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Hydrogels

Smart Materials for Biomedical Applications

Edited by Lăcrămioara Popa, Mihaela Violeta Ghica and Cristina-Elena Dinu-Pîrvu

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Meet the editors

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Contents

Preface

Hydrogels are part of our day-to-day lives. They are components of our food and everyday objects. Most importantly, they are valuable intermediaries in the most innovative and unexpected attempts to cure, reduce the effects of diseases, and regenerate—in other words, to heal.

Hydrogels, as three-dimensional (3D) polymer networks, are able to retain a large amount of water in their swollen state and are the first biomaterials useful for therapy in humans. They are fascinating materials for developing innovative formulations and applications. Hydrogels have unique properties derived from their 3D viscoelastic networks, essentially permitting attachment and later diffusion of particles, molecules, or cells, as well as serving as 3D bioprinting materials for tissue engineering.

The applications of hydrogels cover a range of domains, from the food industry to pharmaceuticals (for example, in controlled drug or cell delivery) to modern medicine (for example, implants, diagnostics, wound dressings, bone regeneration, and soft contact lenses, to name only a few).

In the last few years, new methods have been developed for the preparation of hydrophilic polymers and hydrogels, which may be used in future biomedical and drug delivery applications. Such efforts include the synthesis of self-organized nanostructures based on triblock copolymers with applications in controlled drug delivery. These hydrogels could be used as carriers for drug delivery when combined with the techniques of drug imprinting and subsequent release.

Engineered protein hydrogels have many potential advantages. They are excellent biomaterials and biodegradables. Furthermore, they can encapsulate drugs and be used in an injectable form to replace surgery, to repair damaged cartilage, in regenerative medicine, or in tissue engineering. Also, they have potential use in gene therapy.

Significant advances have been made in the field of hydrogels as intelligent and functional materials. Their application in the biomedical field has been inherently hidden by the toxicity of cross-linking agents. Emerging knowledge in the field of chemistry, as well as the proper understanding of biological processes, has led to the rational use of hydrogels as versatile materials and as matrices helping in minimally invasive therapies. Today, hydrogels appear to have tremendously promising application potentials. However, a number of challenges remain for clinical translation.

This book provides an overview of multidisciplinary hydrogel research and the wider applicability of hydrogels.

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Chapter 1

Introductory Chapter: Hydrogels - From First Natural Hydrocolloids to Smart Biomaterials

Lăcrămioara Popa, Mihaela Violeta Ghica and Cristina Elena Dinu-Pîrvu

1. Introduction

Hydrogels are part of our everyday life. They are components of our food [1–4], our everyday objects [5–7], but most importantly, they are valuable intermediaries in the most innovative and unexpected attempts to cure, to reduce the effects of the diseases, and to regenerate, in a single word—to heal [8–11].

Hydrogels are known as the first biomaterials useful for therapy in humans [12], but they are still fascinating materials and subject for developing innovative formulations and applications [13–15]. They have unique properties derived from its three-dimensional (3D) viscoelastic network [16], essentially permitting attachment and later diffusion of particles, molecules, in controlled drug or cell/gene delivery [17, 18], as well as serving as 3D bioprinting material [19, 20], in modern medicine, for tissue engineering [21, 22], as implants [23], for diagnostics [24], wound dressing [25], bone regeneration [26, 27], and soft contact lens [28], to exemplify only a few.

Today, modern therapy gives a great value to tissue engineering and regenerative medicine (TERM) in various disease treatments [29]. In TERM, a great number of biomaterials are developed, and among them, hydrogels and scaffolds are occupying important places. The interest of the researchers in this subject is enormously expanding.

This book comes to give a small overview for multidisciplinary hydrogel research and widen applicability. That is the reason why the book opens with a board overview of the hydrogel applications in drug delivery over last 10 years.

The chapters of the book were majorly focused on the latest and emergent fields of interest: superabsorbent hydrogels, natural hydrogels based on chitosan, and a clinical grade hydrogel platform for drugs or cell/gene delivery, with potential-derived future organoid culture or bioprinting applications.

An innovated class of recent generation of hydrogels includes superabsorbent hydrogels, and among them, cellulose-based superabsorbent hydrogels are important representative, due to a large availability of cellulose, it being environmental friendly, and its biocompatibility. It is largely presented in one of the chapters of the book. It is noted as smart materials, displaying stimuli-sensitive responsiveness to specific environmental cues.

Among the natural hydrogels, those based on chitosan and chitosan derivatives are described in another important chapter, with their biomedical application. Chitosan, as natural hydrophilic polymer, presents important deal of interest for hydrogel structures due to its biocompatibility and biodegradability. As biological

devices, chitosan-based hydrogels are potentially engineering scaffolds to obtain tissue repair.

A hydrogel platform, designed and obtained at clinic grade, and able to overcome problem of stability of small molecules of drugs, proteins, or cells co-packaged with the hydrogel matrix, is detailed in another important chapter of the book. HyStem® hydrogels are addressed to this issue and solve the problem, mixing the matrix with the active components at the point of administration. It is open the road for incorporating of therapeutic grows factors, antibodies or cells, and by their flexibility, HyStem® hydrogels become a basis for a new generation of therapeutics: patient-derived organoid culture in order to novel drug design, as well as for bioprinting to new organs manufacture.

Significant advances have been made in the field of hydrogels as intelligent and functional materials. Their application in the biomedical field has been inherently hidden by the toxicity of crosslinking agents. Emerging knowledge in the field of chemistry, as well as the proper understanding of biological processes, has led to the rational use of hydrogels as versatile materials, hydrogel matrices helping to minimize invasive therapies, and nowadays, hydrogels appear to have tremendous promising application potentials [30]. However, there are still a number of challenges for clinical translation.

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Chapter 2

Stimuli-Responsive Hydrogels: An Interdisciplinary Overview

Sudipta Chatterjee and Patrick Chi-leung Hui

Abstract

Stimuli-responsive hydrogels formed by various natural and synthetic polymers are capable of showing distinctive changes in their properties with external stimuli like temperature, pH, light, ionic changes, and redox potential. Some hydrogels are developed to exhibit dual responsiveness with external stimuli such as pH and temperature. The stimuli-responsive hydrogels find a wide variety of biomedical applications including drug delivery, gene delivery, and tissue regeneration. The advanced functionalities can be imparted to textile materials by integrating stimuli-responsive hydrogels into them and stimuli-responsive hydrogels including thermoresponsive, pH-responsive, and dual-responsive improve moisture and water retention property, environmental responsiveness, esthetic appeal, display, and comfort of textiles. Stimuli-responsive hydrogels loaded with various kinds of drugs are applied for textile-based transdermal therapy as these hydrogels as drug carriers show controlled and sustained drug release. In this chapter, drug delivery and textile applications of thermoresponsive, pH-responsive, and dual-responsive (pH and temperature) hydrogels are discussed and analyzed.

Keywords: stimuli-responsive, hydrogel, thermoresponsive, pH-responsive, dual-responsive, textile, drug delivery, transdermal therapy

1. Introduction

Hydrogels are three-dimensional polymeric networks of hydrophilic polymers, and the network structure of hydrogel formed by natural or synthetic polymers is capable of holding a large amount of water in it [1, 2]. Hydrogels show the ability to swell and hold a significant fraction of water within its structures without being dissolved in it [3]. The amount of water in the hydrogel, typically in the swollen state depends on the nature of polymer and also on the polymeric network structure [4]. The hydrophilic functional groups attached to the polymeric backbone of hydrogels impart ability to hold water in its structure, and dissolution in water is resisted because of cross-linking polymeric network structures [5]. Physical hydrogels are "ionotropic" reversible hydrogels showing disintegration by changes in the external environmental conditions such as ionic strength, pH, and temperature [6]. Physical hydrogels are formed by the interaction between oppositely charged polyelectrolytes or oppositely charged multivalent ion/surfactant and polyelectrolyte [7]. Chemical hydrogels are formed from covalently cross-linked polymeric network having permanent junctions [8]. Hydrogels are capable of swelling and shrinking reversibly in response to changes in the external environment [9]. Homo-polymeric hydrogels are made of only polymer, whereas copolymeric or multi-polymeric hydrogels are

made of two or more polymers [1]. Hydrogels can be modified in terms of chemical structure, composition, biological functions, biodegradability, and various physicochemical properties such as mechanical and rheological, spectral, and pH stability, and release and loading properties for drugs can be managed to optimize the performances of the hydrogels in multiple dimensions especially for biomedical applications [4, 10, 11]. Hydrogels find a wide range of pharmaceutical and biomedical applications due to their resemblance with the physical properties of living tissues such as high water content, compactness, and low interfacial tension with aqueous media [12–14]. The compactness of hydrogels in aqueous media is maintained by physical cross-linking (e.g., entanglements, crystallites) and chemical cross-linking [15]. Hydrogels are found in the form of matrix, film, microsphere, and nanoparticles depending on the processing parameters of hydrogels [16, 17]. Nowadays, hydrogels are being practically applied for drug delivery, tissue engineering, self-healing process of the body and also as biosensors and hemostasis bandages [18–22]. Hydrogels are being used for developing drug delivery systems as they are possessing good transport properties for drugs and capable of protecting drugs from the external environment, and modifications on the gel structures can be easily introduced according to the route of administration [2].

During the last few decades, a significant amount of research has been performed to develop hydrogels with stimuli-responsive properties where external triggers like temperature, pH, light, magnetic and electrical fields, shear forces, and chemicals cause some changes in the properties of the hydrogel materials such as swelling, porosity, physical structure, and modulus [23–25]. Stimuli-responsive hydrogels are capable of showing switchable sol-gel transition upon application of external triggers. The external stimuli including temperature, light, magnetic and electrical fields, and ultrasonic wave are considered physical triggers, while pH, redox reactions are considered chemical triggers [26]. This stimuli-responsive behavior of hydrogels has opened immense possibilities of extremely diversified applications in biomedical areas especially for drug delivery applications [27–30]. Thermoresposive hydrogels show changes in mechanical and drug release properties with the change in the temperature of the external environment [31, 32]. The schematic representation of formation of drug-loaded thermoresponsive hydrogel as drug delivery system has been given in **Figure 1**.

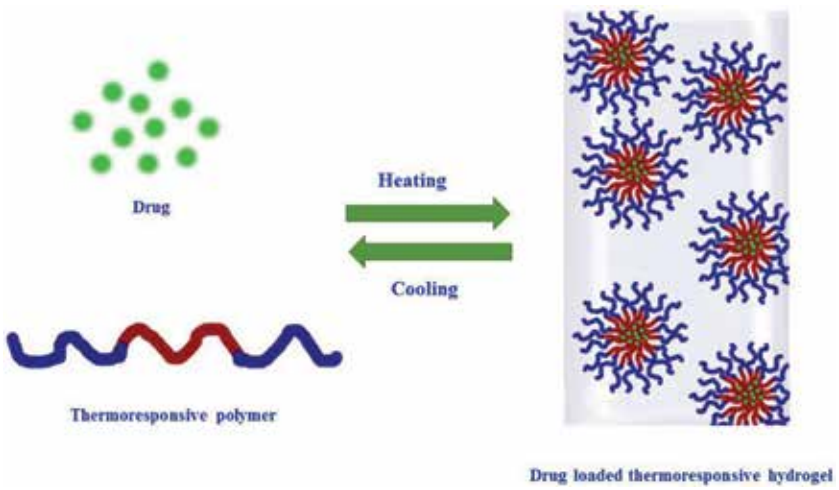

Figure 1.
The schematic representation of thermoresponsive hydrogel formation loaded with drug using temperature as a stimulus.

Thermoresponsive hydrogels are formed above a low critical solution temperature (LCST) where the polymer solutions undergo phase separation to form hydrogels [28, 33]. At temperature below LCST, all the components in the system are completely miscible in all proportions [34]. Polymers with upper critical solution temperature (UCST) get soluble upon heating [35]. The thermoresponsive hydrogels are found to have various hydrophobic groups, and LCST/UCST can be modified by changing the ratio of hydrophilic and hydrophobic groups of the polymers [36, 37]. Thermoresponsive hydrogels are of great interest in the area of biomedical applications especially for drug delivery applications as many hydrogels show gel formation at universally accepted physiological temperature of 37°C, and also, several easy modifications are available to control gel formation at physiological temperature [23, 25, 38–40]. In situ forming hydrogels find biomedical applications as it can provide suitable ways for simple, "custom-made diagnostics" [13]. pH-responsive hydrogels show swelling/shrinking behavior in response to change in the environmental pH [41, 42], and this class of stimuli-responsive hydrogels is of particular interest for biomedical applications as substantial pH changes are found in various organs or locations in the body required for normal body function such as the gastrointestinal tract [43], blood vessels, intracellular vesicles [44], and female genital tract [45]. pH changes within the body also occur due to abnormal body functions or in the diseased state including tumor environment [46] and inflammation [47]. The pH-responsive hydrogel systems have been widely used for developing a wide variety of drug delivery systems [41, 48–50].

Thermoresponsive hydrogels are made from a wide variety of natural and synthetic polymers, and some thermoresponsive hydrogels forming polymers find a lot of interests as these hydrogels have excellent biomedical applications especially for developing drug delivery systems used in cancer therapy, transdermal drug therapy, and oral drug delivery [25, 35, 51, 52]. The thermoresponsive polymers widely used in developing drug delivery systems are poly(*N*-isopropylacrylamide) (pNIPAAm); pluronics® or poloxamers mainly pluronic F127 (PF127), polyoxazoline, and poly(organophosphazenes); and some natural polymers having thermoresponsive properties are gelatin/collagen, cellulose, chitosan, xyloglucan, starch, xanthan gum, carrageenans, hyaluronic acid, dextran, poly(γ-glutamate), and elastin and elastin like polypeptide/oligopeptide [53–55]. The most commonly used polymers to develop pH-responsive behavior in hydrogels include either acidic groups (carboxylic) or basic groups (amino), and the monomers used in pH-responsive polymers are acrylic acid, acrylamide, methacrylic acid, dimethylaminoethyl methacrylate, diethylaminoethyl methacrylate, and ethylene glycol [46, 56]. All pH-responsive polymers contain pendant acidic or basic groups that either accept or donate protons in response to pH change in the external environment [57, 58]. The pendant ionizable groups of anionic hydrogel networks become ionized in solutions at a pH greater than their acid dissociation constant (pKa), and cationic hydrogel networks swell at pH lower than their pKa values as their pendant groups get ionized in that pH. The swelling and shrinking behavior of pH-responsive hydrogels depending on the charge of pH-responsive polymer is schematically presented in **Figure 2**. Natural polymers including chitosan, gelatin, alginate, and albumin can also show pH-responsive behavior [41, 59, 60]. pH-responsive hydrogels made from synthetic and natural polymers are widely used in drug delivery applications [23].

Stimuli-responsive polymers are used as surface modifying systems of textile fabrics to enrich them with advanced functionalities and environmental responsiveness [61]. Thermoresponsive/pH-responsive or any other stimuli-responsive hydrogel present on textile fabric is capable of responding to changes in environmental conditions and giving comfort by actively balancing body temperature and moisture [53]. Stimuli-responsive (thermoresponsive) hydrogels are used as drug

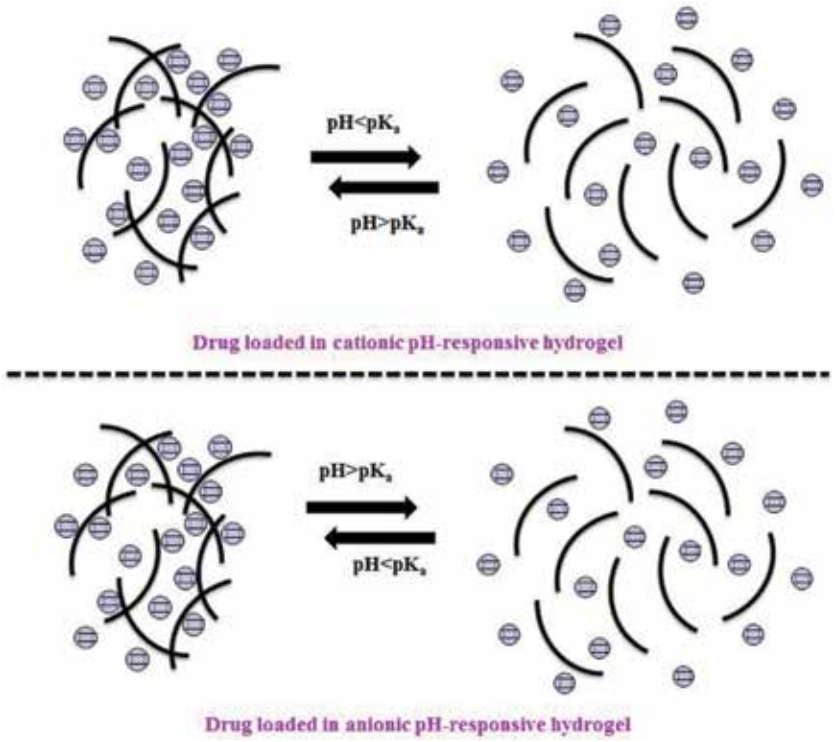

Figure 2.
The schematic representation of swelling/shrinking of pH-responsive hydrogels.

delivery systems for the controlled release of drugs from functionalized textiles applied for skin care [53, 61]. Thereby, drug delivery systems from hydrogels of stimuli-responsive polymers are being developed for textile-based transdermal therapy [61]. The functionalized textiles with stimuli-responsive hydrogels also include esthetic appeal, soft display, wound monitoring, smart wetting properties, and protection against extreme variations in environmental conditions [61].

2. Drug delivery and textile applications of thermoresponsive polymers

A wide variety of natural and synthetic polymers and their derivatives are capable of exhibiting thermoresponsive gelation, and during the last two decades, many drug delivery systems for cancer therapy, tissue regeneration, transdermal drug therapy, and oral drug delivery are developed using thermoresponsive polymers [25, 62]. Thermoresponsive hydrogel-based drug delivery systems have been increasingly gaining attention as thermoresponsive hydrogels can effectively encapsulate drugs and release them in a sustained manner [63]. Also, various methods and easy modifications on both natural and synthetic thermosensitive polymers can be introduced to tailor thermosensitive hydrogel properties in order to achieve the desired drug release profile [64–66]. Several chemical modifications are applied on thermoresponsive polymers to improve stability and drug release properties of the hydrogels [36, 38].

Poly(*N*-isopropylacrylamide) (pNIPAAm) and its copolymers can form thermoresponsive hydrogels which are widely used for developing drug delivery systems with excellent applicabilities and functionalities [67–71]. pNIPAAm shows LCST (32°C) near body temperature in pure water and becomes hydrophobic at the LCST

[72, 73]. pNIPAAm was copolymerized with acrylamide [74] and N-(2-(dimethylamino)ethyl) methacrylamide [75] to work more effectively at body temperature as thermoresponsive hydrogel-based drug delivery systems. The hydrogel made of pNIPAAm and methyl cellulose combined thermoresponsive properties of both materials and LCST of the compound varied with the proportion of constituents forming hydrogel [16]. Also, methyl cellulose addition to pNIPAAm enhanced the mechanical strength of the hydrogel [16]. The thermoresponsive hydrogel system made of pNIPAAm and butyl methacrylate (BuMA) showed a sustained zero-order drug release and showed gelling near body temperature [76]. The thermoresponsive hydrogel made of copolymers of NIPAAm and propylacrylic acid (PAA) by reversible addition-fragmentation chain transfer (RAFT) polymerization method showed tunable properties in a variety of molecular switching and drug delivery applications [77]. This copolymer of NIPAAm and propylacrylic acid (PAA) also showed pH-responsive behavior which is relevant for drug delivery applications [77]. The nanoparticle hydrogel system made of pNIPAAm and a photo cross-linker, poly(ethylene glycol) diacrylate (PEG-DA) showed thermoresponsive hydrogel forming property and was capable of showing in situ photopolymerization to localize at a specific location in the body [78]. The thermoresponsive hydrogel made from copolymer of alginate and pNIPAAm was used as a drug delivery system for anticancer drug doxorubicin [79]. The hydrogel formed from alginate and pNIPAAm showed gel formation at body temperature of 37°C [79]. Thermoresponsive hydrogels made pNIPAAm and poly(ethylene glycol) diacrylate (PEG-DA) were used as ocular drug delivery systems to deliver some bioactive proteins and immunoglobulin G (IgG) [80]. In spite of various drug delivery applications of pNIPAAm-based thermoresponsive hydrogels, there is a doubt on biodegradability of this polymer as it is very relevant to successful and safe drug delivery applications. Nowadays, a wide number of researches are being performed to develop biodegradable copolymers of pNIPAAm. The most promising polymers for biodegradability enhancement of pNIPAAm hydrogels are reported to be poly(ethylene glycol) (PEG) and/or poly(ε-caprolactone) (PCL) [81]. Moreover, the biocompatibility of pNIPAAm-based thermoresponsive hydrogels is mostly achieved by copolymerizing with PEG and/or PCL [81]. A dual-responsive spiropyran-NIPAAm hydrogel (light and temperature) formed by a facile and versatile surface-initiated controlled polymerization method (SI-ARGET-ATRP) showed capability of dimensional changes on cotton fabric upon irradiation with visible light or a temperature stimulus [82]. pNIPAAm-based thermoresponsive hydrogels were applied to develop smart functionalized textiles [61]. The dual-responsive nano-hydrogel made of pNIPAAm and chitosan was applied on cotton fabric as a surface modifying system using 4-butane tetra carboxylic acid (BTCA) as an environmental friendly cross-linking agent [83]. The use of nano-hydrogel on cotton fabric enhanced its water retention capacity [83]. Thermoresponsive hydrogel made of pNIPAAm and polyurethane hydrogel via chitosan modification exhibited antibacterial function against *Staphylococcus aureus* and *Escherichia coli* on nonwoven fabric [84]. pNIPAAm-based thermoresponsive hydrogel applied on fabrics can exhibit reversible swelling/shrinkage and modify water absorption/retention capacity [61].

Another important thermoresponsive polymer pluronic F127 (PF127) is capable of forming hydrogel near body temperature, and PF127 is a triblock copolymer of poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO) [25, 85]. In order to enhance applicability and functionality of PF127-based drug delivery systems, various chemical modifications were done on PF127 [86–88]. The thermoresponsive hydrogel made from PF127 and glycol chitosan was used as drug delivery system for doxorubicin and partially for superoxide dismutase (SOD) [89].

Thermoresponsive hydrogel made from PF127 and hyaluronic acid was used as drug delivery system for human growth hormone [90]. The conjugate was made by photopolymerization, and it formed hydrogel near body temperature [90]. The thermoresponsive hydrogel made from PF127, polyurethane, and Erythrosin B was used as drug carriers and fluorescence imaging probes in biomedical applications as well [91]. The hydrogel also showed pH responsiveness [91]. The thermoresponsive hydrogel microspheres from PF-127 and chitosan cross-linked with glutaraldehyde as a cross-linker were applied for delivery of anticancer drug 5-fluorouracil [92]. Thermoresponsive hydrogels made from PF127-based were used for textile-based transdermal drug delivery [53, 93]. Chinese herbal medicine-loaded PF127-based thermoresponsive hydrogels were applied for textile-based transdermal therapy [94, 95]. The drug-loaded hydrogels developed by a "cold method" were capable of moisturizing the skin and protecting it against the pathogenesis of atopic dermatitis [94, 95]. Thermoresponsive hydrogel system from PF127 and alginate was applied for transdermal delivery of selegiline and the thermoresponsive hydrogel synthesized either by physical mixing of components or chemical grafting showed sustained and controlled release of selegiline [96].

Thermoresponsive hydrogels based on poly(ethylene oxide)-based diblock/triblock copolymers were successfully applied as drug delivery systems [36]. Thermoresponsive hydrogels made of poly(ethylene oxide)-poly(ε-caprolactone) [PEO-PCL] diblock copolymers [97] and poly(ethylene oxide)-poly(ε-caprolactone)-poly(ethylene oxide) [PEO-PCL-PEO] triblock copolymers [98] were used as drug delivery systems. Thermoresponsive hydrogel from [PEO-PCL-PEO] triblock copolymers were coated on nonwoven textile fabric to develop functionalized textile fabric with moisture management property [99]. The hydrogel showed thermoresponsive property with LCST (34°C) close to body temperature and also exhibited controlled and sustained release of drug [99].

Some natural polymers capable of forming thermoresponsive hydrogels and drug delivery systems formed from biopolymer-based thermoresponsive hydrogels find excellent clinical applications [25]. Methylcellulose, water-soluble cellulose derivative, is capable of forming thermoresponsive hydrogels and forms thermoreversible hydrogel in the temperatures range of 60–80°C [25]. The gelation of methylcellulose involves hydrophobic association of polymer molecules and then their phase separation to form gel [100]. Water-soluble methylcellulose was developed by substituting hydroxyl groups on cellulose with more hydrophobic methyl units, and solubility of methylcellulose in water was affected by the degree of substitution [100]. The copolymer of methylcellulose and pNIPAAm combined the thermogelling properties of both materials, and mechanical strength of the hydrogel was enhanced after combining methylcellulose with NIPAAM [101]. The micelle-based thermoreversible gel system from methylcellulose and PF127 exhibited sustained delivery of docetaxel for more than 30 days which resulted in enhanced anticancer effect of docetaxel compared to the free drug [102]. Thermoresponsive hydrogel made of carboxymethyl cellulose and gelatin-loaded with lidocaine was applied for transdermal drug therapy [103]. Thermoresponsive hydrogel system made of PF127 and carboxymethyl cellulose sodium was used as textile-based transdermal drug delivery system with the Chinese herbal medicine (cortex moutan) for the treatment of atopic dermatitis (AD) [94, 95]. This drug delivery system provided both moisture and drug to the skin protecting pathogenesis of AD [94, 95].

