandia frutescens* have been documented to be harmful when used for a long period with HAART [17]. Even chronic herbal derived vitamins and cannabis use were established to negatively influence hepatic metabolism of HAART components [18].

However, some herbal remedies have been documented to be beneficial when used with orthodox medicines. Coumarin derived herbal remedies decreased drug resistance resulting from HIV mutation associated with non-nucleoside analogue-nevirapine [19, 20]. Some herbal remedies have also shown to decreased toxicity associated with HAART. The hepatic toxicity associated with acetaminophen was reduced by Gentiana manshurica [21]

There are many herbal remedies that are used in Nigeria for HIV infection. Many of them are complementary to HAART. Toxicological studies have been done on many herbal products in Nigeria using animal modelels [22]. Unlike the assumptions that herbal remedies are harmless because of the natural source, many have been found to be toxic [23]. Thus, the researchers are trying to identify the safe herbal remedies and encourage its use while discouraged the harmful herbal products [24].

The beneficial effect of combination therapy of HAART and α-Zam an herbal remedy used as alternative therapy for HIV infection had been documented at acute and sub-acute phase [25]. The use of herbal remedy as alternative therapy to HAART had met a lot of criticisms especially when such medication has not passed through all the phases of drug trial. It is not uncommon that some HIV patients taking HAART are also using alternative herbal remedy simultaneously without the knowledge of both herbal therapist and medical practitioners

[26]. However, the benefits in association with potential toxicity of such complementary therapy at chronic phase needed an evaluation.

2. Materials and method

The materials and method had been described in earlier studies [5, 20, 22]

2.1. A-zam

This is herbal concoction that contained alkaloids, saponins, tannins, cardenolides and possibly anthraquinones [5]

2.2. Highly Active Anti-retroviral Therapy (HAART)

The drugs used in this study were Nevirapine (50mg/kg), Lamivudine (100mg/kg) and Zidovudine (300mg/kg) prepared by grinding the tablets into fine powder.

2.3. Drug preparation

10% of the herbal preparation was made to using tepid distilled water as recommended for use by herbal therapist. A fresh preparation was prepared daily.

2.4. Animals

90 male wistar rats (150-200g body weight) were acclimatised for 7 days before the start of the experiment. Throughout the time of the experiment, they were housed under standard environmental conditions, maintained on a natural light and dark cycle. The animals had free access to rat chow and portable water.

2.5. Drug administration

The freshly prepared herb and HAART were administered orally using oral canula to animals once in 24 hours. The herbal preparation and HAART (nevirapine, zidovudine and lamivudine) were administered concurrently to the groups (40 rats) receiving combination Therapy while another 4 groups (40 rats) received graded concentrations of herbal remedy alone. Animals were deprived of food before drug administration after which they were allowed access to food.

2.6. Experimental procedure

A pilot toxicity study was earlier carried out by a single dose administration of herbal preparation to rats. Results showed that neither mortality nor change in behaviour was observed even at 3200mg/Kg body weight (Onifade et al 2011). 90 wistar rats was randomised divided into 9 groups (10 rats per group) and were administered once daily for 84 days with Herbal concoction of 400mg/kg, 800mg/kg, 1600mg/kg, 3200mg/kg, 400mg/kg+HAART,

800mg/kg+HAART, 1600mg/kg+HAART, 3200mg/kg+HAART, 400mg/kg + HAART (Nevirapine, Zidovudine & Lamivudine), 800mg/kg+ HAART (Nevirapine, Zidovudine & Lamivudine) respectively. The 9th group served as control thus received rat chow and water only. All the animals were allowed to free access chow, water, fresh air and move freely. They were monitored daily for feeding pattern, behavioural or physical changes. 24 hours after the last dose (the 85th day), the diethyl ether anaesthetised animals were bled from the retro orbital plexus for haematological (total white blood cell count, red blood cell count, haemoglobin concentration, platelet count and lymphocyte counts), serum biochemical analysis (electrolytes, urea, creatinine, lipid profile, liver and renal functions tests), fertility profiles (follicle stimulating hormone, progesterone, leutenising hormone, Oestrogen, testosterone and prolactin) and sperm motility test. The liver, kidney, spleen, skin, heart and bone marrow were harvested for histological changes.

3. Results

The results were categorised as follows:

OBSERVATION- No physical or behavioural abnormalities were observed in all the groups of animals throughout the study.

LABORATORY- This is outlined into haematological, clinical chemistry (liver and renal function tests and lipid profile), fertility (fertility profile and sperm analysis) and histological results.

Substance administered	Leucocyte	Red blood cell (x 10^6)	Haemoglobin (g/dl)	Platelet (x10^5)	Lymphocyte %	Lymphocyte total
Ratchow& water	6400±334	7.55±1.2	13.4±2.1	3.81±1.9	68±6	4352±124
400mg/kg α-zam	9600±228*	8.34±0.7	15.3±1.8	7.19±2.56	84±9	8064±2365*
800mg/kg α-zam	9400±887*	7.88±0.56	14.3±1.2	9.65±2.85	85.6±8	8084±1890*
1600mg/kg α-zam	9700±934*	7.14±0.34	13.5±1.1	11.09±4.0	72±7.2	6984±459
3200mg/kg α-zam	6200±415	7.42±0.09	14.3±1.5	8.0±2.3	69±4.4	4278±234
0.4g/kg α-zam +HAART	7200±330	7.3±0.8	15.1±1.2	5.7±2.1	72±8.1	4100±350
0.8g/kg α-zam +HAART	8000±720	7.2±0.9	15±1.4	6.8±1.8	70±6.4	4000±270
1.6g/kg α-zam +HAART	6500±635	7.74±0.78	15.0±1.3	7.93±1.8	65±5.9	4225±519
3.2g/kg α-zam +HAART	6600±756	7.22±0.82	14.6±1.6	5.56±1.7	70±3.7	4620±418

*- statistically significant
Rat chow and water only is the control and reference group

Table 1. Showing the Haematological parameters using α- Zam alone and in combination with HAART (mean and standard deviation)

Clinical chemistry- This is divided into 2: Electrolytes, urea and creatinine (Renal functions tests) and Liver functions tests and lipid profile

