Chitosan-Based Hydrogels: Preparation, Properties, and Applications

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Patrycja Domalik-Pyzik, Jan Chłopek, and Kinga Pielichowska

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Abstract

Chitosan is a hydrophilic polysaccharide obtained by partial deacetylation of chitin, which is one of the most popular biopolymers. Chitosan is well known for its favorable properties including biocompatibility, biodegradability,

P. Domalik-Pyzik · J. Chłopek · K. Pielichowska (✉)
Faculty of Materials Science and Ceramics, Department of Biomaterials and Composites, AGH University of Science and Technology, Kraków, Poland
e-mail: kingapie@agh.edu.pl

Md. I. H. Mondal (ed.), *Cellulose-Based Superabsorbent Hydrogels*,
Polymers and Polymeric Composites: A Reference Series,
https://doi.org/10.1007/978-3-319-77830-3_55

antibacterial, and biological activity, as well as its renewable character. Thanks to those features chitosan's popularity in various applications ranging from food industry to tissue engineering is constantly growing. The following chapter will more closely consider fabrication, properties, and specific applications of chitosan-based hydrogel networks. Methods for chitosan preparation will be summarized, followed by detailed characterization of chitosan properties. Strategies for their improvement and fabrication of chitosan derivatives will be discussed as well. Next, attention will be drawn to preparation of chitosan-based hydrogels via chitosan crosslinking. Both chemical and physical crosslinking methods will be considered with special emphasis on comparison between the two crosslinking methods and recent advancements in application of novel biocompatible crosslinkers. This chapter will also take a closer look at formation of stimuli-responsive (especially pH- and temperature-sensitive systems) and injectable hydrogels. Utilization of chitosan hydrogels in tissue engineering will be highlighted together with different techniques for fabrication and construction of three-dimensional scaffolds. Finally, other applications of chitosan-based hydrogels and their composites will be summarized.

Keywords
Chitosan · Hydrogels · Polysaccharides · Biomaterials · Tissue engineering

1 Introduction

Synthetic products, including synthetic polymers, are widely used in many applications due to their favorable and – most importantly – consistent/reproducible properties. However, being mostly derived from fossil fuels, their high overall usage worldwide adversely affects the environment. Given the environmental concerns raised in past years, turning into more eco-friendly polymers is becoming crucial.

Nature gives us many interesting options within polymer family [1, 2], such as collagen, gelatin, alginate, chitin, to name a few most popular, that could reduce negative environmental impact. Unfortunately, the transition from synthetic to bio-based polymers is not easy due to the obvious differences in structure and resulting properties between these two groups. Obtaining bio-based materials with functionality comparable to that of the synthetic ones is currently one of the pressing matters in materials science.

Generally, chitosan possesses structure and properties that are similar to those of glycosaminoglycan (GAG) – natural polysaccharide and a major component of extracellular matrix (ECM). Being nontoxic, antibacterial, biodegradable, and biocompatible, chitosan can be used in many applications ranging from the food industry to tissue engineering. PubMed search of the term "chitosan hydrogel" gives 1382 results, and the term "chitosan" alone appears in the PubMed database 20,041 times (as of mid-2017). That shows how much attention this versatile polymer has attracted recently.

This chapter gives insight into fabrication, properties, and specific applications of chitosan-based hydrogels. Chitosan preparation methods, properties, and example derivatives are presented in more details. Formation of hydrogel networks based on chitosan and features of stimuli-responsive systems is also discussed, followed by examples of recent research on novel chitosan solutions for tissue engineering, drug delivery, wound management, and water purification.