Chitosan produced by the deacetylation of chitin is used to form thermoresponsive hydrogels for biological applications [25]. The commercial

source of chitin is the exoskeleton of shrimp, lobster, and insects, and chitin is converted to more biologically active chitosan using alkali treatment. As chitosan lacks intrinsic thermosensitive properties, thus, other thermosensitive materials need to be introduced into chitosan to make it work as thermoresponsive hydrogels [53]. Chitosan-pluronic (CP) thermoresponsive hydrogel was formed by grafting pluronic onto chitosan using carbodiimide chemistry, and the hydrogel was designed as an injectable cell delivery system for cartilage regeneration [104]. The thermoresponsive hydrogel system using chitosan, hyaluronic acid, and NIPAAm was used as drug delivery system for the analgesic drug nalbuphine, and it showed better controlled release of the drug in vitro than that of hydrogel made of only pNIPAAm hydrogels [105]. Carboxymethyl chitosan-modified pluronic thermoresponsive hydrogel was used for localized delivery of paclitaxel (PTX) [106]. The mechanical strength of the thermoresponsive hydrogel was increased after cross-linking carboxymethyl chitosan with glutaraldehyde, and also the drug delivery system showed sustained drug delivery at the tumor sites [106]. The thermoresponsive hydrogels were made from pNIPAAm and chitosan by interpenetrating polymer network (IPN) technology using a redox initiator system made of potassium peroxydisulfate and sodium hydrogen sulfite [107]. The hydrogel system was applied on cotton fabric using glutaraldehyde as cross-linker to enhance the thermoresponsive behavior and antibacterial activity of cotton fabric [107].

Dextran is a complex-branched glucan made of glucose monomers and synthesized from sucrose by bacterial fermentation using lactic acid bacteria *Leuconostoc mesenteroides* and *Streptococcus mutans*. Thermoresponsive hydrogel system from poly(ε-lysine)-grafted dextran and α-cyclodextrins showed also pH-responsive character and could be used as drug delivery system [108]. The block polymers consisting of dextran, 2-hydroxyethyl methacrylate, oligolactate, and NIPAAm were capable of forming thermoresponsive and completely biodegradable hydrogels which showed LCST near body temperature (around 32°C) and exhibited controlled release of incorporated albumin at environmental temperatures [109]. The multifunctional and biodegradable thermoresponsive hydrogels made from NIPAAm, dextran, and poly(L-lactic acid) were used as drug delivery systems, and it showed gelation (LCST) around 32°C [110].

Xyloglucan, a major component of higher plant cell wall is commercially obtained from tamarind seed (*Tamarindus indica*) and made of a β-1,4 linked D-glucan backbone where α-D xylopyranose residues are partially substituted on *O*-6 position of glucopyranosyl residues. The thermoresponsive behavior of xyloglucan arises after elimination of 35% of its galactose residues, and thermoresponsive hydrogels of xyloglucans have been studied for drug delivery applications [25]. Xyloglucan (3%wt.) in aqueous media after gelation showed three-dimensional macroporous interconnected network with an elastic modulus which was significantly higher than other natural or synthetic hydrogels [111]. β-galactosidase-treated xyloglucan after being partially degraded (45% of galactose residues removed) formed thermoresponsive hydrogel at 27°C to work as drug delivery system for nasal drugs [111]. Xyloglucan-based thermoresponsive hydrogel was developed for delivery of lidocaine HCl in the treatment of periodontosis, and this in situ hydrogel-loaded with analgesic drug offered an alternative to painful injection therapy of anesthesia during dental surgery [112].

The chemical nature and biomedical applications of thermoresponsive hydrogels are briefly summarized in **Table 1**.

Chemical constituents of thermoresponsive hydrogel	Biomedical application of thermoresponsive hydrogel	References
pNIPAAm, butyl methacrylate	Drug delivery application	[76]
NIPAAm, propylacrylic acid	Drug delivery application	[77]
pNIPAAm, polyurethane, chitosan	Textile application (antibacterial)	[84]
PF127, glycol chitosan	Drug delivery application	[89]
PF127, hyaluronic acid	Drug delivery application	[90]
PF127, carboxymethyl cellulose sodium	Textile application (drug delivery and moisture management)	[94, 95]
PEO, PCL	Drug delivery application	[97, 98]
PEO, PCL	Textile application (moisture management)	[99]
Methylcellulose, PF127	Drug delivery application	[101, 102]
Carboxymethyl cellulose, gelatin	Drug delivery application	[103]
Chitosan, pluronic	Drug delivery application	[104]
Chitosan, PF127	Textile application (antibacterial)	[107]
Dextran, poly(ε-lysine, α-cyclodextrin	Drug delivery application	[108]
Xyloglucan	Drug delivery application	[112]

Table 1.
The chemical nature and biomedical applications of thermoresponsive hydrogels.

3. Drug delivery and textile applications of pH-responsive polymers

Anionic hydrogel network formed by polyacrylic acid (PAA) was applied as pH-responsive hydrogel system as it swelled/dissolved at high pH of the upper small intestine of the gastrointestinal (GI) tract but resisted any swelling or remained collapsed while in the acidic pH of the stomach, and thereby, as a drug delivery system, it protected loaded drug from any harsh acidic environment in the stomach [43, 113]. PAA-based pH-responsive biodegradable hydrogels were developed from four types of pH-sensitive PAA derivatives and a biodegradable poly(l-glutamic acid) cross-linker and applied as drug delivery systems for oral delivery of insulin [114]. The wound healing monitoring textiles were developed from pH-responsive hydrogel of polyvinyl acetate cross-linked PAA, and the swelling of the pH-responsive hydrogel resulted in a refractive index change of the hydrogel providing information on the stage of wound healing process [115].

Albumin is a natural protein harvested directly from the human blood plasma, and hydrogels developed from serum albumin are widely applied for drug delivery applications [26]. Albumin formed pH-responsive anionic hydrogel network which swelled in basic pH medium, and pH responsiveness of albumin was developed through reductive reaction followed by oxidative refolding [116]. Furthermore, the albumin hydrogel also showed redox responsiveness. An increase in albumin concentration in the hydrogel enhanced its mechanical and structural stability and improved biodegradability and biocompatibility [116]. The use of the hydrogel as drug delivery system for tetracycline showed its usefulness in drug delivery applications [116]. pH-responsive hydrogel based on bovine serum albumin (BSA) derivative was applied for oral drug delivery, and free radical polymerization technique was applied to develop methacrylate derivatized BSA [117]. Medical textiles for wound healing included coating of BSA hydrogels on textile materials [118].

Poly(ethylene glycol) (PEG) is a highly water-soluble nonionic polymer and widely used in drug delivery applications because of its biocompatibility and low toxicity. The pH-responsive hydrogel made of PEG derivative and α,β-polyaspartylhydrazide loaded with doxorubicin was applied for cancer therapy [46]. It remained as free-flowing fluid before injection but spontaneously changed into a semisolid hydrogel just after injecting into the body [46]. The prepared hydrogel was biocompatible and biodegradable and utilized as a pH-responsive vector for drug delivery [46]. pH-responsive hydrogels made of poly(itaconic acid) with PEG were applied as drug delivery system for oral drug delivery and the hydrogels were prepared by UV-initiated free radical polymerization using tetraethylene glycol as the cross-linking agent and Irgacure 2959 as the initiator [119]. Medical textiles developed from coating pH-responsive hydrogel made of PEG and chitosan on cotton membrane were applied for wound dressing [120].

pH-responsive cationic hydrogels using polymers like poly(dimethylaminoethyl methacrylate) (PDMAEMA) and poly(diethylaminoethyl methacrylate) (PDEAEMA) show swelling in low pH due to the protonation of their tertiary amine groups [121]. Because of pH responsiveness and ability to bind with anionic hydrogels, cationic hydrogels find a wide variety of biomedical application especially for drug delivery [122, 123]. pH-responsive hydrogel-based drug delivery system from poly(vinyl alcohol) and PDMAEMA showed promising drug delivery application, and the molecular weight of PDMAEMA was reported to have significant effect on the structure, swelling ratio, and drug release behaviors of the hydrogels at different pH conditions [124]. A pH-responsive nano-hydrogel was synthesized by copolymerization of PDEAEMA with hetero-bifunctional PEG bearing a 4-vinylbenzyl group at one end and a carboxylic acid group at the other end, and nano-hydrogel was found suitable for endosomal release of anticancer drug doxorubicin [125]. The doxorubicin-loaded nano-hydrogel showed much higher drug in pH 5.3 than that in pH 7.4 [125]. Medical textiles were developed by grafting PDMAEMA onto the cotton surface for low-adherent wound dressing [126].

Chitosan is an excellent example of pH-responsive natural polymer having antibacterial activity, biocompatibility, and biodegradability and a wide range of good biological activities [53]. The pH-responsive behavior of chitosan comes from its primary amine groups which can be protonated/deprotonated depending on pH of the external environment (solutions) [127]. Chitosan forms cationic hydrogel network in water which swells in acidic pH and remains collapsed in basic pH, and thereby, the pH-responsive behavior of chitosan-based hydrogels can be controlled for targeted gastrointestinal delivery of a variety of drugs [23]. pH-responsive hydrogel formed from chitosan and poly(ethylene oxide) was used for oral delivery of antibiotics metronidazole and amoxicillin [128]. The hydrogel network swelled more in simulated gastric fluid than simulated intestinal fluid, and also, the drugs were release more from the hydrogel in gastric pH condition than intestinal pH condition [128]. Physically cross-linked pH-responsive hydrogel with enhanced mechanical strength was developed from chitosan, acrylic acid, (2-dimethylamino) ethyl methacrylate via in situ free radical polymerization for controlled drug delivery of bovine serum albumin, and 5-fluorouracil in cancer therapy [129]. The potential drug carrier from pH-responsive hydrogel of carboxymethyl chitosan and PEG was developed using photo-induced synthesis, and the release of 5-fluorouracil from the hydrogel was investigated [130]. Smart textile fabrics and medical textiles were developed by integrating pH-responsive chitosan hydrogels onto fabrics [131–133].

The chemical nature and biomedical applications of pH-responsive hydrogels are briefly summarized in **Table 2**.

Chemical constituents of pH-responsive hydrogels	Charge of pH-responsive hydrogel	Biomedical application of pH-responsive hydrogel	References
PAA, poly(l-glutamic acid)	Anionic	Drug delivery application	[114]
PAA, polyvinyl acetate	Anionic	Textile application (wound healing monitoring)	[115]
Albumin	Anionic	Drug delivery application	[116]
BSA, methacrylate	Anionic	Drug delivery application	[117]
BSA	Anionic	Textile application (medical textiles)	[118]
PDMAEMA, poly(vinyl alcohol)	Cationic	Drug delivery application	[124]
PDMAEMA	Cationic	Textile application (medical textiles)	[126]
Chitosan, poly(ethylene oxide)	Cationic	Drug delivery application	[128]
Carboxymethyl chitosan, PEG	Cationic	Drug delivery application	[130]
Chitosan, PEG	Cationic	Textile application (medical textiles)	[120]
Chitosan	Cationic	Textile application (medical textiles)	[131, 132]

Table 2.
The chemical nature and biomedical applications of pH-responsive hydrogels.

4. Drug delivery and textile applications of dual-responsive (pH and temperature) hydrogels

The hydrogel system combined both pH-responsive polymer and thermoresponsive polymer-enhanced efficiency of stimuli-responsive hydrogels for drug delivery applications [134]. The swelling behavior of dual-responsive (pH and temperature) hydrogel has been schematically represented in **Figure 3**.

Natural polymer like chitosan was used as pH-responsive polymer to combine with thermoresponsive synthetic polymer pNIPAAm in order to create dual-responsive (pH and temperature) hydrogel-based drug delivery systems [135–137]. Dual-responsive hydrogels based on glycidyl methacrylated chitosan and pNIPAAm via photopolymerization were used as drug delivery systems for

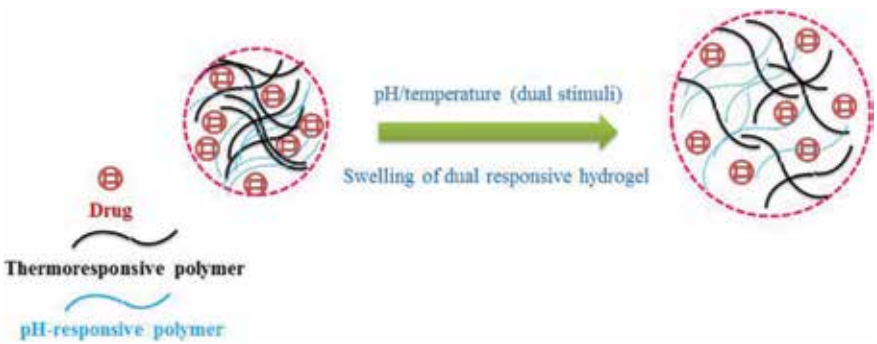

Figure 3.
The schematic representation of swelling/shrinking of dual-responsive (pH and temperature) hydrogels.

acid orange 8 (AO8) and 5-fluorouracil (5-Fu), and the hydrogels showed response to both temperature and pH as external stimuli [135]. Chitosan and pNIPAAm formed dual (pH/temperature)-responsive hydrogel network with semi-interpenetrating polymeric network via radical-induced polymerization of NIPAAm in the presence of chitosan using tetraethyleneglycoldiacrylate as a cross-linker, and this dual-responsive hydrogel was used as drug delivery system for pilocarpine hydrochloride [136]. Dual-responsive hydrogels based on pH-responsive chitosan and temperature responsive pNIPAAm were applied on textile fabrics (cotton fabrics) to modify their surface properties, and these functionalized textiles showed advanced functionalities and environmental responsiveness [83, 138, 139]. Surface modification of cotton fabric with pH and temperature dual-responsive hydrogels of chitosan and pNIPAAm improved air and moisture management activities of functionalized textiles [83].

Dual-responsive hydrogels made of pNIPAAm as thermoresponsive polymer and PAA as pH-responsive polymer found drug delivery applications [77, 140]. The hydrogel system made from copolymer of NIPAAm and itaconic acid [NIPAAm-*co*-itaconic acid] showed dual responsiveness to external stimuli temperature and pH and was proposed as effective drug delivery system [141]. Dual hydrogel system made of pNIPAAm and PDMAEMA by the combination of atom transfer radical polymerization, reversible addition-fragmentation chain transfer polymerization, and click chemistry showed dual responsiveness for temperature and pH, and this dual-responsive hydrogel was used as drug delivery system for ceftriaxone sodium [142, 143]. Dual-responsive biodegradable hydrogel made from thermoresponsive copolymer p(NIPAAm-*co*-hydroxyethyl methacrylate) and pH-responsive poly(L-glutamic acid) was applied as drug delivery system for hydrophilic drugs [144]. Dual-responsive (pH and temperature) hydrogel system was developed using thermoresponsive polymer pNIPAAm and cellulose nanofibril isolated by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation and applied as carrier for drug [145]. Dual-responsive (pH and temperate) hydrogels made from synthetic polymers pNIPAAm and vinyl-capped polyurethane were graft copolymerized onto nonwoven cellulose/PET fabric by ammonium persulfate initiation to modify surface properties of textile material [146]. Dual-responsive hydrogels made of pNIPAAm and polyurethane were grafted onto nonwoven fabric for modifying surface property of textile materials [147].

Chitosan-coated alginate hydrogel beads with pNIPAAm showed pH and temperature dual responsiveness and were applied as drug delivery system with improved encapsulation efficiency and sustained drug release property [148]. The hydrogel consisting of sugarcane bagasse cellulose, carboxymethyl cellulose, and pNIPAAm was applied as a dual-responsive (pH and temperature) drug carrier for BSA, and the drug carrier system showed sustained release of drug [149]. Cellulose-based dual-responsive (pH and temperature) hydrogel was prepared from carboxymethyl cellulose and hydroxyethyl cellulose in an aqueous medium using citric acid (CA) as a cross-linking agent and applied on knitted cotton fabric to modify its surface properties [150]. Chitosan with other biopolymers including β-cyclodextrin (β-CD), arabic gum, guar gum, and pullulan formed four different types of dual-responsive (pH and temperature) hydrogels using glycidoxypropyltrimethoxysilan as a cross-linker, and depending on the nature of biopolymers used in hydrogels, the texture of the hydrogels varied [151]. All the varieties of hydrogels were applied on textile fabrics to modify surface properties like antibacterial, water uptake, and moisture retention, and the hydrogels imparted hydrophobicity to the cotton fabric [151].

The chemical nature and biomedical applications of dual-responsive (pH and temperature) hydrogels are briefly summarized in **Table 3**.

Chemical constituents of dual-responsive hydrogel	Biomedical application of dual-responsive hydrogel	References
pNIPAAm (*temperature*), glycidyl methacrylated chitosan (*pH*)	Drug delivery application	[135]
pNIPAAm (*temperature*), chitosan (*pH*)	Drug delivery application	[136]
pNIPAAm (*temperature*), chitosan (*pH*)	Textile application (water and moisture management)	[138, 139]
pNIPAAm (*temperature*), PAA (*pH*)	Drug delivery application	[77, 140]
pNIPAAm (*temperature*), PDMAEMA (*pH*)	Drug delivery application	[142, 143]
p(NIPAAm-*co*-hydroxyethyl methacrylate) (*temperature*), poly(L-glutamic acid) (*pH*)	Drug delivery application	[144]
pNIPAAm (*temperature*), vinyl-capped polyurethane (*pH*)	Drug delivery application	[146]
pNIPAAm (*temperature*), polyurethane (*pH*)	Textile application	[147]

Table 3.
The chemical nature and biomedical applications of dual-responsive (pH and temperature) hydrogels.

5. Conclusions

Stimuli-responsive hydrogels from a wide variety of natural and synthetic polymers provide a significant contribution in biomedical area especially for drug delivery applications. Over the last 10 years, the potential applications of stimuli-responsive hydrogels in textiles are rapidly advancing. Functionalized textiles integrated with stimuli-responsive hydrogels show improved moisture/temperature management, esthetic appeal, soft display, and enhanced protection against extreme environmental conditions. Stimuli-responsive hydrogels used in textiles are mainly thermoresponsive, pH-responsive, and furthermore dual-responsive (temperature and pH) in nature, and specific drug-loaded stimuli-responsive hydrogels are being applied for textile-based transdermal therapy. The polymers used in thermoresponsive hydrogels vary from synthetic polymers to nature polymers, and often, composites are developed for better functionalities. pH-responsive hydrogels include natural to synthetic polymers, and depending on the charge of polymers, the charge of pH-responsive hydrogel varies. For site-specific delivery of drug by pH-responsive hydrogels, the charge of hydrogels plays a significant role. Dual-responsive hydrogel includes both thermoresponsive polymer and pH-responsive polymer to show response to external pH and temperature changes. With the rapid development of dual responsive (pH/temperature) hydrogels, and their applications in textiles as drug delivery systems will develop smart textiles in near future with more functionalities.

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Chapter 3

Investigating the Structure-Related Properties of Cellulose-Based Superabsorbent Hydrogels

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Abstract

Superabsorbent hydrogels are macromolecular networks able to absorb and retain large amounts of water solutions within their fine mesh-like structure. More importantly, they are capable of multiple swelling/shrinking transitions in response to specific environmental cues (*e.g.*, pH, ionic strength, temperature, presence of given electrolytes), thus exhibiting a stimuli-sensitive behavior, which makes them appealing for the design of smart devices in a number of technological fields. In particular, in the last two decades, cellulose-based superabsorbent hydrogels have proven to be an environmentally friendly and cost-effective alternative to acrylamide-based products. This chapter reviews the relationship between the molecular structure of cellulose-based hydrogels and their physicochemical properties. First, the network formation through the use of different cellulose derivatives and chemical or physical crosslinking agents is presented. Successively, the smart swelling capability of the hydrogels as a function of composition and structure is thoroughly discussed. Finally, several approaches to the hydrogel characterization are reviewed, with focus on the assessment of key mechanical, thermal and morphological properties.

Keywords: smart hydrogels, cellulose, characterization

1. Introduction

A superabsorbent hydrogel is defined as a three-dimensional (3D) matrix formed by hydrophilic polymers in linear or branched configuration and showing the ability to absorb large quantities of water or biological fluids (usually more than 100 grams of water per gram of dry polymer) [1].

The main property of superabsorbent hydrogels is the capability to preserve the stability of their network structure, even in the swollen state and in different media and environments. This feature is the result of the presence of crosslinking nodes [2], which can be induced through two main pathways, chemical and physical crosslinking. The former allows obtaining irreversible covalent bonds among the polymeric chains, *e.g.*, by radical polymerization, reaction of complementary groups, grafting reactions and enzymatic reactions [3]. The latter, instead, leads to the formation of reversible hydrogels, meaning that the matrix can be destroyed in specific environments, since the polymeric chains are held together only by physical

interactions, such as electrostatic attractions, entanglements, Van der Waals forces and hydrogen (H)-bonds [4].

The method adopted for crosslinking, either chemical or physical, also influences some key network properties (*i.e.*, water uptake capacity, swelling kinetics, mechanical and rheological properties, degradation rate, porosity, toxicity) and, consequently, the potential use of superabsorbent hydrogels in different fields [5]. Applicative sectors include, but are not limited to, agriculture, horticulture, hygienic products, wastewater treatment, water blocking tapes and tissue engineering [6, 7]. Therefore, depending on the intended use, the synthesis of hydrogels must be tailored to obtain materials that exhibit the desired responses, *e.g.*, fast swelling, degradability, porosity, *etc*.

Recently, particular focus has been placed on the production of novel superabsorbent hydrogels based on natural polysaccharides such as cellulose, starch and chitosan [8, 9], due to the low cost, biodegradability, availability and renewability of these raw materials. Compared to synthetic polymers (*e.g.*, polyacrylates), polysaccharides allow increasing the biocompatibility, biodegradability and water holding capacity of superabsorbent hydrogels, while decreasing their potential toxicity.

Being susceptible to degradation by microorganisms [10] and by chemical or physical stimuli [11], polysaccharide-based superabsorbent hydrogels are particularly suitable for use in soils as fully biodegradable systems for the controlled release of nutrients. However, although biodegradability permits to avoid the contamination of soils by chemicals, it may still represent a drawback in case of too early degradation, *i.e.*, when the nutrients inside the hydrogel matrix are not released as slowly as would be desired [12]. In fact, the total amount of nutrients should be released in a rate compatible with the plant necessity and occurring during the degradation time of the hydrogel matrix.

The most abundant polysaccharide in nature is cellulose, which has been the subject of academic and industrial studies for many years [13–15]. Although plant cellulose requires several purification steps to eliminate or reduce contaminants (*e.g.*, lignin and pectin), its large availability and low cost make it the preferred choice for the industrial-scale production of cellulose-based materials, including superabsorbent hydrogels. Conversely, the synthesis of cellulose by bacteria, such as *Acetobacter xylinum* and *Acanthamoeba castellanii*, yields a pure product but still on a laboratory scale, unsuitable for industrial uses. An additional source of cellulose may also be algae, *i.e.*, *Valonia ventricosa*, which provide highly crystalline material useful for studying polymorphs of the polymers [16].

In general, the strong hydrogen bonding (both intermolecular and intramolecular) among the hydroxyl groups along the cellulose backbone not only limits the water solubility, but also leads to the poor reactivity of cellulose. For this reason, great interest has been directed to the use of cellulose derivatives, also termed cellulosics, such as ethyl cellulose, propyl cellulose and carboxymethylcellulose (CMC) [16]. In particular, sodium carboxymethylcellulose (CMCNa) is one of the most important water-soluble derivatives currently used, produced by chemical reaction between cellulose and monochloroacetic acid (MCA) in the presence of sodium hydroxide. CMC is widely applied as an additive in a variety of industrial sectors. Examples of products where CMC is used are detergents, oil drilling muds and wall paper glues, while high purity CMC grades are found in pharmaceuticals, tooth paste, cosmetics, food, *etc*.

With specific regard to agricultural applications and the need to control the hydrogel degradation in the soil, some studies reported the possibility to use binary systems based on two cellulose derivatives, *e.g.*, carboxymethyl and hydroxyethyl cellulose (CMCNa/HEC). Such binary systems have been shown to delay nitrogen release [17], improve soil moisture, reduce the use of water, and alleviate

environmental hazards caused by excessive fertilization. As an example, Sannino et al. have recently developed cellulose-based superabsorbent hydrogels [18] with sorption properties similar to those showed by conventional acrylate-based products, by crosslinking CMCNa and HEC in water solution with either divinylsulfone (DVS) or a water-soluble carbodiimide. Furthermore, the possibility to use a component of lemon juice, *i.e.*, citric acid (CA), as a natural cross-linker has been investigated [19]. In this case, the CA crosslinking of CMCNa and HEC, occurring at high temperature by means of an anhydride intermediate, allows to overcome the typical toxicity of chemical crosslinkers and to increase the hydrophilicity and roughness surface of the cellulose hydrogels, thus extending their potential to biomedical applications, including tissue engineering [20].