Substance Administered	Na+	K+	HCO3-	Cl-	Urea	Creatinine
Rat chow and water	142±2.0	4.5±0.31	30±2.2	103±2.4	6.7±0.54	62±2.1
400mg/kg α-zam	140±2.1	4.6±0.22	30±2.1	104±2.3	6.5±0.46	64±2.5
800mg/kg α-zam	144±2.7	4.4±0.27	30±2.0	105±1.9	6.6±0.32	66±1.9
1600mg/kg α-zam	143±1.5	4.5±0.34	28±2.7	103±2.9	6.8±0.45	65±2.3
3200mg/kg α-zam	144±2.0	4.6±0.21	31±0.9	102±2.8	6.9±0.3	63±1.8
0.4g/kg α-zam+HAART	143±1.9	4.6±0.3	27±3.1	104±2.2	6.7±0.5	66±3.2
0.8g/kg α-zam +HAART	142±2.1	4.6±0.2	29±2.9	103±3.4	6.6±0.4	65±2.2
1.6g/kg+HAART α-zam	141±2.9	4.7±0.18	30±1.1	104±2.3	6.6±0.4	64±1.7
3.2g/kg +HAART α-zam	142±1.8	4.7±0.21	31±0.7	103±2.7	6.8±0.55	65±2.1

Rat chow and water only is the control and reference group

Table 2. Showing the Electrolytes, Urea and Creatinine using α- Zam alone and in combination with HAART (mean and standard deviation)

substance administered	albumin	globulins	AST	ALT	Total protein	HDL	Triglyceride	LDL	Total cholesterol
Rat chow and water	31±5	52±5	51±7	11±2	83±13	1.1±0.2	0.8±0.3	0.4±0.2	1.8±0.13
400mg/kg α-zam	35±4	58±4	45±7.8	9±2.1	92±4.9	1.2±0.4	0.8±0.3	0.4±0.2	1.9±0.23
800mg/kg α-zam	34±4.4	59±4	49±6.9	12±2.3	93±2.7	1.3±0.3	0.7±0.29	0.4±0.1	1.8±0.19
1600mg/kg α-zam	32±2.3	57±4	54±4.3	14±3.2	89±4	1.1±0.35	0.8±0.36	0.3±0.1	1.8±0.17
3200mg/kg α-zam	30±3.2	61±3*	56±3	13±1.9	91±5	1.4±0.19	0.6±0.38	0.4±0.1	1.9±0.11
0.4g/kg α-zam +HAART	34±3.2	57±6	49±4	12±2.2	86±6	1.3±0.9	0.8±0.26	0.4±0.1	1.7±0.1
0.8g/kg α-zam + HAART	33±2.7	56±5	52±9	12±1.8	85±7	1.3±0.2	0.7±0.3	0.4±0.2	1.7±0.1
1.6g/kg α-zam +HAART	33±2.9	58±4	52±4	11±0.7	91±5	1.2±0.21	0.7±0.27	0.3±0.1	1.7±0.24
3.2g/kg α-zam +HAART	34±1.8	57±5	53±5	12±1.1	91±6	1.3±0.17	0.7±0.26	0.4±0.1	1.8±0.23

Rat chow and water only is the control and reference group

Table 3. Showing the LIVER FUNCTIONS TEST AND LIPID PROFILE using α Zam alone and in combination HAART (mean and standard deviation)

Substance administered	Follicle Stimulating Hormone (miu/ml)	Leutenizing Hormone (miu/ml)	Prolactin (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)	Testosterone (pg/ml)
Rat chow and water only	2.4±0.2	4.4±0.22	16.2±0.4	110±9	10.1±2.3	7.1±0.1
400mg/kg α-zam	2.8±0.1*	4.9±0.23*	16.9±0.3	114±8	10.2±2.1	7.8±0.2*
800mg/kg α-zam	2.7±0.11*	4.8±0.3	16.3±0.2	111±3	9.9±1.9	7.7±0.2*
1600mg/kg α-zam	2.7±0.12*	4.7±0.23	16.5±0.1	112±4	10.0±1.7	7.8±0.3*
3200mg/kg α-zam	2.9±0.2*	4.9±0.27	15.9±0.4	110±5.6	10.1±1.1	7.9±0.32*
0.4g/kg α-zam+HAART	2.7±0.2	4.5±0.3	16.5±0.3	111±6	10.3±2.2	7.5±0.3
0.8g/kg α-zam+HAART	2.6±0.2	4.5±0.1	16.3±0.1	111±9	10.2±1.8	7.5±0.3
1.6g/kg+HAART α-zam	2.6±0.2	4.6±0.21	16.1±0.3	112±1.8	10.2±2.5	7.6±0.23
3.2g/kg+HAART α-zam	2.5±0.2	4.5±0.18	16.3±0.3	112±2.7	10.3±3.1	7.5±0.21

*- statistically significant
Rat chow and water only is the control and reference group

Table 4. Showing the FERTILITY PROFILE using α-Zam alone and in combination with HAART (mean and standard deviation)

Substance administered	Motility (%)	Live/dead (%)	Volume (ml)	Count (x10^7)
Rat chow& water	66±27	97±2	5.2±0.1	141±21
400mg/kg α-zam	54±22	95±3	5.2±0.1	100±34
800mg/kg α-zam	74±23	95±4	5.2±0.1	130±15
1600mg/kg α-zam	60±12	92±8	5.1±0.1	114±41
3200mg/kg α-zam	72±21	96±3	5.2±0.1	125±31
0.4g/kg α-zam+HAART	67±24	98±4	5.1±0.2	142±27
0.8g/kg α-zam+HAART	69±21	98±3	5.1±0.2	145±36
1.6g/kg α-zam+HAART	66±27	99±2	5.1±0.1	147±27
3.2g/kg α-zam+HAART	64±22	97±4	5.1±0.1	144±9

Rat chow and water only is the control and reference group

Table 5. Showing the **sperm count** using α-Zam alone and in combination with HAART (mean and standard deviation)

Substance administered	Histological Changes-Skin	Histological Changes-Spleen	Histological Changes-Testes	Histological Changes-Liver	Histological Changes-Kidney	Histological Changes-Bone Marrow
Rat chow and water only	No remarkable changes	No remarkable changes	No remarkable changes	No remarkable changes	No remarkable changes	No remarkable changes
400mg/kg α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy and focal necrosis	Hyper-cellular marrow
800mg/kg α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy and focal necrosis	Hyper-cellular marrow
1600mg/kg α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy and focal necrosis	Hyper-cellular marrow
3200mg/kg α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy and focal necrosis	Hyper-cellular marrow
0.4g/kg α-zam+ HAART	No histological changes	Normal splenic histology	Normal testicular tissue	Normal liver anatomy	Mild focal necrosis	Normal cellularity
0.8g/kg α-zam +HAART	No histological changes	Normal splenic tissue	Normal testicular tissue	Normal liver anatomy	Mild focal necrosis	Normal cellularity
1.6g/kg+HAART α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy	Hyper-cellular marrow
3.2g/kg+HAART α-zam	No remarkable changes	Mild congestion of splenic plexuses	No significant changes	Mild atrophy	Mild tubular atrophy	Hyper-cellular marrow

Table 6. Showing the histological changes of A-Zam with HAART

4. Discussion

The effect of combination of drugs may manifest in acute or chronic phase. The acute or chronic manifestation of drug toxicity may be mild or lethal which may even result in death.