2 Chitosan

Chitosan is a derivative of chitin – the second, after cellulose, most widespread natural polymer. The term "chitosan" refers to the whole family of chitin-derived polymers with different deacetylation degree, starting from 50% [1, 3]. Going into details, it is a linear, semicrystalline polyaminosaccharide typically composed of two repeating units: (1 → 4)-2-acetamido-2-deoxy-β-$_D$-glucan (*N*-acetyl $_D$-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-$_D$-glucan (*N*-acetyl $_D$-glucosamine). Chemical structures of chitin and chitosan are shown in Scheme 1. The ratio of the two main units, D-glucosamine and N-acetyl-D-glucosamine, determines the degree of deacetylation – one of the most important parameters affecting final properties of the chitosan material [4–6].

2.1 Preparation Methods

Chitin, poly(β-(1→4)-*N*-acetyl-$_D$-glucosamine), is a polysaccharide available in large amounts in nature, mainly as a component of exoskeletons of crustaceans, fungi, and insects – Table 1.

The most popular sources of chitin raw material are crab and shrimp shells – their rigid shells are composed of complex chitin network with proteins and calcium salts deposits [8]. Therefore, in order to isolate chitin from the shells, those components have to be removed by deproteinization and demineralization steps, respectively. Both can be realized using different methods, namely, chemical and biological extraction, with the latter favored in eco-friendly approach [9, 10]. Their detailed review has been recently given by Younes and Rinaudo [11].

Demineralization is achieved by the acidic treatment that leads to the transformation of mineral constituents (mainly calcium carbonate) into salts that are soluble

Scheme 1 Chemical structure of chitin and chitosan

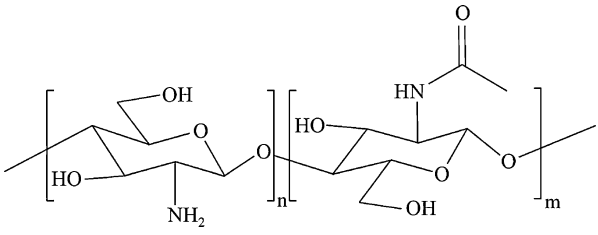

Table 1 Sources of chitin and chitosan. (Reprinted with permission from [7]. Copyright © 1990, American Chemical Society)

Sea animals	Insects	Microorganisms
Annelida	Scorpions	Green algae
Mollusca	Spiders	Yeast (β-type)
Coelenterata	Brachiopods	Fungi (cell walls)
Crustaceans:	Ants	Mycelia Penicillium
Lobster	Cockroaches	Brown algae
Crab	Beetles	Spores
Shrimp		Chytridiaceae
Prawn		Ascomydes
Krill		Blastocladiaceae

in water and thus can be easily removed by filtration and subsequent washing. The reagent is usually diluted hydrochloric acid; however, other options are possible. Next step – deproteinization, i.e., removal of proteins, performed in alkaline conditions, is especially important for the further use of chitin derivatives in biomedical applications, as shellfish are known allergen. Proteins removal is performed in various base solutions, e.g., with sodium hydroxide being the most popular, at different reagent concentration, treatment temperature, and time duration. Reaction conditions need to be adjusted to assure the successful destruction of strong, chemical bonds between chitin and proteins [11, 12].

A biological approach to chitin extraction involves the use of specific enzymes or microorganisms and is considered to be faster, safer, and less destructive in terms of the physicochemical properties of native chitin. During deproteinization and demineralization also some lipids and pigments are eliminated, and if necessary, further decolorization and purification are performed to obtain high-quality, colorless product [11].

The neat chitin is a rigid, highly crystalline polymer with high molar mass from around 200 Da to over 1000 kDa, insoluble in most of the solvents and difficult to process. However, it can be partially deacetylated and thus converted to chitosan – derivative with a larger amount of amino groups and increased solubility. This process can be realized via chemical or enzymatic routes [4].

Chemical deacetylation of chitin is usually performed using alkaline solution, either hetero- or homogeneously. The former is based on the few-hour treatment with concentrated hot NaOH solution. The latter also involves treatment with concentrated NaOH solution but at room temperature and with subsequent dissolution in ice at 0 °C. The products of heterogeneous and homogeneous *N*-deacetylation have different solubility and deacetylation degrees (DD): insoluble with DD as high as 85–100% or soluble with DD around 50%, respectively [11]. Chitosan obtained by hydrolysis via chemical processing of chitin in concentrated NaOH has lower, largely distributed average molar mass.