In this chapter, after a brief overview of the inherent features of cellulose and cellulosics, the synthesis and characterization of cellulose-based hydrogels are discussed, with specific regard to CMCNa/HEC hydrogels, which represent a successful example of 'green' and biocompatible superabsorbents. The focus of the chapter is on the modulation of the physicochemical properties of the hydrogels in relation to their intended use, via changes to the molecular structure.

2. Cellulose backbone properties

The chemical and physical properties of cellulose and cellulosics can be properly interpreted only after acquiring a deep knowledge of the cellulose molecule, in terms of chemical description, structure and morphology [21]. Generally, when approaching the study of macromolecules especially for the creation of crosslinked structures, three structural levels must be identified. The first one is the molecular level, which regards the single polymer chain and is characterized by the following structural parameters: chemical constitution, molecular mass and molecular mass distribution, reactive sites and intramolecular interactions. The second structural level to be identified is the supramolecular level, which accounts for the interactions (*i.e.*, packing, ordering) among macromolecules to form increasingly larger structures, such as elementary crystals, fibrils and fibers. Key structural parameters at the supramolecular level include the crystallinity degree, the degree of order within and around fibrils and the fibrillar orientation with respect to the fiber axis. Finally, the third level is the morphological one, dealing with complex structural entities formed by the macromolecules, *e.g.*, the distinct cell wall layers in native cellulose fibers and the presence of interfibrillar voids.

As highlighted in the following, the molecular and supramolecular levels are those of primary interest for the understanding of the structure-related properties of cellulose and its derivatives.

2.1 Molecular structure

Cellulose macromolecules are linear and rigid homopolymers consisting of D-anhydroglucopyranose units (AGU), linked together by β-(1 → 4) glycosidic bonds formed between C-1 and C-4 positions of adjacent glucose moieties (**Figure 1**). The degree of polymerization (DP) of cellulose depends on both the cellulose source and the method of isolation/extraction. In general, plant cellulose in its native state has a DP higher than 10,000, which then drops down to 800–3000 following the extraction process [21].

The peculiarity of the glycosidic bond (**Figure 1**) is that the two AGU units joined together to form the repetitive unit of cellobiose are rotated by 180° with respect to each other [21]. The relative stiffness of the cellulose chain is partly

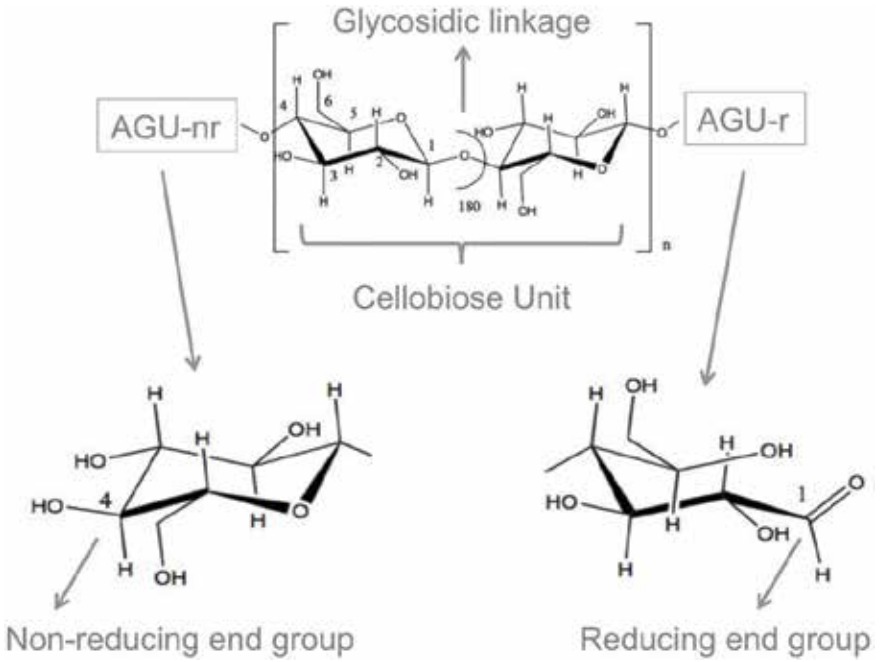

Figure 1.
Molecular structure of cellulose representing the cellobiose unit with its reducing and non-reducing end groups.

attributable to the steric constraints of the β-glycosidic linkage, as well as to the chair conformation of the pyranose ring. With focus on the terminal groups at either side of the cellobiose unit, an aldehyde hydroxyl (OH) group with reducing activity is found in C-1 position (**Figure 1**, right), whereas an alcohol-borne, non-reducing OH group is in C-4 position (**Figure 1**, left). Furthermore, both AGU units have three OH groups located at C-2, C-3 and C-6 positions (one primary and two secondary groups), which are mainly responsible for the poor reactivity and solubility of cellulose. Cellulose is indeed insoluble in water (although the β-glycosidic linkage is susceptible to hydrolytic attack), as well as in common organic solvents. This is due to the formation of an extensive intra- and inter-molecular hydrogen-bonding network among the abundant hydroxyl groups on the cellulose backbone. In particular, intramolecular bonding is ascribable to two different interactions, namely the one between the OH group at C-3 and the oxygen of the pyranose ring (first described in the 1960s by Marchessault and Liang), and the one between the C-6 and the C-2 OH groups of neighboring AGUs (reported by Blackwell et al. in 1977 [22, 23]). Intermolecular hydrogen bonding also takes place via the interaction between C-3 and C-6 groups of adjacent cellulose chains. Intramolecular bonds are those that mainly contribute to the stiffness of the cellulose molecule, which results in the high viscosity of cellulose solutions and the strong tendency of cellulose to crystallize and form fibrillar strands.

2.2 Functionalization

The hydroxyl groups located on the cellulose backbone (**Figure 1**) can be exploited for chemical modification to introduce various functionalities, with the aim of improving the solubility and the reactivity of cellulose. As aforementioned, a number of cellulose derivatives, which display a wide range of solubility in water and water solutions (in presence of alcohol or strong acids or bases), are commercially available in various industrial fields and used for a number of applications,

including coatings, laminations, optical films, absorbents, additives in building materials and also in pharmaceutical, food and cosmetic products [24–26]. Among these derivatives, the most important ones are esters and ethers (*e.g.*, cellulose acetate, methylcellulose, carboxymethyl cellulose and ethyl and hydroxyethyl cellulose), which are prepared on large-scale production by heterogeneous slurry reactions. These reactions most likely result in a random distribution of the substituents along the cellulose backbone. The degree of heterogeneity is determined by the deviations between experimentally measured substituent distributions in the monomers and ideal distributions, which are calculated under the assumption that the samples are prepared in ideal homogeneous reaction conditions [27].

With specific regard to carboxymethyl cellulose (CMC), which is widely used for the synthesis of superabsorbent hydrogels, the average degree of substitution (DS) and degree of polymerization (DP) of available CMC may vary considerably, although DS is generally between 0.5 and 1.5 [28]. In order to control the distribution of the functional groups in CMC, several synthetic pathways have been developed. In this regard, one pathway, developed by Fink et al. [29], includes stepwise etherification using aqueous NaOH solutions at low concentrations. Carboxymethylation is, thereby, primarily achieved in the non-crystalline regions of the cellulose structure [30].

In addition to esterification and etherification, periodate oxidation of the glycol group of glucopyranoside results in the formation of two aldehyde groups (dialdehyde cellulose) [31, 32], which are useful for introducing a variety of substituents such as carboxylic acid [33], hydroxyls [34], or imines [35, 36].

2.3 Degradation features

2.3.1 Effect of enzymes

It is well known that cellulose undergoes enzymatic degradation by specific enzymes, like cellulase and β-glucosidase. Direct physical contact between the enzyme and the surface of the cellulose molecules is a preliminary requirement to enzymatic hydrolysis [37–40]. Since cellulose is an insoluble and structurally complex substrate, this contact can be achieved only by diffusion of the enzymes into the cellulose structure [41]. Therefore, the ability of cellulolytic microorganisms (*Bacillus subtilis* and *Bacillus licheniformis* from bacteria, and *Pleurotus ostreatus*, *Pleurotus florida*, and *Trichoderma viride* from fungi) to degrade cellulose largely depends on the physicochemical characteristics of the specific substrate, which affect the diffusion process and, as a result, the degradation rate [42, 43]. The size and surface properties of the cellulose fibrils, as well as the space between microfibrils and cellulose molecules in the amorphous region, are fundamental factors affecting the permeability of the cellulolytic enzymes [44]. The degree of polymerization and the degree of crystallinity of the cellulose molecules are also important structural parameters that limit the rate of enzymatic degradation. The presence of cellulose contaminants (or components with which cellulose is associated) may also affect the degradability of the given substrate [44].

With focus on cellulosics, the nature, concentration and distribution of substituted groups clearly influence the process of enzymatic degradation, since the chemical substitution may impair the recognition of the substrate by the enzymes [42]. However, it is worth pointing out that the effect of chemical modification on enzymatic resistance is not simply proportional to the number of substituted units. Indeed, neighboring units can also be involved in the enzymatic attack. This is due to the fact that the recognition of polymeric substrates by an enzyme generally implicates the simultaneous involvement of multiple moieties in the polymer chain

[32, 45]. In an interesting study by Kim et al. [32], it has been shown that the chemical oxidation of cellulose not only makes the oxidized glucopyranosides enzyme-resistant (towards both cellulase and β-glucosidase), but also renders the adjacent unmodified glucopyranoside moieties inaccessible to the enzyme. Therefore, the enzyme-resistant portion of cellulose is much greater than the degree of oxidation when the latter is low, while becoming closer to the degree of oxidation when this is increased. The partial oxidation of cellulose can thus be a useful strategy for the design of cellulose-based materials, such as hydrogels, with predetermined degradation rates [32].

2.3.2 Effect of temperature

It is known that native cellulose exists as a mixture of two crystalline forms I*a* and I*b*, having triclinic and monoclinic unit cells, respectively [46, 47]. Cellulose I*a* is thermodynamically less stable than I*b*, as also demonstrated by its conversion at 260°C. In both crystalline forms, cellobiose is the repeating unit with a strong intra-chain H-bond, although the inter-chain H-bonding and packing of the crystal are different in the two forms [48].

Thermal degradation of cellulose is quite a complex phenomenon, involving several chemical reactions whose understanding still appears controversial [49]. At high temperatures (approximately above 250°C), thermal degradation occurs as a first-order reaction. Pyrolysis is thought to take place rapidly via transglycosylation reactions, which lead to the formation of anhydro-sugars or 'anhydrocellulose' [50].

Furthermore, while at high temperature the effect of oxygen on thermal degradation is practically negligible, at lower temperatures oxygen plays a predominant role, so that oxidative degradation in air proceeds faster than pyrolysis in nitrogen. The oxidative degradation process involves the production of free-radical initiators, which then interact with oxygen to lead to autocatalytic oxidative reactions, characteristic of the thermal oxidation of polymers [50].

Along with furanic compounds and gases (*e.g.*, carbon dioxide and carbon monoxide), water is a key product of the thermal degradation of cellulose. This is of great importance in cellulose degradation, as water can cause hydrolytic scission of the bonds between the glucosidic units, leading to diminished degree of polymerization and loss of physical properties [51]. While the physical elimination of water from cellulose occurs at low temperatures (approximately below 220°C), the chemical loss of water takes place in the range 220–550°C. Various chemical reactions have been proposed to explain the observed water loss in this temperature range. Water elimination from cellulose is primarily due to the formation of anhydrocellulose, as mentioned above, through both intra-ring and inter-ring dehydration mechanisms [51]. Intra-ring dehydration involves the intramolecular elimination of water from C-2 and C-3 OH groups, while inter-ring dehydration is due to OH groups from adjacent chains that form crosslinks perpendicular to the chain direction [52]. Another possible mechanism of intermolecular dehydration is a grafting reaction between the C-6 and C-4 OH groups of adjacent chains, leading to the formation of an ether bond [52]. High temperatures (>300°C) may induce further elimination of water from C-6, yielding a vinylene group. Secondary reactions such as ring rearrangement, following the initial elimination reaction, lead to further water loss and production of furanic species [51].

2.3.3 Effect of alkaline environment

Cellulose degradation strongly depends on the alkaline condition of environment. At temperatures <170°C the glycosidic linkages between the glucose units

are stable in alkaline conditions [53]. However, a dramatic decrease of molecular weight is observed when cellulose is boiled in presence of a diluted sodium hydroxide solution, even with the careful exclusion of oxygen.

The principal mechanisms of degradation in alkaline media have been described by several investigators [53, 54], and they include endwise degradation (or peeling) at temperatures <170°C and alkaline hydrolysis of glycosidic bonds at higher temperatures. Endwise degradation is due to a number of isomerizations of the reducing end group of the cellulose molecule, which result in the migration of the carbonyl group along the carbon chain. The ketose or aldose end groups that are produced are then subjected to β-elimination [55]. If β-elimination occurs at the C-4 position, one monomer unit is released from the cellulose molecule, and the next glucose end group can take part in the reaction. In this way, the glucose units are gradually released from the macromolecule, resulting in a depolymerization process commonly known as *peeling-off reaction* or *unzipping reaction*. However, the β-elimination can also occur at positions other than C-4; in that case, the hexose unit remains attached to the cellulose molecule, which terminates the depolymerization. This is called the chemical *stopping reaction*. After either type of elimination reaction, a diketo intermediate is formed: this can undergo benzilic acid rearrangement, which generates the final degradation products. The two most common degradation products generated by peeling-off and chemical stopping reactions are epimers of 3-deoxy-2-C-(hydroxymethyl)-pentanoic acid (ISA) and 3-deoxy-hexanoic acid (metasaccharinic acid) [56].

When the temperature is >170°C, random alkaline scission of glycosidic linkages occurs, resulting in considerable weight loss and marked decrease in degree of polymerization. It is reported that the reaction does not depend on the presence of molecular oxygen and is followed by peeling from any new reducing end group produced by the scission process, thereby resulting in much greater weight losses than alkaline degradation at lower temperatures [57]. Although alkaline scission is normally only associated with alkaline degradation at higher temperatures, it has been also observed in the alkaline degradation of amorphous hydrocellulose at temperatures <100°C.

As mentioned for enzymatic degradation, the cellulose supramolecular structure plays a key role in the degradation process [58]. In general, a high supramolecular order of the polymer chains prevents or delays degradation [58, 59], with amorphous regions being much more sensitive to degradation than crystalline ones. It has been reported [53] that the rate-limiting step for slower chemical attack depends on the rate of mid-chain scission or the reaction of 'inaccessible' end groups. Peeling and chemical stopping are inhibited in fibrous hydrocellulose, which shows an ordered physical structure, and the majority of partially degraded molecules terminate with inaccessible reducing end groups, *i.e.*, by physical stopping. The relative rates of degradation (peeling) and stabilization (stopping) also depend on conditions such as the nature and concentration of the alkali and the temperature; stabilization is favored at high temperature and higher alkali concentrations [60].

3. Synthesis of cellulose-based hydrogels

Cellulosics are interesting precursors for the synthesis of superabsorbent hydrogels, due to the low cost, large availability, biocompatibility and biodegradability of cellulose, along with the responsiveness of some cellulosics (*e.g.*, CMCNa) to external stimuli.

In general, cellulose hydrogels can be stabilized via either physical (reversible) or chemical (irreversible) bonding of the cellulose chains, starting from dilute

aqueous solutions of single or composite cellulosic precursors [15]. Furthermore, natural and/or synthetic derived polymers might be combined with cellulose to obtain composite hydrogels with controlled properties [61, 62]. The number of crosslinking sites per unit volume of the network, called crosslinking degree, is a parameter that affects multiple properties of the hydrogel, including diffusive, mechanical and degradation properties. The control of the crosslinking degree, through adjustment of the synthesis protocol, thus represents a powerful tool to produce tunable hydrogels for the specific application at hand.

Commonly used physical hydrogels are those prepared from aqueous solutions of methylcellulose (MC) and/or hydroxypropyl methylcellulose (HPMC) [63]. The gelation process involves hydrophobic associations among the macromolecules via the methoxy group. While at low temperatures the polymer chains are well hydrated, at higher temperatures they start losing their bound water, so that polymer-polymer hydrophobic associations take place to form a 3D network. The sol-gel transition temperature clearly depends on the degree of substitution (DS) of the cellulose ethers and the presence of salts. A higher DS implies a more hydrophobic character of the cellulose chains, thus lowering the sol-gel transition temperature. Similarly, the addition of salts reduces the hydration level of the macromolecules. Both the DS and the salt concentration can be adjusted to obtain cellulose-based aqueous formulations able to gel at 37°C, for potential biomedical applications [64–66]. Several studies have addressed the use of low viscosity, injectable solutions, which may directly crosslink *in vivo* to deliver therapeutic molecules. However, the reversibility of physically crosslinked hydrogels, *i.e.*, their ability to flow or degrade under given circumstances (such as under mechanical loading) [67], still represents a significant limitation to their *in vivo* use. Conversely, *in vitro* applications of cellulose-based physical hydrogels appear much more attractive. In this context, MC hydrogels have been proposed as novel cell sheet harvest systems [66].

Unlike physical crosslinking, the formation of covalent bonding among the polymer chains creates a stable 3D network with given stiffness. Both physical treatments (*i.e.*, high-energy radiation) and chemical agents can be exploited to create irreversible cellulose-based networks. A number of chemical crosslinkers and catalysts are available to crosslink cellulosics, including epichlorohydrin, aldehydes and aldehyde-based reagents, urea derivatives, carbodiimides and multifunctional carboxylic acids [15]. The crosslinking reactions may occur in water solution, organic solvents or in the solid state (*e.g.*, polycarboxylic acids can crosslink cellulose via condensation reactions occurring at high temperature) [19, 67–69]. It is important to emphasize that some crosslinking reagents, such as aldehydes, are highly toxic, thus impacting negatively on the biocompatibility and eco-sustainable character of cellulose-based hydrogels. Therefore, the use of non-toxic chemicals and/or physical processes is gaining increasing interest in the literature. In this regard, the recent crosslinking of CMCNa/HEC with citric acid has allowed the development of biocompatible, biodegradable and totally eco-friendly superabsorbent hydrogels [19].

Radiation crosslinking of cellulose, using electron beams or gamma radiation, appears also suitable for the production of biocompatible hydrogels, especially for biomedical applications. Although irradiation can lead to scission of the polymer backbone, as demonstrated also for cellulosics [70], mild radiation conditions have been successfully adopted for the crosslinking and simultaneous sterilization of cellulose-based devices [71–73].

Finally, it is also important to highlight that cellulose backbone can be specifically functionalized before crosslinking, with the double aim of producing cellulose-based hydrogels free of potentially toxic contaminants as well as providing a higher

control of the crosslinking process. For instance, several cellulose derivatives have been added with acrylate moieties to enable the photo- or redox- crosslinking of aqueous solutions [74, 75]. Silylated HPMC, which crosslinks in solution by means of pH-driven condensation reactions, is another example of modified cellulose, proposed for the *in vivo* delivery of chondrocytes in tissue engineering applications [76, 77]. Tyramine-modified CMCNa has also been reported for the synthesis of enzymatically gellable formulations for cell delivery [78].

4. Smart swelling capability

4.1 Swelling ratio

The amount of water retained in the hydrogel network is a parameter of crucial importance, since it affects all the properties of the material that are relevant for the selected application (*e.g.*, stiffness, degradation, diffusion, biocompatibility, *etc*.). The hydrogel swelling is therefore the first property to be assessed; this is generally done by measuring the mass of solvent absorbed by the network:

$$Q_m = \frac{W_s - W_d}{W_d} = \frac{M_1}{M_2} \tag{1}$$

In Eq. (1), Q_m is the mass swelling ratio, W_s and W_d are the weights of the network in the swollen and dry state, respectively, while M_1 and M_2 indicate the masses of the solvent (*i.e.*, water) and the polymer, respectively.

The volume swelling ratio (Q) can be calculated as follows:

$$Q = \frac{V_s}{V_d} = \frac{V_1 + V_2}{V_2} = 1 + Q_m \frac{\rho_2}{\rho_1} \tag{2}$$

where V_s and V_d are the volume of the swollen and dry state, respectively; V_1 and V_2 the volumes of water and polymer; and ρ_1 and ρ_2 their densities. The polymer volume fraction in the swollen state is given by:

$$V_{2,s} = \frac{1}{Q} \tag{3}$$

In general, the hydrogel absorption capacity depends on both internal parameters (related to the structure of the polymer network) and external parameters (related to the solution bathing the hydrogel). Superabsorbent hydrogels, in particular, are those that display intrinsic large sorption capabilities (with $Q_m > 100$), together with a marked sensitivity to the external solution. This means that, by changing some environmental parameters (*e.g.*, pH, ionic strength), even slightly, superabsorbent hydrogels are able to undergo notable swelling/shrinking transitions [79].

4.2 Swelling mechanism

Superabsorbent hydrogels can be obtained by crosslinking hydrophilic polyelectrolyte species. Indeed, the presence of ions or fixed charges on the polymer network greatly improves its swelling behavior [80, 81].

Several factors governing the sorption mechanism can be identified. First of all, the polymer hydrophilicity promotes the polymer-solvent mixing, *i.e.*, the swelling,

when the material is placed in contact with water or water solutions. Secondly, there is the elastic retraction force due to the crosslinks, which opposes the swelling of the network. The entity of this elastic response clearly depends on the number of crosslinking nodes in the polymer network. In case of a perfect network with no dangling ends, loops, and entanglements, the number of elastically effective chain elements corresponds to the number of all chemically crosslinked polymer segments. The moles of polymer segments engaged by crosslinks and the moles of crosslinks per unit volume of the network are defined as the crosslink density and the degree of crosslinking, respectively. If ν is the number of units engaged in crosslinks and V is the volume of the network, the crosslink density ρ_x is given by:

$$\rho_x = \frac{\nu}{V} = \frac{1}{\upsilon M_c} \tag{4}$$

where M_c is the number average molecular weight between two consecutive crosslinks and υ is the specific volume of the polymer. Clearly, the higher M_c, the lower ρ_x; consequently, a higher hydrogel swelling is expected at fixed environmental conditions.

In case of polyelectrolyte networks, two additional beneficial contributions to swelling occur: (a) an osmotic mechanism called 'Donnan effect', which is proportional to the number of ionic fixed charges on the hydrogel network and induces the penetration of water into the network to dilute its high charge concentration; and (b) the electrostatic repulsion between charges of the same sign on the polymer backbone, which tends to expand the network, thereby promoting the swelling.

The Donnan effect (also known as the Gibbs-Donnan effect) is related to the behavior of free charged particles in the presence of a semipermeable membrane separating two different solutions. Being the membrane semipermeable, only some charged species are able to pass through it in order to reach the equilibrium between the two solutions. A typical Donnan-type mechanism takes place when a 3D polyelectrolyte network is placed in contact with a water solution, since electrical charges are tethered on the polymer backbone, which thus acts as a semipermeable membrane. The equilibrium of the whole system (composed by the swelling solution and the polymer network itself) is attainable only if a passage of water is established, going from the external solution to the polymer network, thus diluting the concentration of the charges inside the network.

The polyelectrolyte nature of CMCNa explains why this cellulose derivative is widely used for the synthesis of superabsorbent cellulose-based hydrogels, especially in conjunction with HEC [13, 15, 18–20]. The simultaneous presence of HEC has been shown to be fundamental to achieve intermolecular (rather than intramolecular) crosslinking reactions, thus enabling the network stabilization [15]. The hydrogels crosslinked in presence of CMCNa clearly exhibit a much higher sorption capacity if compared with those crosslinked with HEC only, due to the Donnan-type swelling mechanism. The swelling capability of CMCNa/HEC hydrogels has been further increased by the use of difunctional molecules (*e.g.*, polyethylene glycol) as network spacers, able to increase the average distance between two crosslinking sites (*i.e.*, M_c) [82].

4.2.1 Effect of temperature

The hydrogel state can be considered as a peculiar solution composed of water and hydrophilic polymer chains. Because of the presence of crosslinks, which impede the polymer dissolution, the hydrogel solution is characterized by an elastic (rather than viscous) behavior [79].

The polymer-solvent interaction can be described by the thermodynamic theory of polymer solutions. In 1953, Flory [81] showed that the free energy change associated with the mixing process between the solvent and the polymer network can be calculated as follows:

$$\Delta G_{mix} = kT\left[n_1 \ln\left(1 - V_{2,s}\right) + \chi_{1,2} n_1 V_{2,s}\right] \tag{5}$$

where k is the Boltzmann constant, T the absolute temperature, n_1 the number of solvent molecules, and $\chi_{1,2}$ the Flory-Huggins polymer-solvent interaction parameter [81]. This last parameter takes on positive or negative values, for endothermic or exothermic mixing, respectively. In case of complete miscibility of the polymer in the solvent over the entire composition range, $\chi_{1,2}$ is lower than 0.5. The exact value of this parameter can be calculated as follows:

$$\chi_{1,2} = \chi_a + \chi_b V_{2,s} + \chi_c V_{2,s}^2 + \ldots \tag{6}$$

where χ_a, χ_b, etc. are function of the temperature.