Although the combination of herbal medications with orthodox drugs had caused beneficial effects like reduction in side effects of the latter but the chronic toxicity is very fatal [21]. From the result above, none of the animal had physical or behavioural impairment thus only laboratory analysis could indicate the potential harmful effects of the combination therapy of HAART and the herbal concoction.

The fact that the animal is feeding or behaving normally does not guaranteed the total well-being especially when taking medication for a long period [25]. Although the physical and behavioural changes of the animal had to be compared with control group but there may be silent damage to cells, tissues or organs that has not manifested as systemic derangements. The health status of the animal taking potential harmful drugs can be confirmed by assessing the major laboratory parameters that are normally grouped into haematological, renal function, liver functions, lipid profiles, fertility profiles and histology of all the major organs [26].

Haematological profiles normally showed the erythrocyte, leucocyte and platelet but the apparent functionality of the immune system (lymphocyte and granulocyte) can also be obtained. When the herbal remedy was used alone in this study, there was leucocytosis with lymphocyte predominance as shown in Table 1. The lymphocyte predominance gradually changed to granulocytes as concentration increases and peak at 1600mg/kg. However, there was normal leucocytes differential irrespective of the dose when combined with HAART. There was also bone marrow hyper-cellularity when herbal medication alone was administered but normalised with HAART as shown by histological changes in Table 6. This confirmed that herbal induced leucocytosis was ameliorated by HAART induced leucopoenia [21, 22]. This showed that combination of this herbal remedy with HAART for HIV infection is beneficial if used for longer period.

Decrease in erythropoiesis (anaemia) that usually manifest early when potential bone marrow suppressive drug was administered for long period was absent with α-zam alone or combination therapy [25]. It was evident from table 1 above that the herbal remedy did not affect erythropoiesis negatively as evidenced by normal haemoglobin concentration and red cell blood count. However, A-Zam herbal remedy is associated with thrombocytosis. The platelet count increased gradually with increase in A-Zam concentration. The combination therapy of A-Zam with HAART caused significant decrease in platelet count. Thus bone marrow suppression effects of HAART components normalised the potential thrombocytosis as shown in Table 1. Chronic use of A-Zam alone or in combination therapy with HAART has no harmful effect on haematological parameters rather ameliorating side effects of associated traditional and orthodox medicines.

The role of kidney in drug excretion cannot be overemphasised. Any negative interaction of drug(s) may affect its clearance from the body. Thus, it is not unusual to noticed renal impairment while other organs and systems perform normally [26]. The combination of two potential harmful drugs may worsen the renal architecture. It was established that many of the antiretroviral therapy are potential nephrotoxic and becomes more pronounced when

used in combination with another drug with similar deleterious effect [28]. Renal functions were not impaired when A-Zam alone or in combination with HAART as shown in Table 2 although mild histological changes that reduced with complementary therapy in Table 6 were observed. This study depicted a neither harmful nor beneficial effect of combination of 2 potential harmful drugs used for HIV infection [29].

Drug metabolism is majorly handled by liver. Any toxic drug will likely impair hepatic functions. Some drugs induce hepatic enzymes and apparatuses that accelerate metabolism [30]. This increases fast elimination of the toxic drug from the body. However, some drugs inhibit cytochrome P-450 thus delaying its hepatic clearance of such medication [31]. The danger is combination of cytochrome P-450 inhibitors or inducers. Table 3 showed that both HAART and herbal remedy (A-Zam) are not hepatotoxic alone or in combination. Although there were mild atrophic changes in the liver when A-Zam was used alone but the damage did not caused any significant increase in both cytosolic and mitochondrial hepatic enzymes. This result showed one of the silent cellular injuries that resolved favourably therefore not showing any plasma changes.

The metabolic status of individual is very important. The metabolic disorders associated with lipid derangement in adult are fatal. Some drugs have been noted to cause hyperlipidaemia (especially low density cholesterol and triglyceride) therefore potentiating Raeven's syndrome in adulthood [28]. The coronary index is better with hyperlipidaemia of high density proportion [30]. Any drug that lowers HDL will cause harmful effect and increases coronary index [28]. From the result in table 3, neither A-Zam alone nor its combination with HAART caused any significant harmful hyperlipidaemia. This confirmed that complementary therapy (A-Zam and HAART) is cardio-protective.

Average adult male is conscious of his fertility profile and sperm analysis. Infertility has been a major concern especially when the fault is associated with male. Many drugs affect fertility of male therefore complementary therapy of such medications constitute danger [28]. Spermatogenesis is influenced by hormonal changes. Table 4 showed deranged follicle stimulating hormone (FSH) and testosterone in rats with A-Zam therapy portraying a potential danger. However, combination of HAART with A-Zam ameliorated the fertility hormonal changes which was collaborated with sperm sperm analysis (total sperm count) in Table 5. Plasma and semen analysis showed derangement which did not manifested with significant testicular injury as shown in table 6.

The histology of heart and skin as shown in table in Table 4 were not affected by herbal remedy or its combination with HAART. This confirmed the earlier studies that some herbal remedy are not dermato-toxic [25, 32]. Despite there was no significant changes in liver and renal functions parameters, it was clearly evident there were mild injury to kidney and liver with chronic A-Zam administration alone. However, complementary therapy of HAART and A-Zam reduced the toxic injuries on liver, kidneys and bone marrow. This histological result in confirmed the safety of chronic complementary therapy of HAART and A-Zam as documented in earlier studies [20, 33].

5. Conclusion

This study concluded that the chronic complementary therapy of herbal remedy (A-Zam) with HAART is safe and beneficial as evidenced by side effects amelioration of both orthodox and traditional medicines in wistar rat in this study.

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Chapter 18

Nanoparticles Toxicity and Their Routes of Exposures

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Nanotechnology and nanomaterials

Nanotechnology is a new area that presents small sized materials, structures, devices, and systems in last few decades. We hear about many materials and systems that contain nanomaterials nowadays. Most of the producers present their products as materials having excellent characteristics.