Chemical deacetylation, however inexpensive and suitable to mass production, has also some drawbacks including possible damages to the polymer backbone and negative environmental impact due to the high energy consumption, use of large

amounts of concentrated alkalis, and a wide spectrum of soluble and insoluble reaction by-products.

Alternatively, enzymatic-mediated deacetylation can be used [13–15]. This method is based on hydrolysis of *N*-acetamido bonds of chitin substrate, catalyzed by chitin deacetylase, the enzyme found in fungi and insects. The biggest advantage of biotransformation of chitin to chitosan, when compared to conventional chemical process, is the higher molar mass and better molecular structure of the final product. Enzymatic treatment is much less aggressive – it allows to preserve original polymer structure, but at the same time, it is difficult to achieve a high degree of deacetylation, especially considering crystalline nature of chitin chains configuration and limited accessibility of the *N*-acetyl $_{D}$-glucosamine units. Therefore, some modifications of chitin such as aiming at reducing its crystallinity and opening up the structure are necessary [15, 16].

2.2 Chitosan Properties

One of the key parameters characterizing chitosan and distinguishing it from chitin is the degree of deacetylation (DD) [17]. The DD value shows the ratio of $_{D}$-glucosamine to the sum of $_{D}$-glucosamine and *N*-acetyl $_{D}$-glucosamine, i.e., how many (in molar percentage) of monomeric units have amino groups. The DD of chitosan is in the range of 50–100%, with the latter being for fully deacetylated chitin [17, 18]. The average molar mass of chitosan is usually in the range of 50–2000 kDa.

The degree of deacetylation, together with molar mass, directly affects physicochemical (e.g., solubility and chain conformation) and biological properties of chitosan [18]. The degree of deacetylation is directly proportional to the solubility, viscosity, biocompatibility, and other biological properties, such as mucoadhesive, analgesic, antibacterial, and hemostatic activity. At the same time, a decrease of DD leads to decrease of chitosan crystallinity and biodegradability. In the case of molar mass, inverse proportionality is observed for biodegradability and antioxidant activity [19].

Unique properties of chitosan emerge mostly from the presence of amino groups in its structure. Chitosan cannot be dissolved in water, aqueous bases, or organic solvents but dilute acidic solutions (pH < 6) protonate its amino groups, making it a cationic polyelectrolyte. The transition between soluble and insoluble (positively charged and not-charged) chitosan state occurs in the pH range of 6–6.5 at the specific pKa value. The protonation degree depends also on the pK_a of the acid used as a solvent and affects the biomaterial properties [20, 21]. Hence, it is the presence of the amino groups that allows controlling "charged state" and the properties of chitosan [19].

Another advantage arising from the presence of the amino groups is the chitosan's ability to form complexes with, for example, metal ions [4, 22]. Moreover, once the polycation is formed, it can further form ionic complexes with negatively charged synthetic polymers, like poly(acrylic acid) or anionic molecules, such as lipids, proteins, and DNA [22].

Beside amino groups, chitosan chains have also hydroxyl groups. Both of them enable covalent bonding and therefore, by introducing various species along the polysaccharide backbone, widen the possible applications spectrum. The alcohol groups allow nonspecific reactions, such as etherification and esterification, while the amino groups can undergo reductive amination to have aldehyde functions [4, 22].

From the biomaterial perspective, chitosan is an extremely interesting natural polymer exhibiting variety of useful properties. Its remarkable biological features include antibacterial [23], antifungal [24], mucoadhesive [25], analgesic [26, 27], and hemostatic [28, 29] properties, as well as biodegradability [30] and biocompatibility [31].