This means that the polymer-solvent interaction parameter, which in turn affects the polymer hydrophilicity and the hydrogel swelling, depends on temperature (and on polymer concentration). The effect of the temperature on $\chi_{1,2}$ allows to design thermosensitive hydrogels. Most polymers increase their water solubility as the temperature increases (*i.e.*, the $\chi_{1,2}$ parameter decreases), thus they are able to form positive temperature-sensitive hydrogels, which shrink upon cooling below their upper critical solution temperature (UCST). However, negative temperature-responsive hydrogels can be also obtained, which shrink when heated above their lower critical solution temperature (LCST), These include both physical systems, such as the MC and HPMC hydrogels discussed above [63–66], and chemical ones, for example obtained by copolymerizing cellulose derivatives with N-isopropylacrylamide [83].

4.2.2 Effect of ionic strength (constant pH)

Polyelectrolyte networks (*e.g.*, CMCNa/HEC hydrogels) are able to significantly change their volume when changing the composition of the external solution. The fixed charges linked to the polymer backbone drive this peculiar response. In general, the equilibrium solution uptake always diminishes for higher values of the ionic strength. However, polyelectrolyte hydrogels display a marked sensitivity to ionic strength variations. This is due to the osmotic pressure related to the Donnan effect, which is proportional to the difference in concentration of charges between those contained in the gel and those in the external solution. Obviously, increasing the ionic strength of the external solution decreases the difference between the concentration of ion species in the gel and in the external solution and, as a result, the water uptake decreases. This behavior can be ascribed to the neutralization of the fixed charges linked to the polymer backbone by the "free" charges active in the external solution. This neutralization reduces the total active charge of the polymer network, thus reducing both the electrostatic repulsion of the polymer chains and the Donnan-type sorption mechanism. On the other hand, this effect can be explained as a reduction of the chemical potential of the water in the external solution, with a resulting reduction of its capability to penetrate the polymer network.

4.2.3 Effect of pH (constant ionic strength)

In general, the degree of ionization i of polyelectrolyte chains depends on the dissociation constant of the ionizable network groups and the pH of the external

solution. In particular, anionic hydrogels (such as CMCNa/HEC ones) tend to deprotonate and swell when external pH is higher than pK_a of their ionizable groups, while cationic hydrogels protonate and swell when external pH is lower than pK_b of their ionizable groups.

The dissociation of the carboxylic groups fixed on the cellulose chains in CMCNa/HEC hydrogels is strongly affected by the pH of the external solution [18, 80]. A reduction in the number of dissociated carboxylic acid groups in the polymer network is evident at low pH. This mechanism reduces the swelling of the material, in accordance with the reduction of the polyelectrolyte property of the network. At very low pH values, the majority of the carboxylic acid groups are in a non-dissociated state, and the hydrogel seems to be composed of non-polyelectrolyte chains. On the other hand, when the pH of the swelling solution increases, there is a growth of the number of dissociated carboxylic group with a consequent increment in swelling.

5. Mechanical properties

The viscoelastic nature of polymers offers several analytical methods to investigate their mechanical behavior. In addition to 'traditional' mechanical tests (*e.g.*, tensile, compressive, flexural), dynamic mechanical analysis (DMA) is the method of choice to quantify the elastic (*i.e.*, conservative) and viscous (*i.e.*, dissipative) moduli of polymers, *G'* and *G"* respectively, in both the liquid and solid states. Basically, the method consists in subjecting the sample to a sinusoidal deformation, of given amplitude and frequency, and recording the double material's response in-phase (elastic) and out-of-phase (viscous) with the mechanical solicitation. With regard to gels, DMA is widely used to investigate both the progression of the crosslinking reaction and the mechanical properties of the final gel.

In general, the gelation of polymer solutions can be investigated by rheological and dynamic mechanical analyses. As the crosslinking reaction takes place, the viscosity of the polymer solution starts to increase progressively, due to the increase of the average molecular weight, until the gel state, defined as the one where no flow occurs (*i.e.*, infinite viscosity), is reached. Alternatively, the *G'* and *G"* moduli can be monitored during crosslinking via DMA, as a function of frequency, time or temperature [84, 85]. The initial polymer solution, characterized by a modulus *G"* higher than *G'*, undergoes a rapid increase of *G'* (higher and faster than the one of *G"*) as the crosslinking reaction proceeds, so that the final gel state shows a modulus *G'* higher than *G"* for several orders of magnitude. The so-called gel point is defined as the one where *G'* and *G"* curves cross each other and basically it determines the temperature or the time required to obtain a gel from given solutions. In the literature, several investigations are reported exploring the physical or chemical gelation of cellulose-based hydrogels via DMA and are here suggested for further reading [84, 85]. In the following, instead, the authors address the mechanical characterization of final hydrogels, with particular focus on the relationship between the crosslink density and the stiffness of the polymer network.

Since hydrogels show a rubber-like behavior, the theory on the entropic elasticity of rubbers, described by Flory [81], can be exploited to estimate the degree of crosslinking of a hydrogel by measuring its macroscopic mechanical properties. Assuming that the deformation of the polymer chains is affine and that the volume of the polymer network does not change upon uniaxial deformation, Flory derived the following relationship between the uniaxial stress and the uniaxial deformation:

$$\sigma = RT\rho_{xe}\left(\alpha - \frac{1}{\alpha^2}\right) = G\left(\alpha - \frac{1}{\alpha^2}\right) \quad (7)$$

where σ is the stress, R is the universal gas constant, T is the absolute temperature, ρ_{xe} is the elastically effective crosslink density (*i.e.*, the number of elastically effective crosslinks per unit volume, which is equal to ρ_x for perfect networks), $\alpha = L/L_i$ is the deformation ratio, with L the actual thickness of the deformed sample and L_i the initial thickness of the sample ($\alpha > 1$ for elongation and $\alpha < 1$ for compression, respectively), and G is the shear modulus of the polymer network. In practice, Eq. (7) holds for small deformations ($\alpha \approx 1$), in order to assume a constant volume of the network, and states that the plot of σ against ($\alpha - 1/\alpha^2$) is linear, with a slope that is equal to the modulus G and directly proportional to the crosslink density.

If the polymer network is swollen isotropically in a solvent [81], and the crosslinking reaction occurs in the presence of the solvent [86], as for most hydrogels, the modulus G in Eq. (7) can be expressed as follows:

$$G = RT\rho_{xe}V_{2,s}^{1/3}V_{2,r}^{-1/3} = RT\frac{\nu_e}{V_0}V_{2,s}^{1/3}V_{2,r}^{2/3} \quad (8)$$

where V_0 is the dry network volume, $V_{2,s}$ is the polymer volume fraction in the fully swollen state (Eq. (3)), and $V_{2,r}$ is the polymer volume fraction soon after crosslinking and before swelling, *i.e.*, in the relaxed state.

Therefore, the mechanical characterization of hydrogels via uniaxial elongation or compression tests allows estimating not only the gel stiffness and strength, but also the corresponding elastically effective crosslink density. However, it is worth recalling that, due to viscoelasticity, which implies a time-dependent material's response, the rate of gel loading is particularly important in the determination of its mechanical properties. Commonly used strain rates for hydrogel testing are in the range 1–50 μm/s [87–90].

Although derived for uniaxial deformation, Eq. (8) has a general validity. DMA, which directly measures the modulus G through its components G' and G'', might thus be used also to estimate ρ_{xe} [87, 91]. Interestingly, for superabsorbent cellulose-based hydrogels, it has been demonstrated that the calculation of ρ_{xe} from both compression and DMA data provides comparable values, thus suggesting the high potential of DMA as a non-destructive method for the bulk mechanical characterization of hydrogels [87].

For selected types of hydrogels, especially those used in tissue engineering and cell culture applications, the determination of the local mechanical properties of the material, on the nano/submicron scale, is also of great interest for fully understanding the multiscale material behavior, as well as the cell response to the substrate stiffness or elasticity [90, 92]. Such local measurements can be performed via atomic force microscopy (AFM) and nanoindentation techniques [93]. Apart from its use for morphological surface analysis, in recent years AFM has emerged as a well-established method to map the nano-mechanical properties of elastic and viscoelastic materials [94], and to probe the elasticity of cells and biological tissues [92]. AFM can also be adapted to perform nanoindentation tests [94]. Several soft hydrated materials, such as hydrogels, have been characterized via AFM and/or nanoindentation and their local elasticity has been correlated to the bulk one [90, 92, 93]. Notably, the stiffness of single cellulose nanofibres in bacterial cellulose hydrogels has been very recently determined via AFM [95].

As a final consideration on the evaluation of mechanical properties, it is worth noting that Eqs. (7) and (8) are referred to the analysis of non-macroporous

hydrogels. The mechanical behavior of porous gels, which are briefly dealt with in a later section, is clearly affected not only by the precursor polymer(s) and the crosslink density, but also by porosity itself. The determination of the crosslink density of porous hydrogels from mechanical data should thus take into proper account the role of porosity. As for the assessment of the local mechanical properties, so far, AFM has been mostly used for probing of non-macroporous hydrogels, being the analysis of porous substrates particularly challenging. However, the mechanical characterization of porous hydroxypropyl cellulose methacrylate (HPC-MA) hydrogels by means of AFM has been successfully reported in recent literature [96]. The obtained elastic modulus, together with data from microstructural analysis, has also allowed the mechanical modeling of individual pores and the bulk scaffold [96]. These findings pave the way to a more extensive use of the AFM technique in the near future for the mechanical analysis of porous hydrogels.

6. Thermal stability

Thermal stability of hydrogels is commonly investigated by means of thermogravimetric analysis (TGA), a technique that allows identifying the temperatures at which sample weight losses occur, due to heating-induced transitions, such as evaporation of volatile substances (*e.g.*, moisture) and thermal decomposition. In particular, the first derivative of the TGA signal (DTG) is usually calculated to highlight the temperatures of maximum degradation. When comparing the TGA/DTG curve of given hydrogels with those of the respective precursors, very useful information can be obtained related to the quality and efficacy of the crosslinking reaction, as well as the overall stability of the synthesized polymer networks. With regard to cellulose-based hydrogels, the high number of cellulose derivatives and crosslinking protocols currently available for their synthesis (as discussed in the first part of this chapter) make it difficult to draw general or conclusive remarks on their thermal behavior. Therefore, in the following the thermal stability of cellulose-based hydrogels is briefly discussed with reference to specific examples reported in the literature [97, 98], where blends of CMCNa and HEC, used as hydrogel precursors, have been shown to exhibit higher or lower thermal stability in the crosslinked state compared to the native one, depending on the crosslinking protocol used.

6.1 TGA analysis of hydrogel precursors: CMCNa and HEC

As discussed in the previous sections, CMCNa and HEC are widely employed for the synthesis of superabsorbent hydrogels [87, 97–100]. In particular, CMCNa is the polyelectrolyte species that provides the hydrogels with enhanced swelling capability and sensitivity to environmental stimuli (*i.e.*, ionic strength and pH), thanks to the Donnan effect. HEC is adopted to promote the formation of intermolecular rather than intramolecular crosslinks, thus allowing the macromolecules to stabilize into a three-dimensional polymer network [87]. The synthesis of superabsorbent hydrogels, with sorption capabilities up to 500 times the dry weight, is commonly reported for CMCNa/HEC weight ratios of 2/1 and 3/1 (*i.e.*, when an excess of polyelectrolyte CMCNa is used) [87, 97].

When analyzing the thermal behavior of cellulose derivatives, it is worth recalling that, due to the major role of oxygen in cellulose degradation, especially at low temperatures (as discussed previously), the choice between air or nitrogen flow as an atmosphere in which performing the TGA analysis is fundamental and should always be specified.

For analyses in nitrogen flow, typical TGA/DTG curves for CMCNa and HEC (commonly reported in the range 25–500°C) basically show a two-step weight loss, with removal of moisture (up to 10% weight) in the temperature range 40–100°C, and rapid thermal degradation, mostly due to decarboxylation [101], in the range 230–300°C (about 40–50% weight) (**Figure 2**). Weight loss then slowly proceeds at higher temperatures due to further degradation mechanisms, such as those described in Section 2 (*e.g.*, water removal, cleavage of the glycosidic linkage, formation of furanic compounds). Maximum degradation temperatures for CMCNa and HEC have been reported to be 284 and 280°C, respectively [98].

6.2 TGA analysis of CMCNa/HEC hydrogels

As reported in the literature, CMCNa/HEC hydrogels may display two or more steps of thermal degradation, depending on the crosslinking agent(s) used [97, 98]. Additional steps of weight loss in the TGA curve, compared to the curves of cellulose precursors, may be indeed due to the degradation of the crosslinker molecules incorporated in the gel network. As an example, CMCNa/HEC (3/1) hydrogels, crosslinked by either fumaric or malic acid [98], have been recently shown to display a three-step degradation process, including: a first stage, occurring at low temperatures (up to 100°C), due to water/moisture evaporation; a second stage, in the range 200–270°C, related to decomposition of the crosslinker; a final stage, between 270 and 400°C, where thermal decomposition of cellulose backbone occurs. It is worth noting that, in this study, the hydrogels were found to possess a higher thermal stability than the corresponding cellulose precursors. In particular, increased hydrogel degradation temperatures were detected up to 298 and 317°C, when using increasing concentrations of malic and fumaric acid, respectively. This suggested the formation of highly stable cellulose networks upon crosslinking.

Clearly, such a finding is strictly related to the given crosslinking agent/reaction being investigated. For instance, in an independent investigation CMCNa/HEC hydrogels crosslinked by divinylsulfone (DVS) have been shown to be less thermally stable than native polymers [97]. The maximum degradation temperature of cellulose was indeed shifted from about 285 to 276°C, upon DVS crosslinking of a 5/1 CMCNa/HEC mixture. However, in that study the effect of different crosslinker concentrations on the hydrogel stability was not taken into account.

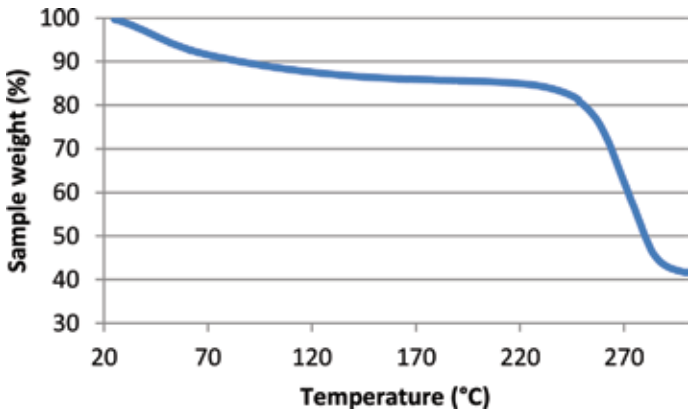

Figure 2.
Exemplificative TGA curve (25–300°C, nitrogen flow) obtained for a sample of HEC (unpublished data by the authors).

7. Hydrogel morphology

In general, the analysis of hydrogel morphology (*i.e.*, porosity, at different length scales) is a fundamental characterization step for the full comprehension of most gel properties, including the thermodynamic and kinetic swelling behavior, the mechanical stiffness and strength and the diffusivity of molecules within the polymer network. However, such an investigation can be challenging, at least via standard experimental techniques (*e.g.*, microscopy), due to the inherent presence of water in the gel state.

In the following, the analysis of the gel structure is briefly discussed, with particular reference to the evaluation of pores at the nano-, micro- and macroscale. Where appropriate, specific examples related to the analysis of cellulose-based hydrogels are provided.

7.1 Nanoscale: hydrogel mesh size

Hydrogel networks show a fine mesh-like structure, where the free space among crosslinked chains can host the diffusion of water and other molecules of suitable size, smaller than or comparable to the mean hydrogel mesh size (**Figure 3**). According to the Canal-Peppas theory for isotropically swollen hydrogels [86], the average mesh size ξ, also referred to as correlation length, can be calculated from the following:

$$\xi = V_{2,s}^{-1/3}\left(\overline{r_0^2}\right)^{1/2} = V_{2,s}^{-1/3} l (C_n N)^{1/2} = V_{2,s}^{-1/3} l \left(C_n \frac{2M_c}{M_r}\right)^{1/2} \qquad (9)$$

In Eq. (9), $\left(\overline{r_0^2}\right)^{1/2}$ is the root-mean-squared end-to-end distance between adjacent crosslinks in the unperturbed state, $V_{2,s}$ is the polymer volume fraction in the swollen state, l is the bond length along the polymer chain, C_n is the characteristic ratio of the polymer, N is the number of bonds per chain, M_c the molecular weight between crosslinks, and M_r the molecular weight of the polymer repeating unit. The higher the crosslink density (*i.e.*, the lower M_c), the smaller the average mesh size. Typical ξ values for swollen hydrogels are reported in the range 5–100 nm [102, 103].

In spite of the fact that most real hydrogels show a broad range of mesh sizes, various studies show that the calculation of the average mesh size according to Eq. (9), starting from theoretical swelling or mechanical models for M_c determination, leads to a reliable and meaningful estimation of the hydrogel nanostructure [104, 105]. From an experimental point of view, the direct visualization or measurement of the hydrogel mesh size distribution is indeed hard to achieve. Standard microscopy techniques, including optical and electron microscopy, often require a too severe processing of the sample (*e.g.*, dehydration), which introduces numerous artifacts, thus deforming the structure of the gel with respect to its native state.

Figure 3.
Schematic representation of a hydrogel network, with its average mesh size hosting multiple diffusing molecules.

Recently, some successful attempts have been made with cryo-transmission electron microscopy (cryo-TEM), for imaging various types of hydrogels (*e.g.*, methylcellulose hydrogels [106]) yet preserving their nanostructure [107, 108]. Cryo-TEM enables the high resolution imaging of the hydrogel under cryogenic conditions, showing the presence of vitrified water in a network of crosslinked polymeric chains. Nonetheless, it is obvious that the extreme care needed in sample preparation to avoid artifacts, still makes cryo-TEM poorly attractive for widespread use. Other methods to evaluate the hydrogel mesh size include confocal laser scanning microscopy (CLSM) [109], small-angle neutron scattering [106, 110, 111], small-angle X-ray scattering (SAXS) [104], pulsed field gradient [112] and low field nuclear magnetic resonance (NMR) spectroscopy [105]. Although accurate, these methods are based on expensive equipment and complex data analyses, which ultimately limit their application. Moreover, in many cases the estimated values of mesh size have been shown to be comparable with those calculated from theoretical models (Eq. (9)). This is why the indirect estimation of ξ via M_c determination (through swelling and/or mechanical tests) is by far the most widely employed method to study the hydrogel structure at the nanoscale. Further solute exclusion/diffusion experiments can be also performed to estimate the average mesh size from the diffusivity of molecules with given hydrodynamic radius [105, 113, 114], often confirming the average ξ values estimated theoretically.

With particular reference to cellulose-based hydrogels, it is worth mentioning thermoporosimetry as another interesting and powerful technique to measure the mesh size distribution, rather than the average mesh size, of polymer networks. The method, based on differential scanning calorimetry (DSC) and first described by Kunh et al. [115], relies on the application of the Gibbs-Thomson equation, which quantifies the freezing point depression of a liquid (*e.g.*, water), when confined in a porous medium, as inversely proportional to the pore size [116, 117]. Furthermore, the melting enthalpy is related to the pore volume, so that the entire mesh size distribution can be determined starting from the DSC curve [116, 117]. However, thermoporosimetry necessitates calibration against a known pore analysis method, proper adjustments of the DSC heating protocol for the given material being analyzed and signal deconvolution when the melting peaks of confined water and bulk water are overlapping [116]. For porous cellulosic materials, that show a low heat transfer and overlapping melting transitions, an isothermal step melting protocol has been recently proposed to estimate the pore size distribution through DSC analysis [116]. Moreover, since the DSC tracing of bound or confined water may be due to water–polymer interaction [118] rather than porosity confinement [119], preliminary experiments in various moisture environments have been suggested to verify whether porosity is the true factor governing the water melting behavior [120]. Several additional studies on the thermal behavior of water in cellulose-based materials are currently available in the literature and are here suggested for further reading [116, 118, 119], since they may provide useful information for the mesh size analysis of selected cellulose-based hydrogels via thermoporosimetry.

7.2 Micro- and macroscale: hydrogel porosity

For an environmentally sensitive hydrogel, the swelling rate, *i.e.*, the rate with which the hydrogel responds to external changes, is significantly affected by its bulk morphology. While reducing the size of the hydrogel in granular or powder form is a simple but rough method to accelerate its swelling kinetics, the creation of an interconnected network of micro- or macropores in the gel represents a sophisticated and accurate way to further control its bulk properties, including the swelling extent and kinetics. First of all, porous hydrogels can retain higher amounts of

water compared to non-porous ones, due to additional capillary retention [87, 99, 100]. Furthermore, they can rapidly swell or shrink in response to given stimuli, due to the convective water transport, taking place in the pore channels. Multiple synthesis methods have been proposed to produce porous hydrogels with given microstructure, which are generally based either on the use of a removable porogen/templating agent [121–123] or on the rapid expulsion of water by phase inversion in a non-solvent [87, 100]. Cryo-gelation, which consists of crosslinking a polymer solution at cryogenic temperatures, is a particular method where ice crystals are the interconnecting porogens, while the crosslinking reaction takes place in concentrated liquid microphases among them. Resulting cryogels exhibit a sponge-like form with remarkable properties compared to other types of porous, soft gels. Indeed, in addition to a very quick swelling/deswelling response, cryogels may also show high elasticity and shape memory, which can make them even injectable for specific biomedical applications [124, 125]. So far several cellulose-based porous hydrogels, including different types of cryogels, have been proposed in the literature as smart materials for a wide range of applications, *e.g.*, absorbents in the agriculture field [126], matrices for controlled drug release [127], stomach bulking agents [18] and scaffolds for tissue engineering [128].

The investigation of hydrogel porosity is primarily performed, with different levels of resolution, via optical methods, such as scanning electron microscopy (SEM) and CLSM, usually followed by software-based image analysis [122, 129]. As discussed above, particular caution is needed in sample preparation to avoid artifacts, especially in cases where preliminary sample dehydration is required (*e.g.*, in SEM). In this regard, cryo-SEM is particularly recommendable to visualize the structure of swollen hydrogels. Alternatively, freeze-drying may be exploited to preserve the gel structure as much as possible, before standard (*i.e.*, under high vacuum) SEM observation. Other methods to analyze the hydrogel porosity include mercury intrusion porosimetry [121] and X-ray computed microtomography (μCT) [123, 130, 131]. While the former still necessitates preliminary sample drying and may not be suitable for the analysis of very soft materials such as hydrogels, μCT represents a powerful and versatile technique for the quantitative analysis of hydrogel porosity. In general, μCT allows the non-destructive visualization and reconstruction of the entire 3D structure of a given material. Then, proper analysis of acquired images/sections provides significant morphological information such as the pore volume fraction and the pore size distribution [123, 130, 131]. Although μCT does not require particular care in sample preparation, in the case of porous hydrogels it is worth mentioning that long scanning times are often required to obtain good quality images, due to the typical low density of the materials. The presence of water in swollen hydrogels may further increase the scanning time, thus implying the simultaneous desiccation of the sample under the X-ray beam. The μCT analysis of freeze-dried hydrogels is thus recommended and is usually reported [130, 131]. Although the μCT quantification of porosity may be particularly challenging for some hydrogel-based materials, *e.g.*, cellulose-based ones, due to their very low density, the successful μCT characterization of various hydrogel types has been recently reported [123], thus suggesting the potential of the technique to be further refined for the analysis of a larger number of hydrogels.

8. Conclusions

Cellulose-based superabsorbent hydrogels are currently explored for a number of technological applications, which range from the traditional use of hydrogels as water absorbents in different contexts (*e.g.*, personal care products, agriculture) to

their latest use in both pharmaceutical and biomedical fields (*e.g.*, controlled drug delivery, tissue engineering). The design of novel hydrogels requires a deep knowledge of the existing correlation between structure and properties of cellulose networks. Multiple cellulose derivatives can be synthesized and crosslinked, via different strategies, for the development of hydrogels with well-defined features, including the swelling capability and sensitivity to external stimuli, the degradation rate resulting from various environmental factors, the mechanical stiffness and strength, the mesh size and the diffusion of molecules within the hydrogel network, and the micro- and macroporosity. Depending on the intended application(s), various experimental tests, such as those briefly reviewed in this chapter, can be performed to highlight the properties of interest. Due to the large availability of cellulose, its environmentally friendly nature and its biocompatibility, it is reasonable to assume that cellulose and its derivatives will drive the evolution of smart hydrogels in different technological fields.