Due to their small size, nanotechnology based materials have unique characteristics such as magnetic, optical, thermal, mechanical, electrical, electron configuration density when compared with macromolecules. Nanomaterials are generally at the 1–100 nm scale and have a vast range of applications such as in medicine, electronics and energy production. Cosmetics, sunscreens, coatings, batteries, fuel additives, paints, pigments, tires and cement are the examples of consumer products that based on nanotechnology. Nanomaterials may also used for special medical purposes such as to produce novel drug delivery systems, to enhance the performance of medical devices, or to produce diagnostic-imaging materials [1].

1.2. Nanomedicine

The European Science Foundation [2] defines 'Nanomedicine' as the science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body. It is discussed under five main sub-disciplines as:

- Analytical tools
- Nanoimaging
- Nanomaterials and nanodevices
- Novel therapeutics and drug delivery systems
- Clinical, regulatory and toxicological issues.

Although most nanotechnology deal with nanoparticles (NPs) sized below 100 nm, in nanomedicine including drug delivery systems particle size is ranged from a few nanometers to 1000 nm. Nanomedicines in practice are generally sized 5–250 nm [3]. In contrast to small sized nanomaterials, this relatively big dimension in drug delivery systems is sufficient to load the drugs onto the particles [4].

1.3. Nanotechnology based novel drug delivery systems

Nanosized drug delivery systems have already entered routine clinical use and Europe has been pioneering in this field [2]. Novel drug delivery systems are improving steadily in recent years. Main goals of these improvements are to achieve targetting of drug more specifically, to reduce toxicity of drug without the interfering of efficacy, to achieve biocompatibility, and to develop safe new medicines [4]. Nanotechnology is extensively used to produce the drug carriers having such kind of advantages, for example, carbon nanotubes (CNTs) are being used in targeted anticancer drug formulations and it has been shown to greatly improve the anticancer activity in animal models. Paclitaxel is another extensively studied anticancer molecule of which NP drug delivery formulation is prepared. This formulation enhanced cytotoxicity of paclitaxel on tumor cells *in vitro* and increased therapeutic efficacy in an animal model [5]

Nanomedicines produced with nanotechnology based engineered materials include proteins, polymers, dendrimers, micelles, liposomes, emulsions, NPs and nanocapsules [3].

2. Nanotoxicology issue of novel drug delivery systems

2.1. Toxicological aspects of NPs

Although nanoscale drug delivery systems are designed to reduce toxicity of drugs and to increase biocompatibility [6], there maight be some risks because of the unique characteristics of them. Due to these challenge, “nanotoxicology” term was adopted and defined as the science dealing with the effects of nanodevices and nanostructures in living organisms [7].

Nanoparticles have intrinsic toxicity profiles. Properties of nanoparticles that might increase the toxicity potential include **i)** particle size, **ii)** surface area and charge, **iii)** shape/structure, **iv)** solubility, and **v)** surface coatings [1]. Small size of NPs give rise to a high surface area per unit mass, and this surface area is often correlated with higher biological reactivity. In addition, formation of free radicals such as superoxide anion or hydroxyl radical may also be increased with high surface area. Accordingly oxidative stress may play an important role in NP toxicity especially for metal-based NPs. For example, inflammatory responses to NPs can be explained with these free radical formation [8].

Data on potential human and environmental exposure and dose-response relationship will be necessary to determine potential risks of nanomaterials following inhalation, oral or

dermal routes of exposure. Significance of dose, dose rate, dose metric, and biokinetics are very useful parameters for the safety evaluation of newly engineered NPs.

One of the most common entry routes for NPs is inhalation. *In vivo* studies have demonstrated lung inflammation as a result of exposure to NPs [9]. Systemic distribution of NPs has been reported into the blood stream and lymphatic pathways [10].

Another important route for NP entry is the skin, from accidental exposure and use of cosmetics and other topical applications. Although the outer layer of the epidermis, the stratum corneum, protects against environmental insults, tittanium dioxide (TiO_2) has been shown to penetrate the stratum corneum and even hair follicles [11]. Penetration of nanosized TiO_2 (5–20 nm) into the skin and its interaction with the immune system has also been demonstrated [12].

Dey et al. [13] have demonstrated that nanosized alumina is internalized and significantly increases manganese superoxide dismutase (MnSOD) protein levels, indicating that the effect of alumina may occur, in part, via alteration of cellular redox status. It was also indicated that NP exposure can cause increased proliferation and anchorage-independent transformation in JB6 cells.

De Jong et al. [4] have summarized more striking toxicological effects of NPs in Table 1.

2.2. Proposed mechanism of NP induced toxicity

2.2.1. Oxidative stress

Through many researches, reactive oxygene species (ROS) production is increased at NP exposure. This phenomenon is called oxidative stress. Knaapen et al. [14] suggested three main factors which cause ROS release: (i) active redox cycling on the surface of NPs, particularly the metal-based NPs [15,16] (ii) oxidative groups functionalized on NPs; and (iii) particle–cell interactions, especially in the lungs where there is a rich pool of ROS producers like the inflammatory phagocytes, neutrophils and macrophages. Overproduction of ROS activates cytokines and upregulates interleukin (IL), kinases and tumor necrosis factor-α (TNF-α) as an indicators of proinflammatory signaling processes as a counter reaction to oxidative stress [17].

Miura et al. reported that the expressions of ho-1 and mt-2A, well-known oxidative stress related genes, were up-regulated by nano-silver treatment. These results indicated that apoptosis induction by silver nanoparticles (Ag NPs) may be created by ROS generation [18].

Potential role of oxidative stress as a mechanism of toxicity of AgNPs were evaluated by Hussain et al. [19]. In this study ROS generation following 6 h of exposure to Ag (15, 100 nm) at 0, 5, 10, 25, and 50 g/mL was investigated. The level of ROS in cells increased in a concentration dependent manner and was statistically increased from 10 g/mL concentration. Ag (15, 100 nm) treatment at 25 and 50 g/mL resulted in an approximately 10-fold increase in ROS generation over control levels.