The biodegradability of chitosan depends mostly on its degree of deacetylation. However, it is also affected by the chitosan molar mass, dispersity, and the distribution of *N*-acetyl $_{D}$-glucosamine residues. In the human body, chitosan can undergo enzymatic degradation by lysozyme, acid, gastrointestinal enzymes, or colon bacteria [30]. Breakage of glycosidic bonds results in the formation of different-length oligosaccharides that, being nontoxic, can be either incorporated in the metabolic pathways or excreted by renal filtration. Interestingly, the amino groups protonation may also occur due to absorption of proton ions released in the inflamed area, what results in chitosan analgesic and anti-inflammatory effects [26, 27].

2.3 Chitosan Derivatives

Chitosan is a remarkable polysaccharide with many interesting properties; however, there is still room for improvement. Researchers are especially interested in increasing its water solubility and finding new applications by altering already favorable properties of the polymer. Chitosan derivatives can be obtained by graft copolymerization, carboxymethylation, thiolation, O-hydroxyalkylation, and quaternarization [32, 33].

Covalent linkage of molecules onto chitosan backbone is possible due to the presence of two types of reactive functional groups, amino and hydroxyl, within its structure. Polymers grafted onto chitosan can form cationic or polyampholyte polymers. Grafting can be realized using redox or photoinitiation. Among grafting initiators, the most common ones are cerium (IV) ammonium nitrate, potassium persulfate, and ammonium persulfate. Grafting usually proceeds via direct oxidation or complex formation [34].

The grafting yield, the chemical structure, and the physical form affect solubility and swelling behavior of the final product. Graft copolymerization of (2-diethylamino ethyl methacrylate) (DEAEM) onto chitosan using redox initiation with potassium persulfate was studied under homogenous and heterogeneous conditions, in three different physical forms [34]. Achieved grafting yield ranged between 31% and 54% and appeared to control the solubility of the final product in acidic solutions.

Carboxymethylation can be realized by different chemical routes resulting in carboxymethyl chitosans (CMCS) with varying properties. The reaction of CS with glyoxylic acid in slightly acidic medium formulates N-CMCS. Strongly alkaline medium is used to prepare O-CMCS – an amphiprotic ether derivative. N,N-di-CMCS is prepared in acidic medium, has the ability to chelate metal ions, and possesses a good film-forming ability and osteoinductive properties. The most desirable for biomedical applications is N,O-CMCS – hydrophilic and amphoteric polyelectrolyte derivative, that is biocompatible, has enhanced antibacterial activity and gel-forming ability [35, 36]. Carboxymethyl chitosan is often used as nanoparticles for applications concerning the controlled delivery of active molecules [37, 38]. For example, Teng et al. [39] developed CMCS-soy protein complex particles that were able to encapsulate vitamin D_3 and release it in a controlled manner. The usability of CMCS nanoparticles crosslinked with calcium ions also for oil-spill treatment was demonstrated by Kalliola et al. [40] in their pH sensitive system.

Thiolation, by introducing –SH groups to the polymer backbone, enhances solubility, mucoadhesive, and cellular permeation properties of chitosan. The covalent attachment between the amine group and the mercaptocarboxylic acid realized using carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) allows chitosan modification with thiol groups. Using coupling reaction of a 3-mercaptopropionic acid and CS/EDAC complex, Ko et al. [41] synthesized thiolated chitosan monolayer for cell-based chips in vitro analysis tool. Later, Esquivel et al. [42] adopted the synthesis method to fabricate thiolated chitosan nanoparticles crosslinked by ionic gelation with tripolyphosphate (TPP).

Quaternized chitosan derivative, *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC), was shown to be antibacterial and antifungal. It was also proven [43] that HTCC possesses the ability to bind high and low molar mass heparin, what may be exploited in controlled inhibition of the anticoagulative activity of heparin. HTCC-heparin complexes were relatively small, with low polydispersity when compared to heparin aggregates formed when protamine sulfate was used. Li et al. [44] synthesized *O*-quaternary ammonium *N*-acetyl thiourea chitosan bearing double functional groups, that has improved water solubility over a wide pH range. Moreover, grafting of double functional groups, i.e., quaternary ammonium and acyl thiourea, enhanced its antibacterial activity.