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Chapter 4

Hydrogels Based on Chitosan and Chitosan Derivatives for Biomedical Applications

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Abstract

Chitosan (CS) is a polymer obtained from chitin, being this, after the cellulose, the most abundant polysaccharide. The fact of (i) CS being obtained from renewable sources; (ii) CS to possess capability for doing interactions with different moieties being such capability dependent of pH; (iii) plenty of possibilities for chemical modification of CS; and (iv) tuning the final properties of CS derivatives makes this polymer very interesting in academic and technological points of view. In this way, hydrogels based on CS and on CS derivatives have been widely used for biomedical applications. Other important technological applications can be also cited, such as adsorbent of metals and dyes in wastewater from industrial effluents. In pharmaceutical field, hydrogels based on CS are often used as drugs' and proteins' carrier formulations due to the inherent characteristics such as the biocompatibility, nontoxicity, hydrophilicity, etc. This chapter is an attempt for updating and joining the plenty of available information regarding the preparation, characterization, and biomedical application of hydrogels based on chitosan and chitosan derivatives. More than 260 references are provided, being the majority of them published in the last 10 years.

Keywords: hydrogels, chitosan, chitosan derivatives, biomedical application, protein and drug delivery

1. Introduction

Among the diverse types of polymeric materials, the biopolymers represent an important class. Extensive research has been conducted on biodegradable polymeric materials, mainly because they are, in a huge majority, from renewable sources. Some of them present wide availability in nature and can be obtained for a low price. Frequently, they exhibit characteristics such as controlled reactivity, low toxicity, biocompatibility, biodegradability, and filmogenic properties [1, 2]. These

materials have gained attention due to environmental issues such as the reuse of organic waste and/or its reduction, preservation of natural resources [3–5].

There are a number of diverse naturally occurring polymers, such as those belonging to the class of polyesters, those obtained from bacterial sources, polymers of animal origin, proteins, and polypeptides. They can be easily used to produce fibers or particles at micro- or nanoscale for diverse and interesting pharmaceutical applications [6, 7]. Polysaccharides, in general, have some abundance in nature and are also biodegradable and nontoxic [8], making them an active subject of study. Because it is a vast field, many other polymers can also be used, and the decision must be according to the sought-after application. Chitosan (CS) is one of most important examples.

Chitosan is obtained from chitin (CT) that is the most abundant polysaccharide after cellulose [8]. CT is a linear, natural, biodegradable, biocompatible, and nontoxic polymer that is insoluble in most solvents [8, 9]. Chitosan is applied in several fields: agriculture, waste treatment, food, textile, and pharmaceutical industries, cosmetics development, and biomaterials such as gels, films, polymer membranes, and nanofibers [8, 9]. The vast and numerous applications are due to the chitosan's interesting properties. In addition to those mentioned, healing, antimicrobial, antifungal, and chelating properties may be also included [10, 11]. Besides, different methodologies and strategies for chitosan applications have been proposed in the literature. Development of hydrogels is one of such methodologies that have gained much attention. Hydrogels are three-dimensional structures formed by hydrophilic polymers that can absorb water or biological fluids [12]. The absorption capacity is due to the presence of cross-linking points, which can be chemical or physical, making these structures insoluble, with the possibility of controlling the pore size in the hydrogels during the preparation method [13]. Because of these characteristics, they have been widely used in various fields of science, such as pharmacy, environmental chemistry, and biomedicine [8, 14].

Hydrogels based on chitosan have attracted lot of attention since this biopolymer is degraded in humans by lysozyme [15], making it highly attractive for the fabrication of dressings. The chitosan dressings can, for example, aid in the absorption of wound secretions and control the hydration of the affected region. Furthermore, for another application in biomedicine, that is, skin regeneration, the polysaccharide chitosan, in addition to exhibiting the aforementioned several characteristics of interest, reduces the healing time of lesions caused by compromises, stimulates cell proliferation, and confers excellent mechanical resistance on the biomaterials [16–18].

In this chapter, the focus was on hydrogels based on chitosan and its derivatives. The study sought to harness the properties of chitosan toward hydrogel applications and to investigate its extensive use in the field of biomedicine.

1.1 Basic concepts and useful properties of hydrogels

Hydrogels are defined as three-dimensional (3D) polymer networks formed by cross-linking hydrophilic homopolymers (or copolymers) that have the ability to absorb large amounts of water and/or biological fluids [19]. Plenty of materials, both natural and synthetic, or mixture of them, fit the definition of hydrogels.

The physical, chemical, and mechanical properties of hydrogels are dependent on intra- and intermolecular interactions among polymer groups or chain segments and solutes/solvents that may be present inside of 3D structure. The interest in this class of materials (hydrogels) has increased significantly in the academic and technological media due, mainly, to the inherent characteristics such as malleability, biocompatibility, nontoxicity, and swelling in the presence of water or biological fluids, without, however, dissolving [20]. The studies show that the most important

characteristic of hydrogels, the swelling, can be significantly altered through variation of external stimuli. Such change is accompanied by alteration in mechanical and morphological properties [21, 22].

Depending on the nature of the 3D network, hydrogels can be divided in two categories: (i) chemical hydrogels and (ii) physical hydrogels. The 3D matrix of chemical hydrogel has cross-links, which are formed by covalent bonds; in the other class, the 3D matrix is formed by physical interactions. Such physical interactions arise due to the presence of (i) groups with opposite electrical charges (electrostatic interactions, as in the case of polyelectrolyte complexes or PECs) and (ii) dipolar or hydrophobic groups (that works for physical hydrogel forming, as is the case of the hydrogels obtained by the process of freezing-thawing) [23, 24]. Thus, the use cross-linking agents is not needed for preparing physical hydrogels. Another characteristic that distinguishes physical hydrogels is the reversibility of cross-links, that is, the 3D matrix of a physical hydrogel can be destroyed by varying pH, temperature, ionic strength, etc. A typical example of physical hydrogel is that obtained by cross-linking alginate through complexation of carboxylic groups (existing in alginate chains) with calcium ions [19, 25]. A very widespread model known as egg box is used to explain this type of gelation [26]. The alginate/Ca^{2+} matrix may be broken down under acidic conditions and/or by the addition of EDTA [27].

In the case of physical hydrogels, polymer-ion (for example, alginate/Ca^{2+} matrix) or polymer-polymer interactions (for example, complexation between anionic and cationic polymers) should prevail in relation to the polymer-solvent interactions. Without such prevalence, the gelation process would be prevented from occurring.

Several methods can be used for the production of chemical hydrogels. Those involve radiation polymerization by free radicals, for example, the polyacrylamide hydrogel made by acrylamide reaction (AAm) in the presence of methylenebisacrylamide (MBAAm); hydrogels can also be made by polycondensation such as the cross-linking of polysaccharides by reaction with dialdehyde that is very frequent in literature. Other important example of hydrogel by polycondensation is the obtainment of chitosan hydrogel by reaction with glutaraldehyde. The disadvantage of such methods is the need for using initiators and/or catalysts. Such a disadvantage ceases to exist if the polymerization/cross-linking is induced by irradiation, for example, gamma radiation, which produces pure, sterile, and residue-free hydrogels and, beyond this, no catalysts or additives are required to initiate the reaction [28]. Because of this, obtaining hydrogels is a very useful method in the preparation of hydrogels for medical applications, where even a small contamination is undesirable [29, 30]. In this case, the disadvantage is the use of high-cost and rigidly controlled equipment that makes this process of little accessibility in many research groups.

The history of hydrogels began in the 1950s when Wichterle and Lim synthesized hydrogels based on 2-hydroxyethyl methacrylate copolymer with ethylene dimethacrylate [31] and applied them as contact lenses. They were the first gelatinous contact lenses with proven biocompatibility. The great commercial success of gelatinous contact lenses stimulated enormous interest in this type of materials. Subsequently, a wide range of studies enabled the development of hydrogels with different chemical structures, morphologies, and properties through several methodologies. The state of the art in the area of hydrogels is the synthesis of "intelligent" or "smart" hydrogels that modifies their properties once exposed to change of external stimulus, such as pH, temperature, light, or electric field [32]. Hydrogels sensitive to pH and temperature have been played an important role in the control of the drug transport and the drug delivery systems, because temperature and pH are important environmental factors in biomedical systems [33–35].

The use of natural polymers in the preparation of hydrogels has attracted the attention of many researchers both for their natural abundance and for their better biocompatibility when used as biomaterials, with chitosan being one of the most commonly used [36]. Hydrogels derived from natural sources are advantageous because of their inherent biological properties and are widely researched for tissue engineering applications. However, as with other naturally occurring materials, variation in batch composition represents an important disadvantage [37].

1.2 Techniques often used for hydrogel characterization

The analysis of morphology, molecular structure, mechanical properties, and sensitivity to pH and temperature can be done through a great number of qualitative and quantitative techniques. The capability of absorbing liquids (swelling) is the main property of hydrogels, which depends on the hydrophilicity of the hydrogel matrix. From this characteristic, another important aspect arises: the liquid retention capacity of the hydrogel matrix. Changes associated to the swelling (or the shrinking), are controlled by various parameters such as temperature, pH, salinity, and ionic strength of the medium [38]. Superabsorbent hydrogels have the ability to absorb and retain large amount of liquid ($S \geq 100$) [39]. They are prepared using highly hydrophilic moieties (polymers or monomers) and have important technological applications, for instance, in environment (as ion and/or dye absorbent) [40] and agriculture (as soil conditioner or nutrient carriers) [41].

The absorbability of the liquid or the swelling (S) capability of the hydrogels is generally calculated by the following equation [39].

$$S = (W_s - W_d)/W_d \tag{1}$$

where W_s is the weight of the swollen hydrogel and W_d is the weight of the dry hydrogel. Thus, this ratio relates the amount of the mass of liquid absorbed to the mass of dry hydrogel. The parameter S can be evaluated aiming to determine the maximum mass of fluid that a giving hydrogel is capable of absorbing (steady state) or can be evaluated at different time intervals to determine the kinetic swelling up to the equilibrium has been reached.

Evaluation of parameters related to swelling kinetics is important for hydrogel characterization during swelling. The swelling process is controlled by diffusion and/or relaxation. Different models for predicting the release behavior of hydrogel matrices dipped in pure solvent, solvent mixture, or solute-solvent solution have been developed. Brazel and Peppas [42, 43] developed a semiempirical equation that is widely used, even if it fits only for the initial 60% of the absorbed consumer liquid. In this way, other mathematical models have been proposed to fit the swelling profile in the whole time scale [44].

Aspects such as the type of functional groups present at polymer chains, the network type, the cross-link density, etc., as well as the intermolecular interactions (e.g., H-bond, ionic interactions, etc.), define the physical and chemical properties of hydrogels. They have been thoroughly investigated by infrared (FTIR) techniques [45, 46], X-ray photoelectron spectroscopy (XPS) [47], nuclear magnetic resonance (NMR), and Mossbauer spectroscopies [48]. Differential scanning calorimetry (DSC) and thermogravimetry (TGA) have been used for analysis of thermal behavior [49], while the crystallinity of the hydrogels have been evaluated mainly by small and wide X-ray diffraction measurements (SAXS and WAXS) [49–51]. Different methods have been effectively used for evaluating mechanical properties of the hydrogels and hydrogel composites. For example, the elastic modulus (E) can be evaluated using data collected on a texturometer equipment

that allows to correlate the necessary force (stress) to induce deformation of these soft materials during compressive tests (for example, strain-stress measurements of compression). In addition, rheological measurements should be cited here [52].

Very important parameters, such as the size of pore and the porous distribution of hydrogel and hydrogel composites, as well as the dispersion of the loads inside the matrix, can be obtained from the images obtained from sample under stress using microscopies techniques. Atomic force microscopy (AFM) in its different modalities is often used to evaluate the surface morphology and topography of hydrogel and hydrogel composites [53, 54]. Finally, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) [54] are also very often techniques for analyzing the structure of hydrogels. In this way, lyophilization of swollen hydrogels and further fracture are important strategies for analysis of pore size and its distribution within the 3D matrix.

2. Chitosan obtainment through chitin deacetylation

Chitosan is a β-(1→4)-D-glucosamine copolymer obtained from chitin [55]. The main sources for chitin extraction and production are crustaceous shells, mollusks, insects, silkworm chrysalides, and microorganisms [56]. Chitin is commonly synthesized in six steps: (i) pretreatment, (ii) demineralization, (iii) deproteinization, (iv) bleaching, (v) deodorizing, and (vi) drying [57]. In the pretreatment processes, washes of the specific chitin source are carried with distilled water, aiming to remove vegetal compounds, organic tissues, contaminants, clays, and soil residues [58]. Demineralization process is carried out using diluted hydrochloric acid solutions under heating, aiming to decrease ash and mineral residues as calcium and magnesium. Other common reagents that may be used for the demineralization of chitin sources are nitric, sulfur, and acetic acids. The deproteinization process is performed using diluted sodium hydroxide solution for the removal of proteins. This process must be performed after the demineralization process. Reagents often employed in the deproteinization processes include sodium carbonate, potassium hydroxide, sodium phosphate, and calcium hydroxide [58]. Bleaching and deodorizing processes are responsible for removing color pigments and improving the taste of the prepared polysaccharide, respectively. Both processes can be performed by using diluted sodium hypochlorite solution [55]. Mixture containing sodium hypochlorite and hydrochloric acid needs sometimes to be used [55]. After each process, subsequent washes with distilled water must be performed, aiming to neutralize the final polysaccharide. Drying processes either by lyophilization under −60.0°C or in an oven at temperatures ranging from 60.0 to 80.0 ± 1.0°C are commonly performed [59].

Chitosan is widely prepared from the chitin deacetylation using concentrated sodium hydroxide solution containing sodium borohydride with the aim of avoiding the polysaccharide degradation [60]. Many primary amino groups are removed from the chitosan molecule during hydrolyze reaction, resulting in polysaccharide chains with different sizes [61]. In this case, the molar mass distribution is influenced for parameters such as extraction time, temperature, reagent concentration, and atmospheric conditions. So, chitosan molecules can have different molar masses as compared to parent chitin. Beyond this, deacetylation degrees and viscosities are influenced by deacetylation process that may significantly affect the performance of the final polysaccharide [62]. Some important parameters that are monitored during the obtainment of chitosan with high deacetylation degree are temperature [63], concentration of sodium hydroxide, and step numbers evolved on chitin modification reaction [64]. Enzymatic deacetylation of chitin for the

production of chitosan is an excellent alternative to offset some disadvantages of the chemical deacetylation process such as high energy consumption, generation of acid and alkaline residues, and environmental concerns [59]. Currently, 1 ton billion of chitin and chitosan are annually produced, around the world, by biological processes. The chitin and chitosan production is industrially important, as these polysaccharides are not found in high amounts in the natural environment due to their high biodegradability. Hydrolytic enzymes such as lysozyme, chitinase, chitin deacetylase, and chitosanase are commonly present in body tissue of animals, plants, and soil, avoiding the bioaccumulation of chitin and chitosan in the natural environment. It is estimated that approximately 50,000 tons per year of chitin is worldly produced with 30,000 ton per year from crustaceous shells, excluding the Krill that has potential to produce 56,000 tons per year of chitin. Depending on the extraction method of chitin and production of chitosan, it is possible to recovery proteins, astaxanthin and carotenoid [65].

The main source of chitosan production is from chitin. For producing 1 kg of chitosan from shrimp shells with 70% deacetylation degree, 6.3 kg of hydrochloric acid, 1.8 kg of sodium hydroxide, and 1400 L of water are necessary. The yield for producing chitin from insects, shrimp shells, and silkworm chrysalides ranges from 1.4 to 2.0, 10 to 15, and 6 to 10%, respectively [55]. Commercial chitosan has deacetylation degree ranging from 70 to 95% and molar mass from 1.0×10^4 to 1.0×10^6 g mol^{-1} [59]. The acetylation reaction can be used for obtaining chitosan with deacetylation degree around 50% and high solubility in water [66]. Chitin and chitosan are commercially produced in India, Japan, Poland, Norway, Australia, and China with costs that are dependent on the physical and chemical properties and desired application [55].

Chitin is extracted from different sources in α-chitin, β-chitin, or γ-chitin forms as indicated in **Figure 1a–c**. The α-chitin is the most abundant form of chitin that is commonly found in crab, shrimp, and lobster shells. It is rarely found in insects and fungi. β-Chitin is a rare form that can be also found in insects, chrysalides, crustaceous, and fungi. However, β-chitin is commonly found in squids. Finally, γ-chitin is found in cocoons of insects [55]. The α-chitin, β-chitin, and γ-chitin are identified by the position of acetyl groups in the molecular structure. The α-chitin is formed by repeating polymer units containing acetyl groups in opposite sides, alternating their position to each monomer (**Figure 1a**). The β-chitin has acetyl groups alternating to each two monomers (**Figure 1b**), while the γ-chitin has two acetyl groups in the same side of two monomers, followed by one acetyl group of the opposite side in the third monomer (**Figure 1c**) [67].

2.1 Properties of chitosan

CS is a nontoxic (DL_{50} of 16 g kg^{-1}, studies *in vivo* using rats), biodegradable, biocompatible, antiallergenic, anticoagulant, antifungal, and antimicrobial polysaccharide. These properties are important to apply CS as a biomaterial in medicine, pharmacy, and so forth [68]. Many biomaterials are developed by using CS as solid support due to its feasible biological properties. For instance, capsules of controlled drug release and adhesive films have been registered by the US Food and Drug Administration (FDA) for human applications. Moreover, CS-based biomaterials can be employed in controlled drug release systems, wound healing, filtration membranes, and so forth [66]. CS has been studied for the synthesis of medical biomaterial [69] due to its alkaline characteristic. As CS can be a zwitterionic polysaccharide that contains cationic/anionic groups in its molecular structure, it is efficient for the controlled drug release systems [69], water and wastewater treatment [70], and immobilization of enzymes [71]. The amino groups in the CS

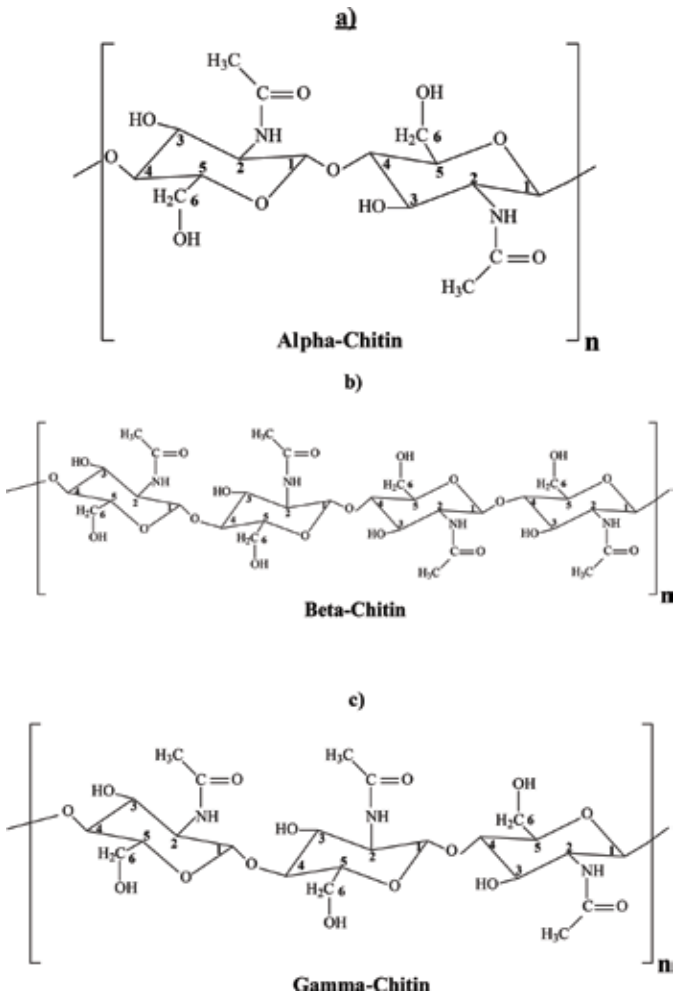

Figure 1.
(a–c) Molecular structures of α-chitin (a), β-chitin (b), and γ-chitin (c).

molecules have pKa values of approximately 6.5, which are deprotonated in higher pHs. Thus, the charge density depends on the pH value and deacetylation degree [55]. CS/silver zeolite composite films can be used for producing burn dressings [72], CS-hydroxyapatite composites can be used for hard tissue regeneration [73], and composite of CS/montmorillonite can be used as matrix for prolonged delivery of some nitric oxide donor compounds [74]. The application of CS as support for the synthesis of composite biomaterials depends on its physiochemical properties such as deacetylation degree and molar mass [75]. Composite materials based on polysaccharides are generally formed by blending either inorganic or organic species to polymer molecules. These composites may be synthesized by electrospray [76], sol-gel process [77], thermomechanical process [78], solvothermal method [79], precipitation and coprecipitation methods [80], solvent casting and evaporation process [81], simple mixing and heating method [75], biomimetic method [82], alternate soaking method [83], and so forth. Composite materials have physiochemical properties different from those ones of initially individual materials and depend on the type of the formed product. For instance, CS-magnetite composite has biodegradability, biocompatibility, mechanical resistance, and magnetic sensibility [84], and the CS-hydroxyapatite composite is pH-sensitive

[85]. CS-iron (III) chloride composite membrane has excellent permeability for different fluids [86]. CS-carbon nanotube composite is mechanically resistant [87]. Carboxymethylated-CS composite protected with ruthenium nanoparticles was synthesized with good thermal stability [88]. Graphene oxide-CS nanocomposite films are formed with excellent mechanical and thermal properties [89], and montmorillonite-CS nanocomposites are produced with excellent biocompatibility and antimicrobial properties [89]. Moreover, CS-based composites were synthesized to be used as microcapsules in drug release systems [90], CS-porous-silica [91] and nanohydroxyapatite-CT derivative composites for bone regeneration [92], CS-zirconium composite as precursor of zirconium carbide [93], CS-barium sulfate composite fibers for endovascular prosthesis, fibrous embolic agent, bone substitution, and X-ray marker [89], CS composite reinforced with carbon nanotubes for biomedicine [78], and CS-hydroxyapatite-glycopolymer/cloisite composite for biomedical applications [94].

2.2 Main chitosan derivatives of technological interest

Considering the vast number of functional groups available on the CS chains and the myriad of chemical compounds that can react with such groups, it is consensual that a plenty of possibilities regarding the use of CS derivatives as precursor materials for the synthesis of hydrogels applicable in drug delivery. There are some methodologies that allow these modifications to occur in CS, such as carboxymethylation, acetylation, and alkylation [7]. In this way, CS derivatives may appear, which seek to improve some properties of this polymer. Among them, the improvement in water solubility is often investigated [6, 95].

From the carboxymethylation reaction, a water-soluble CS derivative can be obtained over a wide pH range [95], and among those obtained so far, carboxymethyl CS stands out because it contains ether groups, and the -COOH and -NH_2 groups [95]. Similarly to CS, the carboxymethylated-CS, also stands out because it exhibits interesting characteristics, such as antifungal activity, low toxicity, and membrane properties, which allows its application for drug release [96].

Quaternized CS through successive methylation in -NH_2 groups [97] has been proposed using different methodologies, methacrylated glycol CS [98] and sulfated CS [99].

The methylation in -NH_2 groups is a very interesting strategy allowing the N atoms be permanently charged with positive charges as -$N(CH_3)_3^+$. Such increase in positive charges allows the material (N,N,N-trimethylchitosan, TMC) be soluble in water in the whole range of pH [100]. Beyond this, the positive charges help for the occurrence of interactions of TMC and the membrane of gram-negative bacteria. So, the TMC has pronounced antibacterial activity [101]. There are several methodologies for synthesis of TMC [101–103]. The following methodology is often used for quaternization of CS: the dispersion of CS in N-methylpyrrolidone containing sodium iodide/methyl iodide in the presence of sodium hydroxide. At final, the iodide counterions of the reaction product are exchanged with chloride for obtaining a more stable salt. Some reviews of CS chemistry are recently published [101–103].

2.3 Methodologies used for obtaining chitosan derivatives aiming at biomedical applications as hydrogels

A large variety of synthetic methods have been utilized for the fabrication of hydrogels based on CS and CS derivatives. The degree of acetylation and/or polymerization of CS-based materials have been widely considered as the critical parameters for controlling their outstanding properties [104–106]. These

structural changes, in principle, lead to completely new properties as well as a significant improvement in water solubility [6, 95]. From the carboxymethylation reaction, a water-soluble CS derivative can be obtained over a wide pH range [95], and among those obtained thus far, carboxymethyl CS stands out because it contains ether, -COOH, and -NH_2 groups [95]. Similar to CS, the carboxymethyl CS derivative also stands out because it exhibits interesting characteristics such as antifungal activity, low toxicity, and membrane properties, which allow its application in drug delivery systems [96]. In general, it is well known that a high degree of deacetylation and a narrow polymer molecular weight distribution are critical parameters for controlling the particle size distribution [107]. Evidently, the size of the particles has significant influence on the biomedical applicability of CS-based hydrogels [106, 108–111].

It is well known that the hydrogel matrix structure is generally created from the hydrophilic groups (or domains) present in a natural or synthetic polymeric network upon hydration in an entirely aqueous environment [112]. On the other hand, it is also well known that the various properties exhibited by hydrogels (such as self-healing, biodegradability, swelling degree, mechanical resistance, and so on) are intrinsically related to the physical or chemical cross-linking methods [113, 114].