Description of finding, *in vivo*	**Particle type**
NPs cause pulmonary inflammation in the rat.	All PSP
Later studies show that inflammation is mediated by surface area dose.	SWCNT, MWCNT
NPs cause more lung tumors than fine particles in rat chronic studies. Effect is surface area mediated.	PSP only.
NPs cause progression of plague formation (ApoE-/-mice)	SWCNT, PM2.5
NPs affect immune response to common allergens.	Polystyrene, CB, DEP
NPs can have access to systemic circulation upon inhalation and instillation.	Specific NP, dependent on surface coating.
Description of finding, *in vitro*	
NPs cause oxidative stress *in vivo* and *in vitro*, by inflammatory action and generation of surface radicals.	PSP, NP general, CNT
NPs inhibit macrophage phagocytosis, mobility and killing.	CB, TiO_2
NPs cause platelet aggregation.	PM, SWCNT, fullerenes, latex-COOH surface
NPs exposure adversely affects cardiac function and vascular homeostasis.	PM, SWCNT
NPs interfere with Ca-transport and cause increased binding of pro-inflammatory transcription factor NF-kB.	CB (<100 nm), ROFA, PM2.5
NPs can affect mitochondrial function.	Ambient NP
NPs can translocate to the brain from the nose.	MnO_2, Au, carbon
NPs do affect rolling in hepatic tissue.	CB

NP: nano particle, PSP: poorly soluble particles, DEP: diesel exhaust particles, SWCNT: single wall carbon nanotube, MWCNT: multi walled carbon nanotube, CB: carbon black, titanium dioxide:TiO_2, PM: particulate matter, ROFA: residual oil fly ash, manganese dioxide: MnO_2, PM2.5: particle mass fraction in ambient air with a mean diameter of 2.5 μm.

Table 1. Toxicological effects of engineered and combustion NPs [4].

In another study intractions of AgNPs with human fibrosarcoma (HT-1080) and human skin/carcinoma (A431) cells was undertaken. When the cells was challenged with AgNPs (6.25 μg/mL), signs of oxidative stress such as decrease in oxyradical scavengers including reduced glutathione (GSH) and superoxide dismutase (SOD) as well as increase in lipid peroxidation were seen. Authors mentioned that observed SOD inactivation might be due to generation of peroxy radicals after AgNPs exposure [20].

Induction of oxidative stress and apoptosis by AgNPs in the liver of adult zebrafish was studied by Choi et.al. The results indicated that the levels of malondialdehyde (MDA), a product of cellular lipid peroxidation, and total GSH were increased in the tissues after treatment with AgNPs. The mRNA levels of the oxyradical scavenging enzymes catalase (CAT) and glutathione peroxidase 1a (GPx 1a) were reduced in the tissues. Authors

concluded that the increased level of hepatic MDA indicates that AgNPs induced oxyradicals in the liver. In addition, the induction of an endogenous antioxidant, GSH, suggests that the liver tissues respond defensively to the increased level of oxyradicals. Also the reduction of the levels of CAT and GPx may thus result in the accumulation of hydrogen peroxide (H_2O_2) and other oxyradicals [21]. The elevated oxidative stress can damage lipids, carbohydrates, proteins and DNA.

Some investigations reported that TiO_2 increased intracellular ROS generation and MDA concentration in a dose-dependent manner [22,23]. The mechanism by which TiO_2 NPs can generate free radicals is through decreasing the activities of antioxidant enzymes such as SOD, CAT, GPx and glutathione reductase (GR) or intracellular levels of antioxidants such as GSH and ascorbic acid [24-27].

Ramkumar et al. reported that the ratio of GSH/GSSG, a good indicator of the levels of cellular oxidative stress, was found to be decreased dose dependently in the TiO_2 treated cells [22] and thus preserving the GSH-mediated antioxidant defense that is critical for cell survival. It is possible that the loss of GSH may compromise cellular antioxidant defenses and led to the accumulation of ROS and free radicals that are generated in response to exposure to NPs or as by products of normal cellular function.

In line with these findings, Sun et al. investigated the generating of superoxide ($O_2^{-\cdot}$) and H_2O_2 after long-term exposure to TiO_2 NPs in mice [28]. Results showed that the production rates of $O_2^{-\cdot}$ and H_2O_2 in TiO_2 treated group were significantly higher than those of control. It is also reported that exposure to TiO_2 NPs elicits lipid peroxidation levels in the mouse lung. Since ROS act as second messengers in intracellular signaling cascades [29], the increase in ROS by TiO_2 NPs exposure may play an important role in the modulation of gene expression and resultant inflammation or apoptosis.

Li et al. investigated the oxidative stress associated with gold NPs (Au NPs) in human lung fibroblast cells [30]. It was observed that the Au NPs treated cells generated significantly more lipid hydroperoxides, a positive indicator of lipid peroxidation, than the control cells. In addition, MDA modified protein adducts were evaluated by western blotting as a further verification of the presence of lipid peroxidation. MDA reacts readily with protein or DNA forming adducts which are considered to be highly genotoxic. The results obtained from this study clearly showed that the amount of proteins alkylated by MDA was significantly more in the AuNPs treated samples than that in control samples.

Jia et al. [31] reported that Au NPs can catalyze nitric oxide (NO), a reactive nitrogen species, generation from endogenous S-nitroso adducts with thiol group (RSNOs) whenever they come into contact with fresh blood serum. RSNOs, such as S-nitrosoalbumin, S-nitrosocystein and S-nitrosoglutathione, is a more abundant and stable form of NO in blood since NO has a relative short lifetime in blood because of its reactivity with various blood components. One notable reaction of RSNO dissociation to yield NO is catalyzed by metal ions including Au NPs. NO reacts rapidly with $O_2^{-\cdot}$ and produces a harmful peroxynitrite ($ONOO^-$) species. ONOO- can disrupt lipids, DNA, and proteins.

Another study, conducted by Tedesco et al. [32], investigated the oxidative stress and toxicity of AuNPs in *Mytilus edulis* (blue mussel). In this study *M. edulis* was exposed to 750 ppb AuNP (average diameter 5.3 ± 1 nm) for 24 hours to investigate in vivo biological effects of nanoparticles. Traditional biomarkers and an affinity procedure selective for thiol-containing proteins were used to study toxic and oxidative stress responses. Protein thiols can play a role in antioxidant defense and absorption of ROS. *M. edulis* that was exposed to Au NP were displayed a decreased amount of thiol containing proteins in comparison both to controls and those treated with cadmium chloride ($CdCl_2$) (a well known pro-oxidant). This result was consistent with direct oxidation of thiols by ROS induced by AuNP. Also this was supported by more traditional independent measures of biological and oxidative stress such as lysosomal membrane stability and lipid peroxidation. Lysosomal membrane stability measured as neutral red retention time showed a decrease for both Au NP and $CdCl_2$ treatments confirming significant biological stress. The effect was stronger in the case of Au NP than $CdCl_2$. This study also showed that Au NP caused significant lipid peroxidation in digestive gland of *M. edulis*. This study suggested that *M. edulis* is a suitable model animal for environmental toxicology studies of nanoparticles.