3 Hydrogel Formation via Crosslinking of Chitosan

Hydrogels are three-dimensional (3D) polymer networks characterized by high water content and the resulting high flexibility. Hence, being soft and pliable, they naturally mimic many tissues of the human body. In terms of mechanical properties, hydrogels are especially similar to soft tissues. Their viscoelastic nature prevents adverse effect on the surrounding host tissue after implantation, and together with other desirable properties like biocompatibility, functionality, and reversibility, makes them important and extremely interesting part of biomaterial group [19, 45].

Hydrophilic groups within hydrogel polymer network enable high absorption of water-based liquids. Increased water content results in swelling and expansion of the hydrogel volume. Water molecules in hydrogel structure exist in three different states: as free, intermediate, and bound molecules [46]. The amount of free water molecules is dictated by the hydrogel structure – the more compact structure, the less amount of free water it contains. Weak interactions between functional groups of polymer chains and water molecules form intermediate water, while bound water is a result of strong hydrogen bonding between the polymer and water molecules.

In general, crosslinking improves mechanical properties and increases hydrogel stability by interconnecting molecules. At the same time, it reduces the number of available functional groups, thus limiting modification possibilities, decreases polymer degradability, and changes rheology properties that can cause processing difficulties [47]. There are many different methods to prepare chitosan-based hydrogels, but each one requires some steps to ensure hydrogel network stability, i.e., chitosan hydrogels have to be physically or chemically crosslinked. The stability and reversibility of hydrogel system are determined by the crosslinking method.

3.1 Physical Crosslinking

Formation of stable, however nonpermanent, hydrogel networks exploits physical interactions, resulting from physical domain junctions, hydrogen bonding, hydrophobic association, chain aggregation, crystallization or ionic complexation [45, 46]. Physical crosslinking of chitosan hydrogels usually occurs in response to specific conditions, e.g., temperature or pH and might be affected by the polymer concentration and the number of other components. Gelation is a complex problem; due to specific nature of the chitosan molecule, many interactions are involved at the same time, affecting sol-gel transition.

In the case of thermogels, the temperature change is the stimulus triggering gel formation. It was reported [48] that by adjusting the degree of deacetylation, pH of the hydrogel solution, the glycerophosphate concentration, and the chitosan's molar mass, it is possible to modulate gelation temperature. Depending on the temperature, the polarity of the chitosan chains and other possible moieties introduced into the hydrogel system changes. Therefore, also possible hydrophobic and hydrogen bonding interactions are affected, and the gel is formed by heating of the solution. Chitosan/glycerophosphate (CS/GP) hydrogels have physiological pH and gelation temperature optimal for the human organism (37 °C) – that is why they have been tested in various biomedical applications. However, despite this popularity, recent studies [49] suggest that the GP concentration used for rapid CS gelation (i.e., 0.4 M) is cytotoxic. Glycerophosphate may be replaced with sodium hydrogen carbonate and phosphate buffer [49]. This formulation transformed into the gel at 37 °C but had better mechanical characteristic and improved cytocompatibility.

Physical gelation can occur due to the formation of polyelectrolyte complexes between oppositely charged molecules. As it was mentioned previously, in mildly acidic conditions, chitosan is a polycation, and therefore, it can form complexes with

many polyanionic polymers and various ions. Chelation of amino groups can cause adsorption of metal cations and thus formation of complexes between chitosan and metal ions. It has been reported that ionic complexation results not only in molecular changes but also affects the structure of in situ prepared chitosan hydrogels. Nie et al. [50] studied the influence of metal ions on the hydrogel structure. Their results showed that ions with a strong affinity towards chitosan, e.g., Cu^{2+}, act as ionic crosslinking agents, increase volume shrinking tendency of the polymer, and cause transformation of the hydrogel structure from oriented fibers to multilayers. On the other hand, ions such as Ca^{2+}, that have a weak affinity with chitosan, in the presence of hydroxide ions, tend to form precipitates. Hence, inorganic particles are introduced to the polymer matrix, but the structure of the chitosan hydrogel is not affected.