Hence, the physical cross-linking of hydrogels leads to the formation of a non-permanent network due to noncovalent interactions (e.g., hydrogen or electrostatic bonds and physical entanglements) [22, 115–117]. Consequently, physically cross-linked hydrogels can be formed via ionic interactions of charges, that is, by utilizing the graft copolymers, crystallization, as well as the formation of different stereo-complex forms [117, 118]. Notably, CS can be self-cross-linked when the initial polymer concentration is beyond the critical concentration (C*) for chain entanglement. In this condition, a precise balance between the hydrophilic and hydrophobic interactions is reached [114]. These values may be achieved after decreasing the apparent charge density by solvent evaporation or changing the dielectric constant of the medium [119]. Phosphate-bearing molecules such as polyacrylic acid, sodium alginate, heparin, and polyglutamic acid are the common anions used for physically cross-linked CS [120]. Sarmento et al. have reported the preparation of alginate/CS nanoparticle hydrogels by ionotropic pregelation of an alginate core followed by CS polyelectrolyte complexation, for biomedical applications [121].

On the other hand, it is well known that chemical cross-linking methods, including free-radical polymerization, condensation reactions, and addition reactions, provide good mechanical strength while preserving the hydrogel properties [114, 122–125]. Particularly, chemically cross-linked hydrogels have more uniform properties as compared with those of physically cross-linked hydrogels. For biomedical applications, immense attention must be paid to the cleavage of the cross-linker (which can be done either by chemical or enzymatic methods) to avoid the release of toxic compounds [114]. However, the concentration of the cross-linking agent and the cross-linking reaction time are the main factors that could affect such an approach [126]. For example, the photopolymerization process is started by free radicals produced by radiation (e.g., UV, visible light irradiation, gamma irradiation, or electron beam) that, in turn, promote attacks on the double bonds of monomers and propagate the radical attack, creating a chemically cross-linked polymer network [114, 127–129]. Furthermore, the cross-linking reactions can occur more efficiently on the surface than in the polymer center, probably due to the steric effects [130]. However, the principal disadvantage of using CS-based hydrogels, mainly with regard to their biomedical applicability, is probably the poor reproducibility of the particles formed [106]. Hence, it is believed that a deeper understanding of these methods as well as the development of new strategies is fundamentally necessary and represents

a prerequisite to obtaining optimized CS-based hydrogels with entirely new functionality and properties for a broad variety of applications in emerging biomedical technologies.

3. Uses of hydrogels based on chitosan and chitosan derivatives

The well-known and attractive properties of CS rank this polysaccharide as a safe choice to engineer novel materials applicable as biomaterials. Proof of this is the great volume of studies and review papers published in the literature dealing with this subject [131–133]. As noticed, various research groups are focused on the development of materials based on CS or CS derivatives, aiming their use in the most varied areas and subareas of pharmacy, medicine, and biochemistry [134–136]. In particular, hydrogels synthesized from CS or CS derivatives have been extensively utilized as delivery systems (for drugs, gene factors, and/or protein delivery), dressing devices, and scaffolds for cell/tissue culture [134–137]. Considering these applications, some notable finds and characteristics related to CS and CS-derivative-based hydrogels will be discussed below.

3.1 As carriers of drug

Drug delivery systems that make use of carriers based on CS are of particular interest because this polysaccharide exhibits three paramount features: a mucoadhesive nature, ability to transiently open epithelial tight junctions, and biodegradability [138]. The mucoadhesion ability can be assigned to different interactions (electrostatic attraction, hydrogen bonding, and hydrophobic effects) that take place between CS and mucosa [139]. Biodegradability property is attributed to CS because it is degraded by the two naturally produced enzymes: lysozyme (present in various mucosal surfaces) and chitinase (present in the intestinal flora) [117]. Due to this, CS-based carriers are able to deliver drugs across various well-organized epithelia (e.g., ocular, nasal, buccal, pulmonary, and intestinal) in a controlled manner [140].

In light of this, carrier systems based on CS have been formulated in different forms (tablets, particles, films, membranes, gels, and so forth) using a vast number of protocols [141, 142]. CS hydrogels can be prepared via physical and/or chemical cross-linking processes, and its functional groups allow grafting synthetic monomers on its backbone [143, 144]. Generally, the association of CS with other synthetic polymer or its grafting with vinylic monomers (acrylic acid, acrylamide, etc.) is architected in order to increase the liquid uptake capacity and to enhance the mechanical properties of the hydrogel [117, 145]. Of course, the cross-linking process has a direct relationship with the final properties of the hydrogel, which allows, for example, tailoring hydrogel properties according to the application. Furthermore, CS hydrogels usually show responsive properties, mainly pH-dependent properties [146]. In acidic condition, CS hydrogels show high liquid uptake capacity favoring the drug release by diffusional processes [147]. On the other hand, hydrogels synthesized from the polyelectrolyte complexation of CS (polycationic) with polyanionic polymers (e.g., alginate, pectin, chondroitin sulfate, among others) or anionic salts (e.g., potassium tripolyphosphate) can be disrupted by changing the pH of the release medium [148–150]. In this case, the drug loaded into the hydrogel is released owing to erosion/disruption process. This pH-sensitive is useful to modulate the drug release profile, which prevents unwanted side effects such as burst or time lag release, and to promote targeted drug release.

CS hydrogels also show versatility regarding the drug loading process because drugs can be encapsulated *in situ* during the hydrogel synthesis procedure (this increases the loading efficiency) or they can be loaded in the hydrogel by sorption processes. Generally, drugs adsorbed in the hydrogel are easily released due to the weak interaction forces between the drug and the hydrogel matrix [151]. More recently, the incorporation of filler materials (e.g., clays, metallic particles, graphene, etc.) within the CS matrix has been investigated, aiming to increase the drug encapsulation efficiency (mainly for hydrophobic drugs) and a better control of the release profile [114, 152, 153]. Another strategy adopted by several researchers to enhance the encapsulation efficiency and to improve the release profile is the synthesis of hydrogels using CS derivatives. In general lines, CS derivatives have been synthesized in order to solve some issues related to the use of raw CS in the design of delivery systems. For example, CS shows poor solubility under neutral conditions, which limit its processability and reactivity in biological solutions (pH 7.4) [154]. Moreover, CS is insoluble in the most part of organic solvents hindering the loading and delivery of hydrophobic drugs from CS-based hydrogels [58].

Currently, various studies report the synthesis of hydrogels from water-soluble CS derivatives, such as carboxymethyl CS, quaternized CS, (such as TMC) zwitterionic CS, oligomerized CS, and so on, and their use as drug delivery systems [155, 156]. The chemical modification of the CS backbone without modification of its initial backbone (to preserve the original properties) is a reliable strategy to overcome the shortcomings related to the use of raw CS. For instance, owing to their properties, CS derivative hydrogels can offer prolongation of the contact time between the drug and the absorptive sites in the mucosa and slow and continuous drug release [157]. Furthermore, the grafting of specific chemical modifiers on CS makes possible new cross-linking routes (click reactions, self-assembling, etc.) allowing, for example, the synthesis of *in situ* hydrogels [158, 159]. The use of CS derivatives to synthesize hydrogels may impart new properties to this material (antioxidant or bactericidal properties, for instance), which enlarge the field of the potential applications of such materials, especially as carriers for drug delivery [160, 161].

3.2 As wound healing

Any internal or external stimulus that damages the anatomy of a tissue and compromises its function generates a wound [162]. In general, the healing process of a wound can be described in four phases named vascular response, inflammatory response, proliferation, and maturation [163]. Therefore, a proper wound dressing should present specific features to act in each abovementioned phase to promote a satisfactory healing process. Such features include blood clotting, inflammation fluids absorption, barrier against infection, protection against friction, support for cell attachment and growth, hydration, air permeability, and others [164].

CT, CS, and CS derivatives have been studied in several preparations for treatment of wounds and in tissue regeneration especially due to their biological properties including hemostatic, antibacterial, antifungal, biocompatibility, biodegradability, lack of toxicity, adhesive, and more [165, 166], all those required by a proper dressing. Currently, distinct manufacturers make available wound dressings based on CT and its derivatives under many trade names (Syvek-Patch®, Beschitin®, Tegaderm®, Chitodine®, Trauma DEX®, and Talymed®, among several others) [164].

CT/CS-based wound dressings have been reported in a variety of forms, for example, membranes, sponges, scaffold, fibers, and so on. However, hydrogels are probably the most promising materials for wound dressing because of their similarity and physical chemical properties to the extracellular matrix, which allows cell

diffusion and proliferation. Either chemically or physically, hydrogels can be readily prepared from CS, as extensively reported [167–169].

Zhang et al. demonstrated the wound dressing based on CS loaded with superoxide dismutase (SOD) enzyme could effectively improve the healing process in chronic wounds in rat models [170]. Hydrogels based on electrostatic interaction of the cationic CS and the anionic heparin and poly(γ-glutamic acid) were successfully prepared and characterized. The best composition was loaded with SOD and applied to the wounds. The continuous release of SOD prevented cell oxidative damage due to excess of O_2^- and improved the healing process.

Hydrogels based on CS and Ag nanoparticles were proved to be efficient restructuration of epithelium and collagen deposition, effectively accelerating the wound healing [171]. CS hydrogel was prepared by the freeze-thawing process using the system LiOH:KOH:urea:H_2O as solvent. In this case, the Ag nanoparticles worked as both fillers for improving the mechanical properties of the hydrogel and antimicrobial agent. The mechanism of bactericidal activity was a combination of cell membrane disruption and DNA binding, preventing bacteria replication.

Other study reported hydrogels based on freeze-thawing of solution of CS with poly(vinyl alcohol) PVA, sodium alginate (SA) or Pluronic F68 [172]. The dressings based on CS and Pluronic were more effective in the healing probably due to its porous morphology and the level of moisture based on comparative histology of healing effects of hydrogels after 15 days of inflicted wounds. Chemically crosslinked hydrogel based on glycol CS and glycidyl methacrylate was obtained via visible light radiation [173]. The endothelial and fibroblast growth factors-loaded hydrogel accelerated the wound healing *in vivo* models. In general lines, several reports have demonstrated the CS-based hydrogels either unloaded or loaded (bactericidal agents, growth factors, etc.) played an important role in the wound by direct acting in different phases of the healing process. The authors also refer to the following review papers focusing on CT/CS hydrogels as wound dressing for further reading [162, 174, 175].

3.3 As protein delivering

Mucoadhesive systems such as CS-based matrix are used in order to increase the protein residence time at the activity site [176]. However, CS can be dissolved in the stomach due its solubility at low pH condition (pKa 6.5) [177], causing release and denaturation of protein [178]. Therefore, the solubility of CS can be prevented by its association with anionic polymers including alginate [179], pectin [180], gelatin [181], and carrageenan [182] to create hydrogels as oral protein delivery carriers.

When proteins are physically incorporated in CS-based hydrogels, their release can occur by diffusion, erosion/degradation, swelling, or a combination of these mechanisms [183]. In order to slow down the degradation rate of hydrogels and prevent burst protein release, polycaprolactone can be incorporated in CS-based hydrogels. Shamloo et al. [181] developed poly(vinyl alcohol)/CS/gelatin hydrogel incorporating polycaprolactone microspheres for delivery of basic fibroblast growth factor (bFGF). Poly(vinyl alcohol) and gelatin were used to improve mechanical properties and increase cell adhesion, respectively. The bFGF release accelerated the wound healing process with polycaprolactone incorporation into hydrogel [181].

The control protein release can also be enhanced with use of mineralized inorganic compounds combined with CS-based hydrogel network [184]. Salama et al. [184] reported the synthesis of CS-g-poly(3-sulfopropyl methacrylate) hydrogel mineralized with calcium phosphate for bovine serum albumin (BSA) release. The mineralization decreased the permeability of the loaded protein and controlled the release proteins [184].

Hydrogels prepared from polysaccharides containing -COOH groups such as carboxymethyl CS may undergo shrinking in acidic condition (e.g., gastric juice) and swelled in neutral or alkaline environment (e.g., intestinal juice) due to electrostatic repulsion between the ionized acid groups present on polysaccharides chains [185]. Therefore, carboxymethyl CS-based pH-sensitive hydrogels can be used to direct protein release, including BSA and insulin (INS), into intestinal region [178]. Zhang et al. [185] synthesized carboxymethyl CS-g-polyacrylic acid for insulin delivery. The results showed that 16.3 and 93.2% of insulin was released at pH 1.2 and 7.4, respectively [185].

Carboxymethyl CS can also be associated with xanthan to prepare hydrogel to fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA) release [186]. Huang et al. [186] developed aldehyde xanthan/carboxymethyl CS hydrogel to FITC-BSA release. Moreover, a signaling protein, endothelial growth factor (VEGF), was loaded to accelerate abdominal wall reconstruction. The BSA-FITC release was stable within 10 h. After VEGF incorporation, the abdominal wall reconstruction was accelerated [186].

Besides carboxymethyl CS, other CS derivatives such as quaternized CS [97], methacrylated glycol CS [98], and sulfated CS [99] have been used as oral protein delivery carriers. Quaternized CS has been used in hydrogel preparation for protein delivering due to its several properties such as absorption enhanced across intestinal epithelial for hydrophilic drug delivery [187], low toxicity [188], capacity to open the junctions between epithelial cells, which allow greater transport of hydrophilic compounds [187], better mucoadhesive [189], and antibacterial activity [190] than CS. Wu et al. [97] developed hydrogel-based N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride (HTCC) and poly(ethylene glycol) (PEG) for insulin release. Hydrogen bonds among amino groups present in insulin and hydroxyl groups present in PEG or HTCC allowed slowed drug release. The results showed that the hydrogel can be used as nasal delivery carrier for protein or peptide drugs [97].

The controlled protein release from heparin-based hydrogels has also been studied due to its strong binding capacity, which attenuates the burst protein release such as rhBMP2 [191]. Therefore, some CS derivatives have been used to mimic heparin to protein delivery, including sulfonated molecules incorporated into methacrylated glycol CS hydrogels [98] and sulfated CS hydrogels [99]. Kim and Chung [98] developed methacrylated glycol CS (MeGC) hydrogel used to mimic heparin to stabilize bone morphogenetic protein-2 (BMP-2) and to enhance osteogenesis by the addition of poly-4-styrenesulfonic acid (PSS) or poly-vinylsulfonic acid (PVSA) into hydrogel. The addition of PSS or PVSA reduced the initial burst and increased the recombinant human BMP-2-induced osteogenesis differentiation, indicating efficient protein delivery [98].

However, sulfated CS, 2-N,6-O-sulfated CS, besides to mimic heparin, has been shown to enhance BMP-2 bioactivity than heparin [192]. In this context, Cao et al. developed rhBMP-2-loaded 2-N,6-O-sulfated CS nanoparticles and hydrogel photopolymerizable incorporating rhBMP-2-loaded 2-N,6-O-sulfated CS nanoparticles. The composite gel system showed gradual and more release than nanoparticle system. The use of 2-N,6-O-sulfated CS enhanced the bioactivity of released rhBMP-2 [99].

The protein residence time at activity can also be improved by thiolation of CS due to the covalent bond formation between subdomains with high cysteine content in the mucus glycoproteins and thiol groups [193]. Liu et al. [194] developed thiolated CS-TBA/hydroxyapatite(HA)/beta-glycerophosphate (β-GP) hydrogel. The BSA protein residence time at activity using such hydrogel was higher than unmodified CS system CS/HA/β-GP [194].

In order to increase protein half-life and to improve the biocompatibility of CS, polyethylene glycol (PEG) can be used to prepare chemically modified CS hydrogels [195, 196]. Farahani et al. [197] developed semi-interpenetrating polymer network CS-PEG-acrylamide hydrogels for closed-loop insulin delivery. Moreover, catalase and glucose oxidase were loaded into hydrogel to make an intelligent protein carrier. The increasing of PEG increased the swelling ratio, protein loading capacity, and entrapment efficiency. The increase in insulin release was observed with increase in the glucose level, indicating that the hydrogel has a good responsiveness to the glucose concentration [197].

3.4 As scaffolds for cell growth in tissue engineering

Scaffolds provide an intermediary template for neotissue/organ formation as well as temporary artificial extracellular matrices [198, 199]. Scaffolds-based extracellular matrices are characterized by pore volume fraction of typically 0.90–0.95 or higher and pore diameter in the range of 5–500 μm [199]. Generally, the use of scaffolds in tissue engineering should have several properties, such as biocompatibility, cell proliferation, controlled swelling, antimicrobial, biomineralization, biodegradability, stability, porosity, adhesion, and protein absorption [200–202].

3.4.1 Chitosan hydrogels in bone tissue engineering

CS has been widely used for bone tissue engineering due to its capacity in providing growth and deposition of matrix with high mineral content by osteoblast cell culture [203]. As already mentioned in this chapter, CS can be associated with anionic polymers, such as pectin to create physical hydrogels [204]. Moreover, metallic nanoparticles (NPs) coupled to hydrogels can be used to induce cell growth [205, 206]. Tentor et al. related scaffolds-based CS/pectin/gold nanoparticles for bone tissue engineering [204]. The hydrogels were cytocompatible with several cell types, such as normal kidney epithelial cell, HPV-16-positive human cervical tumor cells, epithelial colorectal adenocarcinoma cells, and murine macrophage cells. Regarding the cell viability assay, such hydrogels possess potential for applications in the bone tissue engineering to promote proliferation and growth of bone cells (e.g., MC3T3-E1).

Hydroxyapatite (HA) is the major inorganic component of bone [207]. So, nanohydroxyapatite (n-HA) has been used in bone tissue engineering due to its osteoconductivity and bioactivity [208]. However, n-HA has poor shape ability [208]. Mechanical property of HA can be improved by its association with hyaluronic acid [209] and glycol CS [210], for fabricating scaffold hydrogels to apply in bone tissue engineering. Huang et al. [210] developed n-HA/glycol CS/hyaluronic acid hydrogel composite as scaffold for bone tissue engineering. The porosity of hydrogel increased with increase in the HA concentration. *In vitro* cytocompatibility tests were carried out using MC-3T3-E1 cells. After 7 days co-incubation, cells were attached, and spreading on scaffolds and increasing in cell aggregation were observed. The scaffolds were cytocompatible and nontoxic, so these results are suitable for bone tissue engineering application [210].

3.4.2 Cardiac and nerve tissue engineering

CS and CS associated with biopolymers [211], including gelatin [212], collagen [213], and alginate [214], have been used in developing hydrogel scaffolds for cardiac tissue engineering applications [215]. Gelatin scaffolds are susceptible to fast degradation, while gelatin/CS composite scaffolds are structurally stable in cell

culture media [212]. However, these polymers may be associated with polycaprolactone (PCL), to provide sufficient tensile strength to work in the ventricular wall [216]. Pok et al. [216] developed 3D scaffolds composed of self-assembled PCL sandwiched in a gelatin-CS hydrogel for reconstruction of congenital heart defects. The compressive modulus of the hydrogel was similar to native tissue, and migration of neonatal rat ventricular myocytes (NRVMs) was observed [216].

Quaternized CS can also be used in tissue engineering due to its enhanced antibacterial activity and more solubility than CS [217]. Similar to CS, quaternized CS possesses properties of biocompatibility, low toxicity, biocompatibility, and biodegradability [188]. Zhao et al. [218] developed antibacterial conductive hydrogel scaffolds using quaternized CS-grafted polyaniline with oxidized dextran as cross-linker. The use of polyaniline into quaternized CS copolymer decreased the cytotoxicity, enhanced the antibacterial activity, and stimulated proliferation of C2C12 myoblast cells, by a synergistic effect, as compared with quaternized CS hydrogel. These scaffold showed great potential as scaffold for muscle, nerve, and cardiovascular repair [218].

3.4.3 Cartilage and skin tissue engineering

The cartilage tissue engineering can involve the seeding chondrogenic cells in scaffolds for cartilage repair [136]. Glycosaminoglycans and type II collagen are components commonly found in the cartilage-specific extracellular matrix, which may stimulate the chondrogenesis [219, 220]. CS has structural characteristics similar to glycosaminoglycans and can mimic their functional behavior [221]. Several polymers, such as alginate [222], glycosaminoglycans [223], collagen [224], and carrageenan [225], have been associated with CS for cartilage engineering tissue applications. Hong et al. [224] developed an injectable composite scaffold obtained from collagen-coated polylactide microcarriers/CS hydrogel. Collagen-coated polylactide microcarriers enhanced the mechanical properties of the scaffold. The cell metabolic activity increased rapidly before 9 days of *in vitro* chondrocytes growth within the scaffold. After 9–12 days, confluent cell layers were formed. The composite scaffolds showed great potential for tissue engineering applications, particularly in orthopedics [224]. Liang et al. [225] developed rubbery CS/carrageenan hydrogels prepared by electroneutrality system as cartilage scaffold. The results showed pH- and salt-responsiveness, hierarchically porous architecture, and great mechanical properties. The hydrogels enhanced the viability and the adhesion of TDC5 cells [225].

Mechanical properties and biological activities may be altered by chemical modification of CS. *N*-succinyl CS possess biocompatibility and long-term retention *in vivo* [226]. Kamoun [227] developed *N*-succinyl CS-dialdehyde starch hydrogels for cartilage repair. The hydrogel was relatively stable, and the hydrolysis rate was limited with a high N-succinyl hydrogel composition without any by-products in physiological conditions. The adhered human gingival fibroblast cell number on hydrogel surface was improved by *N*-succinyl CS content in hybrid hydrogels. This hydrogel showed great potential to be used as injectable scaffold for cartilage repair [227]. *N*-succinyl CS can also be associated with other polysaccharides such as hyaluronic acid to prepare hydrogels, as an injectable scaffold, to improve biocompatibility and biodegradation [228].

Regarding skin tissue engineering, CS has been used in the preparation of scaffold hydrogel due to its biocompatibility [229], biodegradability [230], antibacterial properties [231], and hemostatic activity [232], stimulating fibroblast growth and accelerating tissue regeneration [233]. Franco et al. [234] developed CS/gelatin hydrogel scaffolds for skin engineering. The hydrogel showed a high porosity and

supported fibroblast cell proliferation. Notably, the scaffold with lowest cross-linker content swelled more than 600% of its dry weight [234].

Carboxymethyl CS can also be used in skin tissue engineering. This polysaccharide is biodegradable, biocompatible, and more bioactive than the raw CS [235]. Jaikumar et al. [236] developed alginate/O-carboxymethyl CS composite hydrogel scaffolds for adipose tissue regeneration with incorporation of fibrin nanoparticles. The use of fibrin nanoparticles promoted adhesion and proliferation on hydrogel. Human adipose-derived stem cells cultured on this hydrogel scaffold supported cell growth [236].

3.5 Other technological applications

The main reasons to propose different applications of hydrogels is attributed to their considerable volume change capacity in response to little surrounding alterations, such as electric and magnetic fields, solvent, pH, ionic strength, and temperature. Specifically, in biomedical application, it is desirable that the hydrogels may mimic the performance of human organs in response to some alterations in the environmental conditions such as pH, temperature, enzymes, and electric field [237].

Beyond technological applications mentioned in this session, hydrogel and nanocomposites based on CS and their derivatives have been studied for other several applications, such as contact lenses [238, 239], enzyme and cell separations and immobilizations [71, 240–243], DNA delivering [244], cartilage and skin regenerations [245–249], biosensors [250–252], submucosal fluid or injections [253–257], tissue engineering [258–263], postoperative adhesion prevention [264–266], cancer treatment [267–270], orthopedic applications [271, 272], artificial muscles [273], and others. Important characteristics of some cited applications are discussed as follows.

Among the different areas that the hydrogels would be used, the chemotherapy treatment is one of the most important due to great side effects that chemotherapy treatment provokes for patients, such as highly toxic, poor specific drugs, insufficient availability of drugs to the tumor, and others. In this way, the usual chemotherapy treatments have been changed to controlled/localized drug release technology using polysaccharide hydrogel as carrier vehicles [274]. In that work, the authors concluded that the CS/polyvinyl alcohol hydrogels have great potential to be used for the treatment of cancer because these 3D matrices had an antiproliferative effect and great capacity of the inhibit angiogenesis. In the work described by Pattavarakorn et al. [273], the authors found that the electroactive performance of the polythiophene/CS/carboxymethyl CS (PTh/CS/CMCS) as conductive hydrogel is dependent of the hydrogel composition, and the hydrogel prepared with 3:2 CS:CMCS ratio exhibited highest electric field response sensitivity. According to Tan et al. [275], CS and its derivatives are one of the most appropriate materials for enzyme immobilization because of a high specific surface area (high enzyme loading), nontoxicity, and biocompatibility, improving their stability and reusability.

Ulutürk and Alemdar [252] reported that the electroconductive hydrogel has great applicability as biosensor because this material can overcome some disadvantages of the inherently electroconductive polymers like toxicity and can also contribute to decreasing the release of the conductive polymer to the body. As mentioned before, hydrogels have been used in the biomedical area such as contact lens due to the possibility of increase up to 50% of drug bioavailability, which contributes to minimizing the collateral effect that this drug would provoke in the patient. However, the main disadvantage is that these carrier vehicles presented burst release in the first or couple of hours after application. In this way, Åhlén et al. [238] observed that contact lenses based on CS-poly(acrylic acid) nanoparticles and poly(vinyl alcohol) (PVA) hydrogels had greater potential for extended release

during 28 h. Postoperative peritoneal adhesion is one of the serious damages after surgeries, reaching until 67–93% after general surgical abdominal procedures, and as consequence, the patient may have various complications like chronic pain, female infertility, bowel obstruction, and others. *In vivo* studies realized by Song et al. [276] showed that the injectable N,O-carboxymethyl CS-aldehyde hyaluronic acid hydrogel had significant antiadhesion efficacy in a rat repeated-injury model. They observed that after 14 days, the peritoneum is completely recovered without adhesion aspect.