Due to the interesting magnetic and electrical properties with good chemical and thermal stabilities, spinal ferrite nanoparticles such as nickel ferrite are used in bioapplications including magnetic resonance imaging, drug delivery and hyperthermia [33-37]. However, little is known about the toxicity of spinal ferrite nanoparticles at the cellular and molecular levels. Ahamed et al. investigated oxidative stress mediated apoptosis induced by nickel ferrite nanoparticles in human lung epithelial (A549) cells [38]. In this study the potential of nickel ferrite nanoparticles to induce oxidative stress was assessed by measuring the ROS and GSH levels in A549 cells. Results showed that the nickel ferrite nanoparticles significantly induced the production of ROS and reduced the level of intracellular GSH in these cells. Further, co-treatment with the antioxidant L-ascorbic acid migrated the ROS generation and GSH depletion due to nickel ferrite nanoparticles exposure. These results indicated that nickel ferrite nanoparticles induced oxidative stress in A549 cells by induction of ROS and depletion of GSH.

2.2.2. Phagocytosis of NPs and inflammation

In respiratory tract, mucociliary clearance removes particulate matter (PM) in <6 μm diameter. Alveolar macrophages engulf and process particles that are not cleared by mucociliary action and coughing. Upon phagocytosis macrophages are activated to release substantial amounts of oxygen radicals, proteolytic enzymes, proinflammatory mediators and growth-regulating proteins. These mediators may lead to both acute and chronic lung inflammation [17].

As other NPs, the toxicity of Ag NPs appears to be driven by their oxidative and inflammatory nature, which then drives genotoxic and cytotoxic outcomes [39].

Carlson et al. [40], investigated the size-dependent cellular interactions of Ag NPs in rat alveolar macrophages (NR8383) cell culture. In this study cells were exposed to 0, 5, 10 and

25 μg/mL Ag NPs (15 nm, 30 nm and 55 nm) for 24 hours and levels of characteristic markers of macrophage activation such as TNF-α, macrophage inhibitory protein-2 (Mip-2), IL-1β and IL-6. The results demonstrated significant levels of TNF-α, Mip-2, IL-1β at 5, 10 and 25 μg/mL for all sizes of Ag NPs comparing to the control group. However, there was no detectable level of IL-6 upon exposure to Ag NPs. Also the cytotoxicity of Ag NPs were evaluated by MTT metabolic activity assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and membrane integrity (lactic dehydrogenase (LDH)) assays. The results of the MTT viability assay showed a significant decrease in mitochondrial function of alveolar macrophages exposed to Ag NPs at 15 nm, 30 nm, or 55 nm for 24 h at concentrations ranging from 10 to 75 μg/mL. It was noted that compared to the smaller nanoparticles (15 and 30 nm), 55 nm did not exhibit significant toxicity until 50-75 μg/mL. Similarly to the MTT data, the results of LDH assay showed a dose-dependent decrease in cell viability compared to control cells after 24 h of exposure to Ag NPs. Ag NPs at 15 and 30 nm exhibited significant cytotoxicity at 10-75 μg/mL, whereas 55 nm required a concentration of 75 μg/mL to markedly decrease cell viability according to LDH assay results.

In another study, Park et al. [41] measured secreted NO levels, as a second messenger in inflammatory signaling, to investigate the correlation of nitrosative-oxidative stress and cytotoxicity induced by Ag NPs. The mouse peritoneal macrophage (RAW264.7) cell line were exposed to 0.2, 0.4, 0.8 and 1.6 μg/mL Ag NPs (68.9 nm) for 24, 48, 72 and 96 hours. Results showed that NO secretion was increased 2-fold over the control group by Ag NPs at 1.6 μg/mL. Also TNF-α level was increased almost 2.8-fold and GSH level was decreased by the same concentration comparing to control group. Ultimately, the phagocytosis of Ag NPs stimulated inflammatory signaling through the ROS generation in macrophages followed by the induced secretion of TNF-α. The increase of TNF-α can cause damage of cell membrane and apoptosis. Authors concluded that ionization of Ag NPs can be a major factor for all these results in cells.

Park et al. [42] also conducted a 28-day oral administration study in mice to investigate toxicity and inflammatory responses of 0.25, 0.5 and 1 mg/kg Ag NPs (42 nm). Evaluation of inflammatory responses by repeated administration of AgNPs were conducted by measurement of pro-inflammatory cytokines (IL-1, TNF-α and IL-6), Th1-type cytokines (IL-12 and interferon-gamma), Th2-type cytokines (IL-4, IL-5, IL-10) and transforming growth factor beta (TGF-β) concentrations in serum. The results showed that IL-1 was significantly increased by Ag NPs. TNF-α and IL-6 were increased almost 2.8-fold and 9.5-fold of the control group, respectively. Also both Th1-type cytokines and Th2-type cytokines showed a significant increase. TGF-β which is known as tissue damage-related cytokines, was also increased in a dose-dependent manner.

Also some studies reported that exposure to TiO_2NPs results in pulmonary inflammation, pulmonary edema, macrophages accumulation and pneumonocyte apoptosis [28, 43-45].

Jacobsen et al. [46] studied inflammatory potential after intratracheal instillation of 5 different types of nanoparticles (CB, gold clusters, fullerense C60, SWCNT and quantum

dots) for pulmonary effects in apolipoprotein E knockout mice. Results indicated significant increases in mRNA levels of Mip-2, IL-6 and macrophages/monocyte chemoattractant protein-1 (Mcp-1) in lung tissue following 3h and 24h instillation of SWCNT, CB and quantum dots. Also gold and fullerense C60 were found less potent at the three end points when compared to others.

Gosens et al. [47] administered a single dose of 1.6 mg/kg bw of single (50 nm) and agglomerated (250 nm) gold particles in the rat lung by intratracheal instillation. Findings showed that both single and agglomerated particles were taken up by macrophages. Both particles increased inflammatory cells and pro-inflammatory cytokine production. The effects were the least for 50 nm Au NPs.

In line with these findings, Cho et al. [48] reported that Au NPs sized at 13 nm induced acute inflammation and apoptosis in the liver of BALB/c mice after intravenous administration.

Downs et al. [49] measured TNF-α and IL-6 in plasma samples of the rats intraperitoneally treated with silica NPs (15 nm and 55 nm) and crystalline silica (quartz) particles (400 nm). The largest increases in the plasma levels of cytokines were found in the animals exposed to 125 mg/kg (the highest dose) of both the 15 nm and 55 nm silica NPs and the quartz particles. A remarkable increase in the levels of both TNF-α and IL-6 was found in the rats treated with the 15 nm silica NPs at 50 mg/kg dose (middle dose), but not at the 25 mg/kg dose (low dose). Treatment with the 55 nm silica NPs resulted in a 1.5-fold and a 2.3-fold increase in TNF-α and IL-6 levels at 125 mg/kg, respectively, but no change was observed in IL-6 levels at the 25 mg/kg doses. Quartz particles showed a 2.3-fold and 2.1-fold induction of TNF-α and IL-6 production, respectively, at the 100 mg/kg dose.