Chitosan can be successfully crosslinked with tannic acid, a natural nontoxic molecule which has the ability to form ionic, hydrophobic, and hydrogen interactions with the polymer [51]. Sionkowska et al. [52] reported the influence of tannic acid crosslinking on the photochemical stability of chitosan in view of possible sterilization of chitosan biomaterial via UV irradiation. It was shown that absorption of UV light by tannic acid may affect the polymer macromolecule and lead to the scission of chitosan chains and/or crosslinking.

Bioactive chitosan hydrogels were obtained by crosslinking with laponite – platelike synthetic clay [53–55]. Dense polymer network was formed as a result of electrostatic interactions between negatively charged clay molecules and protonated amine groups of chitosan. Formation of the crosslinked structure limits the swelling behavior of the hydrogel [54]. Moreover, laponite acts not only as a crosslinker but also increases the bioactivity of the system and is a good alternative for other commonly used crosslinking agents, that are often toxic to some extent [53].

Han and Yan [56] prepared supramolecular hydrogels of chitosan and graphene oxide (GO) by changing CS/GO ratio, GO concentration, and temperature. In this system, GO worked as a two-dimensional crosslinker and the self-assembly of chitosan chains and GO nanosheets was observed due to noncovalent interactions. The hydrogel was formed at room temperature when higher amounts of GO were used and the as-prepared hydrogel exhibited self-healing properties. With lower concentrations of GO, high temperature (95 °C) treatment was necessary to obtain reversible hydrogel (Fig. 1).

3.2 Chemical Crosslinking

Permanent bonds within hydrogel network are formed via chemical crosslinking by photopolymerization, radical polymerization, enzymatic reactions, and covalent linking with, for example, aldehydes. Nature of chemical hydrogels is permanent and irreversible due to chemical processes altering molecule configuration. Chemically crosslinked chitosan hydrogels can form hybrid polymer networks (HPN), semi-interpenetrating polymer networks (semi-IPN) and interpenetrating polymer networks (IPN) [46].