4. Future trends and perspectives

CT, CS, and CS derivatives have been used for wide technological applications, from metallic ions and dye absorbents in environmental to drug carriers in biomedical field. Another important application of CS derivative, for example, the trimethyled CS, is due to its antibacterial properties [277] and, at same time, no toxicity for human and animals. Most of the derivatives are soluble in the whole range of pH, for instance, the trimethyled CS, CS sulfate, and others. So, the drawback related to solubility of chitosan that is limited to acidic conditions (due to the pKa ca. 6.5) is overcome.

The use of CS and its derivatives as hydrogels is one very important issue. The fact of CS being easily cross-linked or doing complexes, forming chemical or physical 3D matrixes, induced plenty of researchers to target themself for producing new materials through different methodologies/strategies/formulations, aiming to intensify some desired properties improving new applications. This is also due mainly to the relatively low cost, abundance, renewability, and biodegradability, among other advantages for using CS and its derivatives. In the last two decades, important technologies have been developed mostly for chemically modifying CS enabling to the preparation of hydrogels with a wide range of desired properties. A lot of examples were given in this review, but a very large window in this issue remains opened [278]. Some highlights, among others, can be given as trends in this field:

- Studies show that functional properties of CS and its derivatives clearly depend on their molecular weight. So, many studies need to be performed to investigate this aspect, because the molecular weight of chitosan depends strongly on the methodology used for CS obtainment from CT or the one used for preparing the CS derivatives.

- Besides the chitosan and its derivatives are not toxic to human or animals, current matter of discussion is whether these biopolymers may have the potential to influence physiological functions or metabolism in the microorganisms [277]. So, huge enforces need to be done in this issue because the molecular weight of CS is dependent on the methodology used for CS obtainment from CT or for CS derivative preparation from raw CS.

- Another important issue is to evaluate if the mixture of chitosan and chitosan derivatives with other polymer (synthetic or natural) affects their low toxicities.

- The future of materials based on CS and CS derivatives is still more promising due to the lack of petroleum. In this way, eco-friendly extraction methods need to be developed. It was mentioned in this review that 1400 L of water is used for extracting and purifying 1 kg of chitosan. So, in the future, water will also suffer eminent lack.

Of course, the understanding of structure-properties-applications relationship in application of CS and CS derivatives as hydrogels will be expanded with more comprehensive studies. Certainly, this will increase the significance of these important soft materials, considering their application.

5. Conclusions

The objective of this review is to update and discuss important aspects related to chitin extraction from different sources and methods for obtaining and purifying chitosan (CS) and for chemically modifying chitosan to obtain CS derivatives with adequate properties. The particular position of the CS and its derivatives is due to the possibility of oil-based products replacement. Several chemical modification methods for CS have also been described in this review as well as for the preparation of hydrogels based on CS or CS derivatives are widely used in the last decades because of the multiple properties allowing many applications. The state of the art is the use of CS and its derivatives combined (or not) with synthetic or natural (sometimes nanostructured) moieties. Although patents and papers mentioning new structures and properties in materials derived from CS and CS derivatives appear in the literature almost every day, this review demonstrates that the window of opportunities in research and development is still opened. The influence of the molecular weight of the CS and CS derivative hydrogels mainly on biological properties can be pointed out as one of the future trends in this field. In addition, environmentally correct methods for extracting CS should be developed taking into account the fact that the water, extensively used in CS extraction/purification, will run out quickly. More comprehensive and in-depth studies will expand the understanding of the structure-properties-applications relationship of CS and CS derivative hydrogels, which will certainly further enhance the importance of this soft material class.

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Chapter 5

HyStem®: A Unique Clinical Grade Hydrogel for Present and Future Medical Applications

Thomas I. Zarembinski and Aleksander Skardal

Abstract

Medicine needs targeted, minimally-invasive delivery of protein-based and cell-based therapeutics to increase efficacy and reduce occurrence and severity of side effects. Local delivery requires a matrix to sequester and protect the medicine until its effect can be realized. The problem is, unlike stable small molecule drugs, proteins and cells cannot be co-packaged with a matrix in a prefilled syringe—they must be mixed with their matrix at the point of care. HyStem hydrogels fix this problem: They are arguably the first commercially available, GMP-qualified biodegradable hydrogels both with the ability to formulate with either proteins or cells in the hospital/surgical suite and with a history of safe use in humans. HyStem is designed to be protein, cell-friendly and *in situ* crosslinkable, permitting homogeneous mixing of therapeutics. One HyStem formulation is 510(k) cleared and another the subject of two European clinical trials. Key applications include localized delivery of therapeutic growth factors, antibodies, and cells. In the future, we envision HyStem's flexibility and clinical use history forming the basis for a new generation of therapeutics. Two examples described here include HyStem's use for patient-derived organoid culture to develop new drugs as well as for bioprinting to manufacture new organs.

Keywords: HyStem, GMP, hyaluronic acid, extracellular matrix, delivery, organoid, bioprinting

1. Introduction

A hydrogel is a crosslinked, water-rich network that can be fabricated from a wide variety of hydrophilic, biological or bio-inspired polymers. Human beings are in fact largely hydrogel: cellular cytoplasm is a hydrogel as is the extracellular matrix that surrounds them [1, 2]. Many body substances are also hydrogels (e.g. mucus [3], blood clots [4]). It can be argued that hydrogels in the human body carry out many of their functions as a result of being water swollen and that the functionality of manufactured hydrogels also relies heavily on this capability They can heal through hydrating and protecting wounded surfaces, fill empty spaces where there used to be tissue in younger days or prior to disease or injury, seal surgical wounds that cannot reintegrate on their own, and deliver therapeutics to places that cannot easily be accessed from systemic routes. A variety of animal and plant-based hydrogels (e.g. gelatin, pectin, carrageenan) are also used in our food supply.

Despite their restorative potential, relatively few hydrogels are currently marketed for human medical use. This is because commercialization is neither cheap nor easy: product development demands years of effort to design a stable product that is user-friendly and cheap enough to sustain commercial requirements [5]; cGMP manufacture is expensive, especially if aseptic vial fill filling is required. Commercialization also requires the requisite quality system to support the cGMP manufacturing as required by FDA 21CFR820. The regulatory path can also be onerous: while hydrogels are generally considered medical devices (Class II or III) by the US FDA, obtaining FDA approval in combination with therapeutics (drugs or cells) can increase the complexity and length of the regulatory journey due to the need for oversight by multiple FDA agencies [6].

A growing focus is in local administration of therapies to not only deliver disease-modifying drugs but also to rebuild damaged tissues [7–9]. Given the arduous path to the clinic, it is tempting to short-circuit the normal manufacturing/quality/regulatory/clinical path by improvising by using marketed hydrogels approved for different indications and derived from commonly-used biological polymers (see **Table 1**). While this approach may be attractive in the short-term, there is little gained in the long term. This is because hydrogels must be re-optimized for each therapeutic. In addition, the regulatory and clinical paths will not be circumvented: these medical devices, like drugs, must be approved independently for each new indication [10–12].

If a short-term fix is required, what is the harm in selecting a hydrogel from **Table 1** to locally deliver a therapeutic? Upon closer inspection, it becomes clear that a non-optimized hydrogel adds additional risk to the success of the therapeutic. HA-only hydrogels (Dermal Fillers, Viscosupplements, Opthalmic and Gynecology hydrogels), must be excluded for anchorage-dependent cells like mesenchymal stem cells (MSC)—they do not provide the requisite cellular attachment sequences required to prevent anoikis [13]. Many hydrogels (Dermal Fillers, Viscosupplements) are also pre-crosslinked within its sterile syringe and are dubbed monolithic [9], preventing homogeneous mixing with drugs or cells. Tissue sealants made from fibrin actually fulfill two key criteria: they contain fibronectin, an effective cellular attachment protein for many cell types [14]. They also form a gel after its components are combined, allowing homogeneous mixing of cells. These sealants however suffer from their lack of staying power:

Class	Example	Key polymer	Marketed state	Cellular attachment sites present
Wound management	Biozel®, Integra® Wound Matrix	Hyaluronic acid (HA), collagen	Uncrosslinked	Yes
Tissue sealants	Evicel®, Tisseel®	Fibrin	Uncrosslinked	Yes
Dermal fillers	Juvederm®, Restylane®	HA	Crosslinked	No
Viscosupplements	Synvisc®, Hyalgan®	HA	Crosslinked	No
Opthalmic	Healon®, Provisc®	HA	Uncrosslinked	No
Gynecology	HyaloGyn®	HA	Uncrosslinked	No

Table 1.
A sample of currently marketed hydrogels.

the endogenous plasmin degrades the hydrogel within a week, providing only brief shelter for the therapeutic [15]. Fibrin is also difficult to use for indications outside of tissue sealing. This is because it is formulated to gel in 3–5 min [16], placing a short and rigid timeframe for mixing with other therapeutics and injecting in the operating room. Collagen-containing matrices (wound management) have collagen which provides requisite cellular attachment sites [15]. The challenge with some wound management devices (e.g. *Integra* flowable wound matrix, **Table 1**) is that the collagen can be granulated. These insoluble collagen particles will not homogeneously mix with its matrix, providing suboptimal cellular attachment. Many also do not form covalent crosslinks, deteriorating their staying power [15].

What is needed is a biodegradable, biocompatible hydrogel platform that is not only customizable but also provides the requisite foundation for streamlined regulatory approval. The HyStem hydrogel platform, a clinical grade customizable hydrogel matrix, fulfills these criteria. While a discussion of key aspects to hydrogel selection and commercialization was described previously [17], this review serves to pick up where it has left off. This book chapter will have three parts: First, an introduction to the history and basics of the technology; Second, a description of the successful uses of the HyStem platform based on our customers and collaborators' published experiences for the past 10 years for both drug and cell delivery. Third, an introduction and description to recent studies in what we believe will provide the springboard to future therapies: HyStem's use for preparing tissue-specific and patient-derived organoid culture to develop new drugs as well as HyStem's use as a bioink for bioprinting to manufacture new organs.

2. Background

2.1 History

The HyStem technology was developed in the laboratory of Dr. Glenn Prestwich in the Department of Medicinal Chemistry at the University of Utah. The goal was to develop a hydrogel platform designed to recapitulate the minimal composition necessary to obtain a functional extracellular matrix [ECM] while using specific design criteria for both function and future commercialization [9]. Since the first publication in 2002 [18], over 200 articles have been published describing novel uses of the technology for drug and cell delivery. Glycosan BioSystems (Salt Lake City, Utah) obtained the exclusive rights to the technology to certain fields of use for medical and research applications in 2006. Glycosan commercialized the technology in late 2006 for research use with the ultimate goal of manufacturing and providing clinical grade material to a growing cell therapy market. BioTime (Alameda, CA) acquired glycosan in 2011, manufactured cGMP-qualified HyStem, and subsequently performed the requisite ISO-10993 biocompatibility experiments and stability experiments in support of regulatory clearances and approvals. In 2014, one HyStem formulation was 510(k)-cleared for wound management (tradename Premvia™; 510(k) number: K134037). In 2013, BioTime also embarked on two European clinical studies using another HyStem formulation (tradename Renevia®) as a delivery vehicle for autologous fat-derived cells to treat HIV-associated facial lipoatrophy (HIVLA). BioTime successfully completed its pivotal clinical study in 2017 and met its primary clinical endpoints.

2.2 Composition and reaction mechanism

The elegance of the HyStem platform is in its three building blocks. They covalently bind to each other like Lego® blocks and they can be used to make highly customized and complex matrices: these blocks are called Glycosil®, Gelin®, and Extralink® (**Figure 1**). Each has a specific role in the platform (**Table 2**). The most basic form of the platform is called HyStem-C where the concentration (w/v) ratios of Glycosil:Gelin:Extralink are equal in concentration (w/v) (**Table 2** and **Figure 2**). Upon mixing, Extralink's acrylates react with the former two components' thiol groups via click chemistry (Michael addition reaction) [13, 19]. Crosslinks form in trans (e.g. Glycosil molecules can link to Gelin as well as to neighboring Glycosil molecules). In addition, given Glycosil's large molecular weight and its ability to adopt semiflexible random coil configurations, it can likely loop back on itself and bind in cis (**Figure 3**) [20]. The final clear, transparent, viscoelastic hydrogel forms at physiologic pH and temperature in approximately 20 min and is greater than 98% water. This time frame allows an investigator to both customize the hydrogel with drugs or cells and to load and deliver the mixture through a cannula.

Also like Lego® blocks, these three base blocks can be used to make highly customized and complex matrices in large part due to its thiol chemistry. First, the number of blocks can be changed. For example, the addition of thiolated or thiol-reactive species allows a user to fundamentally change the character of the matrix. One case is the addition of thiolated heparin which provides a negatively charged component crosslinked into the matrix. This added character can aid in significantly increased sustained release of proteins non-covalently incorporated into the matrix prior to gelation [21, 22]. The marketed version of this mixture is called HyStem-HP (**Table 3**). In addition, the incorporation of either acrylate or maleimide-tagged molecules allows facile covalent linkage directly to the thiolated species [23–25]. Sometimes, however, less is more: removal of the Gelin component from HyStem-C provides an *in situ* crosslinkable adhesion barrier [26] (**Table 3**).

Second, the levels of each component can be adjusted to change the hydrogel properties. HyStem is a soft gel and can be tailored to stiffnesses comparable to tissues such as endoderm, nerve, liver, and smooth muscle (G' 20–3500 Pa) (**Table 3**). [17, 27, 28]. In more basic terms, HyStem can be made to be as soft of flavored gelatin (G' 35 Pa) or moderately stiffer than Greek yogurt (G' 1900 Pa) (B. Lohman and T. Zarembinski, unpublished). Its stiffness is modulated primarily by increasing Glycosil and/or Extralink concentrations [28]. Its pore size (less than 15.9 nm based on the hydrodynamic radius of trapped polymers (**Table 3**)) can also be modulated

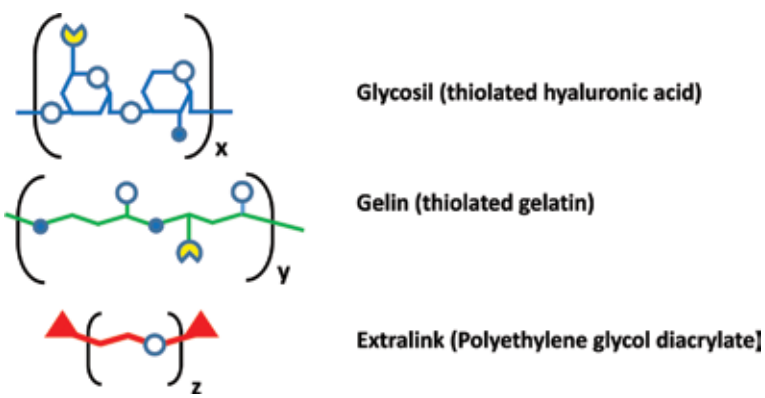

Figure 1.
HyStem-C building blocks. Shapes are as follows: unfilled circles, oxygen; blue circles, nitrogen; yellow partial circles, sulfur; red triangle, acrylate.

	Glycosil	Gelin	Extralink	References
Identity	Thiol-modified hyaluronate	Thiol-modified porcine gelatin	Polyethylene glycol diacrylate	[18, 19, 85]
Purpose	Backbone of gel	Cellular attachment sites	Crosslinker	[18, 19, 85]
Substitution density	30% of HA repeating units	42% of available carboxylates	>65% bis-acrylation	[85]
Mw (kDa)	160	Heterogeneous	3.4	[19]
Final concentrations in gel (mg/ml)	4	4	4	Unpublished

Table 2.
HyStem-C component characteristics.

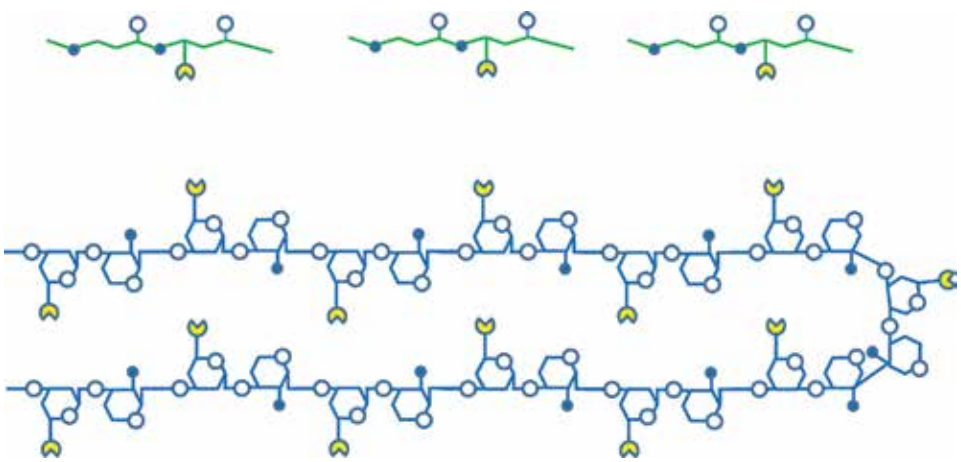

Figure 2.
Glycosil and Gelin prior to gelation. Shapes are as same as those for **Figure 1**.

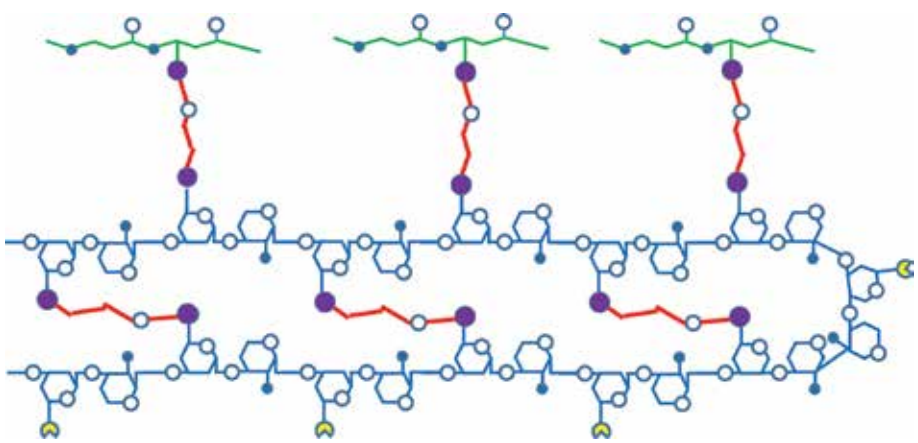

Figure 3.
Covalent bonding in HyStem after gelation upon addition of Extralink. Shapes are same as for **Figure 1** *with addition of purple circles (new bond resulting from Michael addition reaction).*

	Attribute	References
Appearance	Clear	[19]
Gelation time	Approx. 10–20 min	[19]
Versions	1. HyStem-C (Glycosil, Gelin, Extralink) 2. HyStem-HP (Glycosil with thiolated heparin, Gelin, Extralink) 3. HyStem (Glycosil, Extralink)	[9]
Stiffness (G' Pa)	20–3500	[28]
Pore size (nm)	< 15.9	[29]

Table 3.
Final hydrogel characteristics.

from by increasing or decreasing the density of crosslinks of the HyStem-C hydrogel [29, 30]. Third, HyStem composition can be easily altered by adding in any component (e.g. drug, protein) by incorporating it prior to full gelation. This is possible since HyStem has a medium rate of gelation (10–20 min) compared to other *in situ* crosslinking hydrogels [17] (**Table 3**). This non-covalent addition of any therapeutic cargo paves the way for drug and cell delivery to be discussed next.

3. Drug delivery

Sustained release of drugs of both small molecules and protein drugs occurs when they are incorporated into HyStem. While a compilation of drug release results and conclusions for individual HyStem formulations is presented below, this is not the point. The key is HyStem's flexibility: it can be leveraged to adjust release. This modulation can be done by either changing its crosslinking density by increased Extralink or Glycosil concentrations or by including different polymers with affinity for the drug such as heparin [21] or increased Gelin (M. Onorato, unpublished). In the end, this ability to optimize formulation can be crucial for maximizing efficacy *in vivo* [31].

HyStem utility for drug delivery was first described in 2005 with the sustained release of human serum albumin (HSA, 66 kDa) and human basic fibroblast growth factor (bFGF, 16 kDa) from the prototype to the HyStem-HP product (containing thiolated heparin crosslinked into the matrix). bFGF was released over 35 days at different velocities depending on amount of thiolated HP present [21]. This publication was important not only because it was the first illustration of HyStem's capabilities in drug delivery but it underscores the complexity of drug release: several variables affect speed and extent of release. These include identity of protein, molecular weight, base gel polymer (hyaluronic acid (uncharged) or chondroitin sulfate), presence of hyaluronidase (HAse), and release solution composition [21]. Sustained drug release can also occur by using HyStem to immobilize MSC engineered to express and secrete therapeutics like diabodies [32] or by covalent linkage of a small molecule to HyStem to further slow its release [33].

Generally, drugs release from hydrogels is diffusion-driven [34]. HyStem hydrogels are no different *in vitro*: they typically display first order release directly proportional to the drug concentration in the hydrogel [8]. There are two exceptions: Celecoxib and BMP-2 which display near zero order release independent of concentration [8, 31]. Celecoxib is poorly water soluble and the possibility exists that molecule may have precipitated or crystallized in gel [35]. In this event, the molecule's release may be dependent gradual disintegration of the solid [36]. Slow

Drug	Matrix	Release time (days)	MW (kDa)	Final concentration (mg/ml)	Area of research	Reference
1. Lenalidomide	HyStem	0.25	0.26	1.33	Cancer	[8]
2. R848	HyStem	0.25	0.31	1.33	Cancer	[8]
3. Celecoxib	HyStem	18	0.38	10	Cancer	[8]
4. 2′3′-cGAMP	HyStem	0.25	0.67	0.67	Cancer	[8]
5. BMP-2	HyStem or HyStem-HP (no Gelin)	28	26	0.17	Orthopedic	[31]
6. IL-15sa	HyStem	2	29	0.06	Cancer	[8]
7. Anti-PD1	HyStem	5	150	2	Cancer	[8]

Table 4.
Listing of drugs released from HyStem hydrogels (in vitro).

Drug	Matrix	Release time (days)	MW (kDa)	Final concentration (mg/ml)	Area of research	Reference
1. Dexamethasone	HyStem-C*	3	0.39	10	Auricular	[86]
2. IGF-1	HyStem-HP	28	7.6	0.02	Stroke	[87]
3. BDNF	HyStem-C	21	27	0.1–0.17	Stroke	[88, 89]
4. TrkB-Fc	HyStem-HP	5	125	0.005	Stroke	[88]
5. Ascorbic acid and platelet-rich plasma	HyStem-HP	n/a	Multiple	0.05 (ascorbic acid)	Myocardial infarction	[90]
6. miR-29B	HyStem	n/a	7.5	1.0	Myocardial infarction	[41]

**A version of HyStem-C that was used with four-fold lowered Gelin concentration.*

Table 5.
Listing of drugs released from HyStem hydrogels (in vivo).

release of BMP-2 has been described before from HA-based hydrogels and the mechanism for its release is unclear [37].

Another general rule is the smaller the molecule, the faster the release. Small organic molecules less than 1 kDa are usually fully released from hydrogel after 6 hours and larger proteins like antibodies are released on the order of days (**Tables 4** and **5**; **Figure 4**), compare data points on left and right of vertical dotted line. Red data point 1 (dexamethasone) and blue data point 3 (Celecoxib) are outliers for reasons described in this section). The relationship is not linear; this is particularly true of proteins whose release from HyStem hydrogels shows little correlation between molecular weight and release time (**Figure 4**, right of vertical dotted line). This result suggests that their different shapes and surface charges may affect rate of release. There is also a difference in release depending on whether it is measured *in vitro* or *in vivo*; Proteins *in vivo* release rate tend to have slower release rates (**Figure 4**, blue compared to red data points). This increase could be due to the increased resistance to release in the protein-rich host fluids and tissue. In the end, the optimal release rate for *in vivo* efficacy must be empirically determined since it is difficult to predict. For example, BMP-2 is released faster in HyStem compared to HyStem-HP, leading to a more complete release for HyStem at day 28 (84%) compared to the latter (68% for HyStem-HP).