2.2.3. Genotoxicity

NanoGenotoxicology is yet another new term that was coined to represent the growing trend of research into NP-induced genotoxicity and carcinogenesis [17]. Although there is still no exact correlation between NP-induced genotoxicity and lung cancer from epidemiological studies and *in vivo* rodent experiments, it is pointed out in literature that long-term inflammation and oxidative stress present in tissue can eventually induces DNA damage in cells and tissues. Continuous ROS production in the cell can cause gene mutations/deletions leading to mutagenesis, carcinogenicity, and subsequently development of tumors and cancer. Particularly the metal based NPs like Ag NPs [19], Au NPs [30] and TiO_2 NPs [22] are important for that kind of ROS production and genetic damage. As a result of DNA damage induced by NPs, single-strand DNA breaks, double-strand breaks, DNA deletions and genomic instability in the form of increase in 8-hydroxy-2-deoxyguanosine levels are formed [50]. According to Mroz et al.[51] long-term exposure of cells to NPs displayed genome instability under comet assay analysis, altered cell cycle kinetics in flow cytometry and induced protein expression of p53, having a critical role in responding to various stresses that cause DNA damage, and DNA repair-related proteins.

Li et al. [52] studied the genotoxicity of 5 nm Ag NPs using two standard genotoxicity assays, the Salmonella reverse mutation assay (Ames test) and the *in vitro* micronucleus assay. Results demonstrate that 5 nm AgNPs did not induce mutations in five different *S. typhimurium* strains (TA102, TA100, TA1537, TA98 and TA1535). However, Ag NPs displayed concentration-dependent genotoxicity in the human lymphoblast TK6 cell micronucleus assay. Ag NPs produced statistically significant increases in micronucleus frequency in the assay. The data suggest that the *in vitro* micronucleus assay may be more appropriate than the Ames test for evaluating the genotoxicity of the AgNPs.

Ahamed et al. [53] investigated the ability of uncoated or polysaccharide-coated Ag NPs (25 nm) to elicit DNA damage within two types of mammalian cells; mouse embryonic stem cells (mES) and mouse embryonic fibroblasts (MEF). mES and MEF cell lines were exposed to AgNPs at a concentration of 50 μg/mL for a duration of up to 72 hours. Results showed that the proteins p53 and Rad51, responsible for DNA double-strand repair, were up-regulated in two types of mammalian cells. Also in this study DNA double strand breakage induced by AgNPs was confirmed by both immunofluorescent and immunoblot analysis of phospho-H2AX which is ordinarily induced by DNA double-strand breakage. Results indicated that phosphorylation of the histone H2AX were induced by AgNPs. Also this study suggested that the polysaccharide coated AgNPs are more individually distributed and exhibited more severe damage than uncoated AgNPs. This finding may be related with the agglomeration of the uncoated particles and restriction of their cellular distribution.

In an *in vivo* study, Kim et al. [54] conducted a 90-day whole-body inhalation study (6 hours day/5 days a week) to AgNPs (18 nm) in rats at low (0.7 × 106 particles/cm^3), middle (1.4 × 106 particles/cm^3) and high (2.9 × 106 particles/cm^3) doses. After sacrificing of rats, micronucleus induction was measured in the bone marrow according to the test guideline 474 issued by OECD. Authors found out that AgNPs did not affect either the frequency of micronucleated polychromatic erythrocytes (PCE) as an indicator of DNA damage or the PCE / (PCE+NCE) ratio as an indicator of toxicity to bone marrow cells (NCE: normochromatic erythrocytes) in male and female rats. The authors concluded that exposure to AgNPs by inhalation for 90 days does not induce genetic toxicity in male and female rat bone marrow *in vivo*.

Also the same authors conducted an *in vivo* micronucleus assay after 28-day oral administration of Ag NPs. The results were similar to those of inhalation study [55].

A number of studies have shown that TiO_2 NPs exhibited genotoxicity in cultured cell lines [56-58]. Kang et al. [59], studied the genotoxic effects of TiO_2 NPs (20-100 μg/ml) in human peripheral blood lymphocyte cells using alkaline single cell gel electrophoresis (Comet) and cytokinesis block micronucleus (CBMN) assays. The CBMN assay results showed that the micronuclei frequency increased in a dose-dependent manner. Also cells had a significant olive tail moment which indicates unrepaired DNA strand breaks in comet assay.

Similarly, Wang et al. [60] investigated the toxicity of ultrafine TiO_2 particles in cultured human B-cell lymphoblastoid cell line (WIL2-NS). Significant increases in the micronuclei

frequency were detected by the CBMN assay in a dose-dependent manner. In the comet assay, 3-fold increase in %Tail DNA were found when the cells were treated with ultrafine TiO_2 at a dose of 65 μg/mL for 24 hours exposure. In the olive tail moment a 5-fold elevation was found at the same dose and exposure duration. Also a linear relationship was determined between the mutation frequency and concentration in the clonal selection assay for the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene.

Toyooka et al. [61] examined the genotoxicity of TiO_2 NPs (5 nm) and microparticles (<5000 nm) in the lung adenocarcinoma epithelial cell line (A549) based on the phosphorylation of histone H2AX (γ-H2AX) as a new sensitive biomarker for DNA damage. Results showed that TiO_2 particles have the ability to generate γ-H2AX and this was more remarkable with nanoparticles than microparticles.

Contrary to above studies, a number of investigations showed that TiO_2 NPs didn't induce DNA damage and mutation using the Ames test, micronucleus assay, comet assay, and etc. [62-68].

Au NPs are also recognized in their ability to contribute in genotoxicity. Schulz et al. [69] investigated two genotoxic endpoints, alkaline comet assay in lung tissue and micronucleation in PCE of the bone marrow, 72 hours after a single instillation of 18 μg uncoated Au NPs in different sizes (2, 20 and 200 nm) into the trachea of male adult Wistar rats. Results indicated that AuNPs in the different sizes were non-genotoxic and showed no systemic and local adverse effects at the given dose.