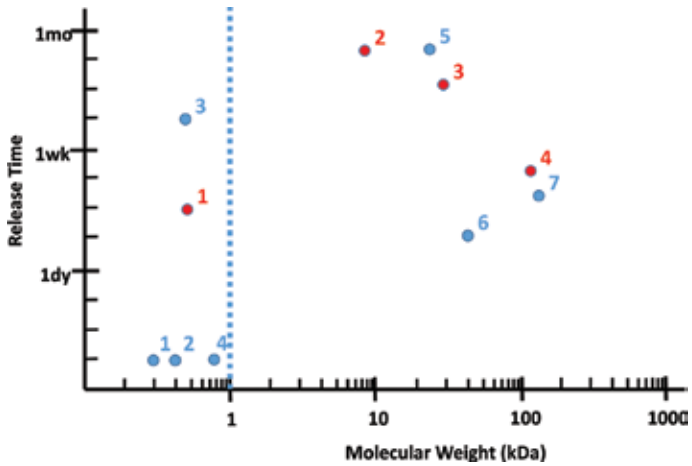

Figure 4.
Release rate of different sized molecules from HyStem hydrogels. Numbered, blue dots refer to in vitro molecules listed in **Table 4***; numbered, red dots refer to in vivo released drugs in* **Table 5***). Blue dotted vertical line separates low molecular weight small molecules (left) from proteins.*

Unexpectedly, faster release corresponds to better efficacy since the former yields 50% more ectopic bone formation [31].

In addition to small molecules and proteins, future opportunities for HyStem will be for the sustained release of a new class of polymer therapeutics, nucleic acids. In 2016, 69 antisense oligonucleotides and 37 siRNAs were in clinical trials [38]. These numbers have grown in 2018 to 87 and 57 trials, respectively (search terms: antisense oligonucleotides and siRNA in clinicaltrials.gov). HyStem can add value especially by protecting the nucleic acids from the host immune system [39]. So far, delivery of the agomir version of miR-26A and of miR-29B have been reported [40, 41]; in addition, double-stranded phosphorothioated DNA oligonucleotides have also been released slowly over 7 days at 10 mg/ml and higher; this rate of release can be modulated by adjusting the levels of Gelin in the final HyStem hydrogel (M. Onorato, unpublished).

4. Cell delivery

HyStem is a versatile tool for the implantation of cells. Beyond the ease of use mentioned previously, the biomaterial constituents provide two key functions that can be used to improve cell-based therapies. First, because these biopolymers are normally found in ECM and can be easily remodeled [19], HyStem can provide a more habitable environment for implanted cells and thereby improve engraftment. Second, HyStem's base biopolymers, hyaluronan and collagen, are ubiquitously found in tissues and the implant composition and structure is not considered foreign. This characteristic plus the small pore size of the hydrogel excluding host cells allows HyStem to shroud the implanted cells from the host's immune system while providing cellular attachment sites [29, 42]. Both of these aspects used together, provides a versatile and tailorable platform for implantation of cells into many tissues for a variety of indications. The combination of these attributes likely explains HyStem's ability to support cell survival post-implantation and to ultimately provide better *in vivo* efficacy in rodent models (**Table 6**) [43, 44].

Herein, HyStem will be presented in two different contexts for cellular delivery. (1) HyStem as a synthetic ECM for cellular remodeling and tissue regeneration

Cell type	Matrix	Last data point (days)	Area of research	Reference
Endothelial progenitor cells	HyStem-C	14	Nephrology	[42]
Neural progenitor cells	HyStem-HP	14	Stroke	[45]
Placenta-derived adherent cell	HyStem-C	56	Orthopedic	[91]
NSC and MSC expressing sTrail	HyStem-C	28	Glioblastoma	[44]
Cardiosphere-derived cells	HyStem-C	1	Myocardial infarction	[43]
Neural stem cells	HyStem-C	14	Imaging	[92]
Retinal progenitor cells	HyStem-C and variations thereof	7	Ophthalmic	[93]
MSC expressing oHSV	HyStem-C	12	Glioblastoma	[94]
NSC expressing Pseudomonas exotoxin	HyStem-C	21	Glioblastoma	[95]
MSC/GDF5 (growth differentiation factor 5)	HyStem-C	42	Bone formation (dental)	[96]
Islet beta-cells	HyStem-C	560	Diabetes	[29]
Cardiomyocytes	HyStem-C	28	Myocardial infarction	[97]

Table 6.
Listing of cells delivered in HyStem hydrogels (in vivo).

(e.g. implantation and remodeling into native tissues) and (2) HyStem as an encapsulating matrix to maintain cellular fitness, localization, and isolation (e.g. paracrine effects of implanted cell and/or immuno-isolation of implanted cells).

The first publication describing the utility of HyStem hydrogel for cell delivery appeared in 2004 [19]. Hydrogels seeded with T31 human tracheal scar fibroblasts implanted in nude mice showed two important milestones 8 weeks post-implantation: the cells were proliferating and also producing their own extracellular matrix. By these measures, the cells had made a home for themselves and were going about populating the space provided by the hydrogel. This work has since been extended to a variety of cell types such as cardiac, neural, mesenchymal based cells [32, 43–45].

While most animal experiments so far reported were performed for several weeks to show improvement in *in vivo* function across a variety of indications (**Table 6**), the possibility exists that this improved efficacy can further be amplified with lengthened time points. One case in point is the transplantation of pancreatic islets in diabetic rats using HyStem-C [29]. Surprisingly, the diabetic rats maintained normal glucose levels and remained insulin-independent for at least 80 weeks (1.5 years) and may have been studied over still longer term had the animals not succumbed to old-age related diseases. The longevity of cellular response is attributed at least in part to reduce fibrosis which is well known to occur in the alginate-based biomaterials more popular for this application.

Successful use of HyStem in animals portended successful use in humans. A major challenge in HIV patients is the disappearance of facial fat resulting from highly active antiretroviral therapy (HAART) and is known as HIV-associated lipoatrophy [46, 47]. One approach to treat this HAART complication is to transplant a patient's own fat-derived cells (stromal vascular fraction cells, SVF)

subcutaneously in the facial deficits. A clinical version of HyStem, Renevia, was used to deliver these cells harvested by liposuction and reinjected subcutaneously. The pivotal trial was a European, multi-center, randomized, evaluator-blinded, delayed-treatment-controlled study of the effectiveness and safety of Renevia in combination with the autologous SVF. The primary endpoint in this 56 patient trial was the change in hemifacial volume at 6 months in treated patients compared to patients in the delayed treatment arm as measured by 3D photographic volumetric assessment. Renevia successfully met the primary endpoint with treated patients retaining 100% of transplanted volume at 6 months. In addition, treated patients retained 70% and 64% of the transplanted volume at 12 and 18 months, respectively. All Renevia transplants were shown to be well tolerated and there were no device-related serious adverse events noted during this trial (investor.biotimeinc.com).

5. Future technologies

5.1 Introduction to organoids

In the current drug development pipeline, preclinical testing of novel drug compounds in 2D cell cultures is well established, but is not always accurately predictive of clinical outcomes in human subjects. [48]. This result is not surprising since cells grown in 2D using plastic dishes experience drastically different surface topography, mechanical properties, cell–cell interactions, cell-matrix interactions, and nutrient diffusion properties compared to a 3D architecture.

Organoids (also called organ-specific 3D cultures) are three-dimensional constructs comprised of tissue-specific cells with the intention of recapitulating the cellular microenvironment and function of their originating tissues. While organoids can be formed through self-aggregation to form spheroids, they are also formed using biomaterial hydrogels that suspend cells in 3D within polymer or protein-networked matrices (refer to the different methods shown in **Figure 5**). Biomaterial-based approaches have an advantage used over spheroid-based approaches as they allow for heightened control of the organoid and organoid microenvironment composition with regard to cellular, biochemical, and physical parameters, such as stiffness, addition of ECM components, and spatial organization of cell types [49, 50]. Organoids often contain multiple cell types that are representative of those typically found within their target tissues [51]. Importantly, these differences result in significant phenotypic and gene expression changes that is much more reflective of their *in vivo* origin. For example, when grown metastatic colon carcinoma cells are cultured in 2D culture, they exhibit an epithelial morphology and expression profile. In contrast, when the cells are introduced into a 3D liver organoid environment supported by HyStem, they "switched" to adopt a mesenchymal and metastatic phenotype [52, 53].

5.2 Novel platforms for making and using organoids

HyStem has been employed to create a wide variety of 3D tissue constructs and organoid form factors since its composition and gelation time can be customized for each model. An example of composition customization is in a recently-developed liver model: primary human hepatocyte spheroids are embedded into HyStem hydrogels modified to include liver-specific ECM extract [54]. The inclusion of liver ECM increases long-term hepatocyte viability, stabilizes albumin secretion, and supports cytochrome p450 activity [55]. These organoids have been tested in environmental toxin screening [56], and are currently being used to screen a range

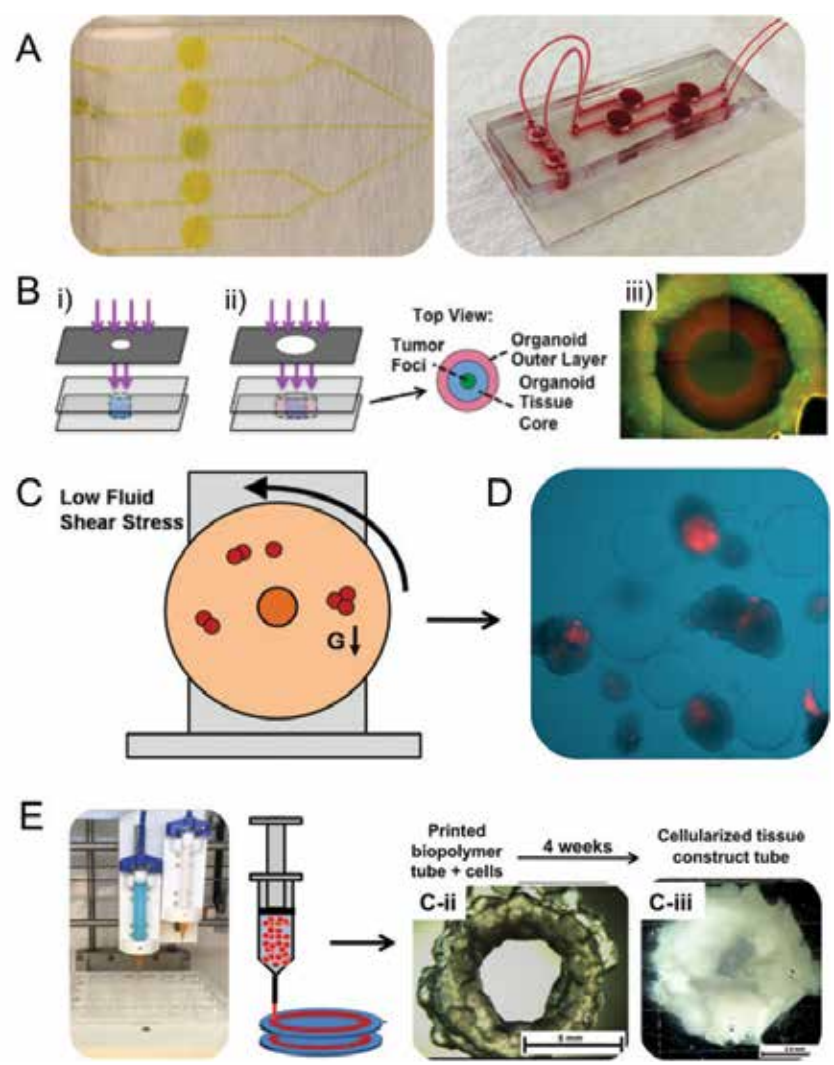

Figure 5.
Organoid biofabrication methods and form factors. (A) Examples of microfluidic devices that house organoids that are (B) photopatterned in situ through hydrogel precursor exposure within the devices through photomasks to UV or blue light, initiating crosslinking. (C) Rotating wall vessel bioreactor culture of (D) tumor organoids. (E) Extrusion bioprinting of cell-laden laden hydrogel bioinks.

of previously recalled drugs as further validation. This model can also be integrated into multi-tissue organoid, body-on-a-chip systems to test drug or toxin kinetics in the context of multiple organs [54, 57]. Lastly, organoids can also be formed by bioprinting technologies (discussed below).

Customization of HyStem's gelation time also enables novel approaches to generating and using organoids. Two examples include using bioprinting technologies (**Figure 5E**) to be discussed in the next section) and organ-on-a-chip technology. Organ-on-a-chip is a nascent 3D-based technology which employs microfluidics to more accurately model *in vivo* tissues by simulating its hydrodynamic flow as well as its placement of multiple cell types in close proximity with respect to one another [58]. This technology can also be used to make organoids by customizing HyStem's crosslinking for spatio-temporal control of gelation. For example, the recent creation of *in vitro* cancer models employ a range of biofabrication approaches, all which employ HyStem in a variety of methods. Since many tissue- and tumor-on-a-chip

platforms are based on closed microfluidic devices with no direct access to locations in which tissues will reside, introduction of cells is performed generally through fluid flow channels. To introduce 3D tissue and tumor constructs within such sealed microfluidic devices (**Figure 5A**) 3D hydrogel photopatterning strategies were developed. By adding light activated photoinitiator molecules (e.g. Irgacure 2959), HyStem is able to form solid structures by shining light through photomasks to yield defined shapes and locations *in situ* within microfluidic devices (**Figure 5B**) [59]. By harnessing control over the extracellular matrix components and adding healthy cells, the resulting organoids can have more complex stroma and extracellular matrix architectures, which provide additional components that contribute to overall tissue-tumor physiology [60]. Additional complexity can be realized by creating multiple tissue and tumor organoids and combining them in a single closed system. This facilitates study of phenomena such as metastasis, where events take place in two locations—a primary tumor site and a downstream site of metastasis. We recently demonstrated a metastasis-on-a-chip platform to model metastasis of colorectal cancer cells from a gut organoid to a liver organoid [53]. We have been able to encapsulate dissociated tumor populations from these biospecimens in 3D using HyStem or its derivatives, forming patient-derived tumor organoids for precision medicine applications for patient specific diagnosis and treatment [61, 62].

5.3 Bioprinting

The shortage of donor organs for implantation in patients has been a significant problem for years, [63, 64] and does not appear to be improving quickly [65]. For example, as of July of 2017, over 117,000 patients were still on waiting lists for donor organs, and there are only 8096 currently identified available donors [66]. Bioengineered tissues and organs have the potential to address this need for implantable tissues for patients waiting on donor lists. Bioprinting technologies have advanced in recent years, holding immense potential to 1 day be employed for biomanufacturing of transplantable tissues and widely adopted human testbeds.

Bioprinting is a multi-disciplinary technology that has emerged in recent years as a tool with immense potential in regenerative medicine and tissue engineering that combines 3D printing, biomaterials, and cell biology. [67–70] Bioprinting is a relatively new field within biotechnology and biomedical research (less than 2 decades old) that can be described as robotic additive biofabrication with a goal to create viable and functional 3D organ or tissue structures [71–75]. A number of bioprinting approaches have been recently explored, encompassing use of inkjet-like printers, extrusion devices, and laser-assisted devices [72, 73, 76]. Biomaterials play an integral role in bioprinting, as they act as the "glue", figuratively and literally, that connect the fabrication approaches with the biological components [72, 73]. Currently, few biomaterials exist that both integrate seamlessly with bioprinting hardware and are optimally compatible with living cells. Most biomaterials used in bioprinting employ terminal covalent or physical bond formation during sol–gel transitions, requiring precise timing or control over chemical reactions to facilitate printing. Put simply, if one bioprints too soon, the result is a puddle of the bioink material and cells; if one bioprints too late, the bioink is too stiff and clogs the printer. Being able to control this transition to enable successful printing of 3D structures has been a major focus in bioink development.

HyStem and its individual components have played important roles in the development of bioinks for bioprinting since the early days of bioprinting, where our group published several of the first studies in which novel biomaterial bioinks were developed specifically for 3D bioprinting [77, 78]. The key is in its compatibility with the bioprinting process. More specifically, the same characteristics that enable organoid

Crosslinker/crosslinking strategy	Key attributes
Gold nanoparticles	1. Slow gelling (24–96 hours) 2. Reversible
Four-armed PEG acrylate	Stiffer gels
Addition of photoinitiator	Rapid crosslinking using light initiation
Addition of tetraorthosilicate	Thixotropic quality

Table 7.
Different crosslinking chemistries with HyStem for bioprintin.

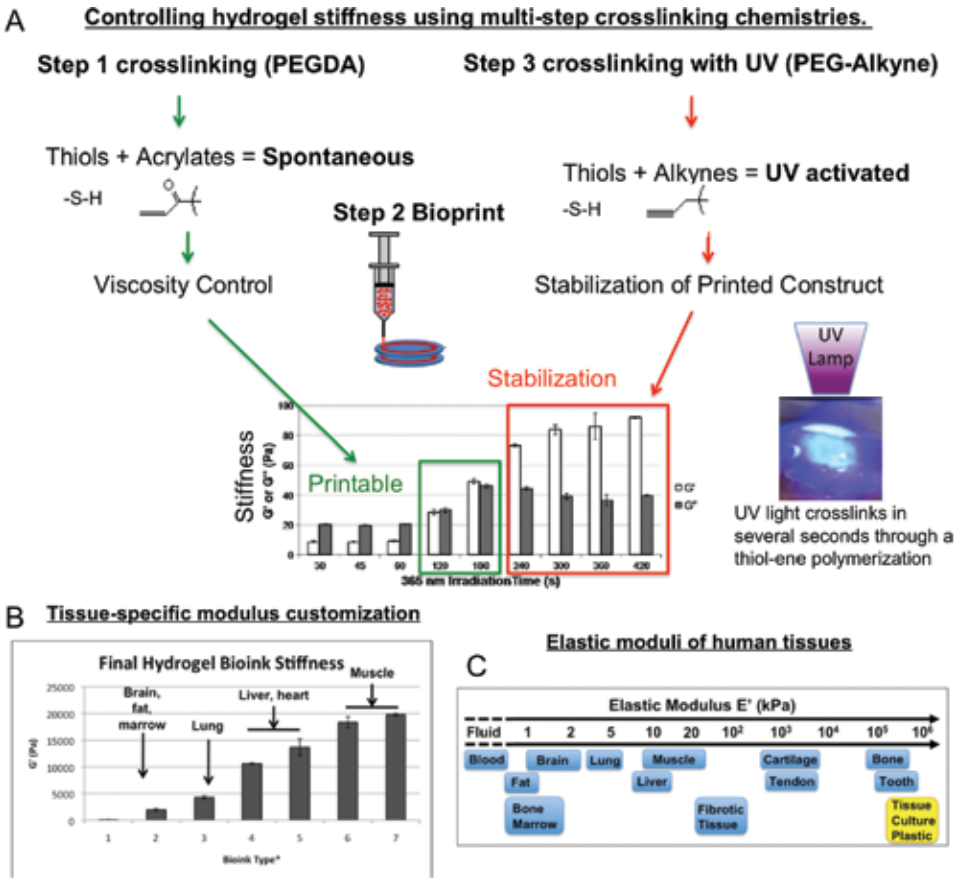

Figure 6.
Description of a multi-step crosslinking, HyStem-based hydrogel bioink. (A) Controlling G' and G" using a multi-crosslinking approach to facilitate extrusion bioprinting of thiolated HA and gelatin-based hydrogels through thiol-acrylate and thiol-alkyne reactions. (B) Data from rheological testing of bioink formulations, demonstrating the capability to mimic the elastic modulus of many soft tissues in the body. (C) Tissues occupy different ranges of elastic moduli.

culture apply here to bioprinting: HyStem can be customized easily while providing a cell-friendly matrix. For example, HyStem forms very soft hydrogels that are not structurally robust, and the crosslinking methods employed do not facilitate effective extrusion bioprinting. This result can be a limitation in terms of scalability. To address these problems, new crosslinking and molding techniques were developed which increase the breadth of the bioprints formed (**Table 7**). These technologies include the ability to form supporting matrix allowing the printing of fragile structures and which can be removed (gold nanoparticles, AuNPs). The use of multi-armed PEG

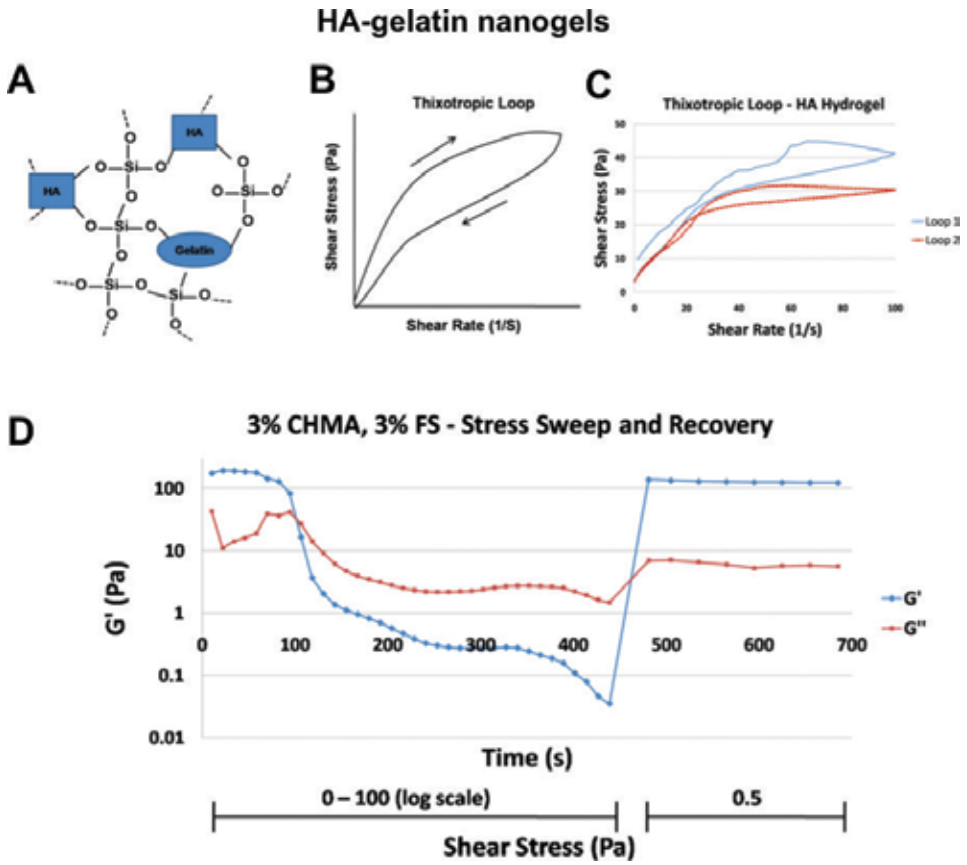

Figure 7.
Thixotropic HA hydrogels. (A) Hyaluronic acid and gelatin thixotropic hydrogel, formed using complexing with tetraorthosilicate (TEOS). (B) A depiction of a thixotropic loop mechanical test and (C) the HA-gelatin thixotropic hydrogel under such a test, indicating thixotropic characteristics. (D) A stress sweep and recovery demonstrating thixotropy.

acrylate crosslinkers to stiffen the gel [78, 79]; multistep spontaneous and light-initiated crosslinking to spatio-temporally regulate crosslinking (**Figure 6**, **Table 7** [80, 81]); and inclusion of tetraorthosilicate to render HyStem thixotropic (allowing extrusion due to reversible liquefaction of the hydrogel (**Figure 7**)).

6. Conclusion

In summary, HyStem's strength is in its clinical roots, its protein and cell compatibility, and its flexibility to enable optimized drug and cell delivery. These attributes make HyStem well-suited for present applications such as local delivery of protein and autologous cells as well as future applications are also such as organoid culture for better drug discovery and development as well as bioprinting of tissue and organs. in the longer term, we envision HyStem to be a platform for using these new technologies to develop the next generation of made-to-order therapies and tissues for an increasing tissue and organ starved human community, whether it be an individual requiring an organ transplant due to disease, a tissue transplant due to injury, or the multiple needs of the wounded warrior.

We also envision HyStem eventually becoming a standard building block for a wide variety of future therapies so that it is clinically available for a physician as an

off-the-shelf, general delivery vehicle. This is because new technologies based on 20–100 nm nanoparticles for drug delivery and for theranostics will require a delivery vehicle for local and/or sustained delivery [82, 83]. In addition, local cellular delivery of nucleic acids will grow in need especially with the rapid development of CRISPR/Cas9 technology for delivery to specific organs and tissues [84].

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Conflict of interest

Dr. Zarembinski is an employee of and owns stock options in BioTime, Inc.

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Hydrogels, as three-dimensional polymer networks, are able to retain a large amount of water in their swollen state. The biomedical application of hydrogels was initially hampered by the toxicity of cross-linking agents and the limitations of hydrogel formation under physiological conditions. However, emerging knowledge in polymer chemistry and an increased understanding of biological processes have resulted in the design of versatile materials and minimally invasive therapies.The novel but challenging properties of hydrogels are attracting the attention of researchers in the biological, medical, and pharmaceutical fields. In the last few years, new methods have been developed for the preparation of hydrophilic polymers and hydrogels, which may be used in future biomedical and drug delivery applications. Such efforts include the synthesis of self-organized nanostructures based on triblock copolymers with applications in controlled drug delivery. These hydrogels could be used as carriers for drug delivery when combined with the techniques of drug imprinting and subsequent release. Engineered protein hydrogels have many potential advantages. They are excellent biomaterials and biodegradables. Furthermore, they could encapsulate drugs and be used in injectable forms to replace surgery, to repair damaged cartilage, in regenerative medicine, or in tissue engineering. Also, they have potential applications in gene therapy, although this field is relatively new.

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