Also the genotoxicity of zinc oxide nanoparticles (ZnO NPs), widely used in cosmetics and sunscreens, was evaluated in some studies. Sharma et al. [70] investigated the genotoxicity of these nanoparticles in primary human epidermal karatinocytes using comet assay. Results showed a significant induction in DNA damage in cells exposed to 8 and 14 μg/mL ZnO NPs for 6 hours comparing to control group. Finding demonstrated that ZnO NPs are assimilated by the human epidermal karatinocytes and induce cytotoxic and genotoxic responses.

Also in another study Sharma et al. [71] highlighted the *in vitro* genotoxicity of ZnO NPs in human liver cells (HepG2). Similarly significant increase in DNA damage was observed in cells in the comet assay.

Fen Song et al. [50] analyzed the induction of reticulocyte micronuclei and oxidative DNA damage in ICR female mice after intraperitoneal injection of metal oxides (CuO, Fe_2O_3, Fe_3O_4, TiO_2) and Ag NPs at various doses (0, 1, 3 mg/mouse). The results of the micronucleus assay demonstrated significant increases in micronucleated reticulocyte formation after the intraperitoneal administration of CuO, Fe_2O_3, Fe_3O_4, TiO_2 and Ag NPs. Also the levels of 8-hydroxydeoxyguanosine (8-OH-dG) which is one of the most well studied biomarkers to measure the oxidative damage in DNA was evaluated in liver and bone marrow DNA and urine after the administration of metal oxide and Ag NPs. The urinary level of 8-OH-dG was significantly increased by the CuO at each time point of the urine analysis. Although the increases in the urinary levels of 8-OH-dG for the other

nanoparticle treatments were not significant, all of the other metal compounds showed higher levels of urinary 8-OH-dG than the control. The 8-OH-dG levels in the bone marrow immediately increased after the injection of CuO, and continued to increase up to 24 h after administration. Also the 8-OH-dG levels in the liver DNA of the mice treated with 3 mg CuO were significantly higher than those in the non-treated control. The increase of 8-OH-dG levels in the liver DNA continued for 72 h after the administration of 1 and 3 mg doses of CuO. The other nanoparticles did not cause an increase in the liver 8-OH-dG level at 24 h after administration. Authors concluded that metal oxide nanoparticles can cause genotoxic effects *in vivo*. Among them, the CuO NPs were the most potent, iron oxide NPs also showed relatively high toxicity and TiO_2 and Ag NPs showed low toxicity.

3. Toxicity assessment of novel drug delivery systems

Lack of full toxicological knowledge about nanomaterials including novel drug delivery systems lead to the misperception regarding all nanomaterials pose a significant health risk. Under such realistic conditions, many engineered NPs are unlikely to induce adverse effects although effects of chronic and low level exposures are still largely unknown. Owing to extensive toxicological studies it will be possible to do exact risk assessment related with NPs.

Identification of potential health risks is a prerequisite for assessing the safety of the new products that are being developed. That is why, nanotoxicology area is gaining increasing importance with the growth of nanotechnological applications. Safety evaluation of nanomaterials through toxicological research will also provide information about their undesirable properties. These information will also help to avoid their possible adverse effects [7].

Toxicity studies on nanoparticles are generally conducted at very high doses. With high doses, any NP can be identified as toxic in living systems. A more realistic approach will be to discriminate high doses tested and tests under real exposure conditions. Therefore non *in vivo* assays for the purpose of extrapolating the responses to *in vivo* results may reduce and avoid a lot of laboratory animals. Beside occupational exposure to NP where they are produced and intentional use of consumer goods containing NP should be evaluated. With respect to nanomedicine, *in vivo* tests will always be mandatory for nanotechnology-based therapeutics and diagnostics [72].

As mentioned by Oberdorster [72], a tiered testing system to assess NP toxicity was suggested by a working group of the International Life Sciences Institute (ILSI) [73]. Table 2 lists the tiered testing strategy including **physico-chemical characterization prior to and during testing in cell-free, cellular** and ***in vivo* assays**. Studies designed to determine whether *in vitro* assays are predictive for *in vivo* effects have come to opposite conclusions.

4. Dose concepts in NP toxicology

Classical mass dose trend applied in conventional toxicological research may not be sufficient in nanotoxicological testing due to the extensively large surface area compairing with large particles. Oberdorster [74] studied the toxicity of ultrafine and fine TiO_2 measuring polymorphonuclear neutrophils in lung lavage fluid as an index of inflammation and they found ultrafine TiO_2 as more toxic than fine TiO_2 considering mass unit dose. When considered surface area, the toxicity was equivalent.

- **Physico-chemical characterization**
- **Cell-free assays** (solubility; ROS generating potential; chemreactivity; agglomeration/aggregation; zeta potential; other)
- **Cellular assays** [primary cells; cell-lines; (primary and secondary organs); co-cultures]
- **In vivo assays** [generally rodents; diverse methodologies (resp. tract; skin; GI-tract)]
- **Question**
  Can any of the in vitro tests be used to predict in vivo toxicity?

Table 2. Tiered testing system to assess NP toxicity (ILSI Report) [73]

In vivo and *in vitro* test correlation is critical point in safety evaluations. The dose unit considered in the evaluations may affect the correlations between *in vitro* and *in vivo* tests directly. Say et al. studied fine and nanoparticle toxicity assessing in alveolar macrophages and a pulmonary epithelial cell line and then comparing them with the in vivo pulmonary inflammation induced by the same particles in rats [75]. They could not find a significant *in vitro – in vivo* correlation and suggested more sophisticated in vitro cell culture systems to gauge the relative toxicity of nanoparticles *in vivo*. When these results were analyzed according to the new approach suggested by Rushton et al. [76] who used NP surface area as a dose unit, the *in vitro* and *in vivo* results of Say et al. were correlated significantly.

5. Conclusion

As said by Oberdorster [72], nanotechnology, nanomedicine and nanotoxicology are closely related disciplines aimed at the improvement of human life. As a result of introducing of nanotechnology based nanomaterial into nanomedicine, novel and superior diagnostic, therapeutic and preventive systems have emerged. The safety assessment of these new materials is mandatory to recognize risks and avoid potential hazards. This assessment also provide useful information to avoid disinformation about the toxicity potential of nanosized systems. At this point nanotoxicology will have a crucial role. Material scientists, physicians, pharmacists and toxicologists should be included in nanotechnological developments as a team aproach.

As a result, it is important that safety assessments and health risks of novel drug delivery systems including nanomaterials should be made with the available and produced data. Nanomaterials should be evaluated on case-by-case basis in place of general evaluations for NPs.

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This contribution book collects reviews and original articles from eminent experts working in the interdisciplinary arena of novel drug delivery systems and their uses. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentialities of different drug delivery systems. 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 the 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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