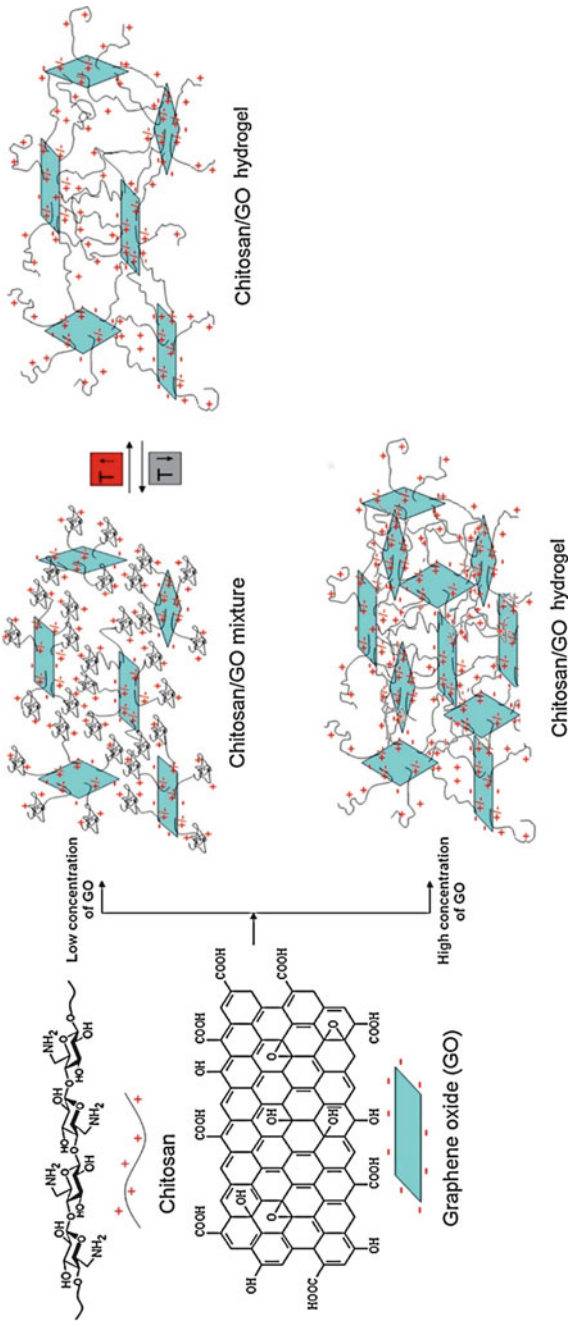

Fig. 1 Schematic mechanism for the supramolecular hydrogel formation at different conditions. (Reprinted with permission from [56]. Copyright © 2013, American Chemical Society)

Crosslinking agents used for chitosan hydrogels preparation include: glutaraldehyde (GA) [57], *N,N*-methylene-bisacrylamide (MBA) [58], glyoxal [59], and genipin [60]. At the beginning, GA gained much popularity as a crosslinker for systems where chitosan was combined with the second polymer; however, GA toxicity can impair the biocompatibility of the crosslinked polymer, and thus GA application in the biomedical field is limited. Currently, the research focus is on naturally derived crosslinkers that offer reduced or no toxicity and higher biocompatibility. One of the most popular is genipin, a compound found in the *Gardenia jasminodes Ellis* fruit. Genipin reacts promptly with chitosan and serves as a crosslinking agent even at a relatively small molar ratio. Chitosan hydrogels crosslinked with genipin are blue-colored and fluorescent [61].

Photopolymerization allows forming chemical gels by using photoinitiators and visible or UV irradiation. Photoinitiators, due to exposure to visible or UV light, produce free radicals, that induce radical polymerization and hence polymer crosslinking. The distance from light source and the duration of exposure are the main conditions controlling the reaction. Chitosan hydrogels that are photocrosslinkable have the distinct advantage of possible in situ preparation. Photopolymerizable chitosans are especially useful for fabrication of 3D scaffolds using rapid prototyping techniques. Recently, Kufelt et al. [62] fabricated N-succinyl-chitosan using succinic anhydride and then conjugated its carboxyl groups with glycidyl methacrylate (Fig. 2).

As a result, they obtained photosensitive chitosan appropriate for two-photon polymerization (2PP) processing. The 2PP method allows creating 3D micropatterning by initiating polymerization only within the laser beam focus on the photosensitive material.

In a novel approach, based on a combination of advantages of physical and chemical crosslinking, Chen et al. [63] prepared hydrogel using ethylene glycol chitosan crosslinked with a new crosslinker synthesized by reacting 1,6-diisocyanatohexan (HDI) and poly(ethylene glycol) (PEG) (Fig. 3). Only small amount of crosslinking agent was necessary to obtain semitransparent gel through the reaction between the amino and hydroxyl group of ethylene glycol CS and the crosslinker.

4 Stimuli-Responsive and Injectable Chitosan Hydrogels

4.1 Temperature Sensitive Systems

Thermosensitive hydrogels can undergo a phase transition in response to a variation of the temperature. This phenomenon is often employed when developing new biomaterial systems that should be responsive at the human body temperature. Neither parent chitin nor chitosan possesses thermosensitive properties in their native form; however, some chitosan derivatives can be designed to respond to the temperature changes. To yield thermoreversible behavior, chitosan can be grafted with poly(N-isopropylacrylamide) (PNIPAAm) [64] or poly(N-vinylcaprolactam)

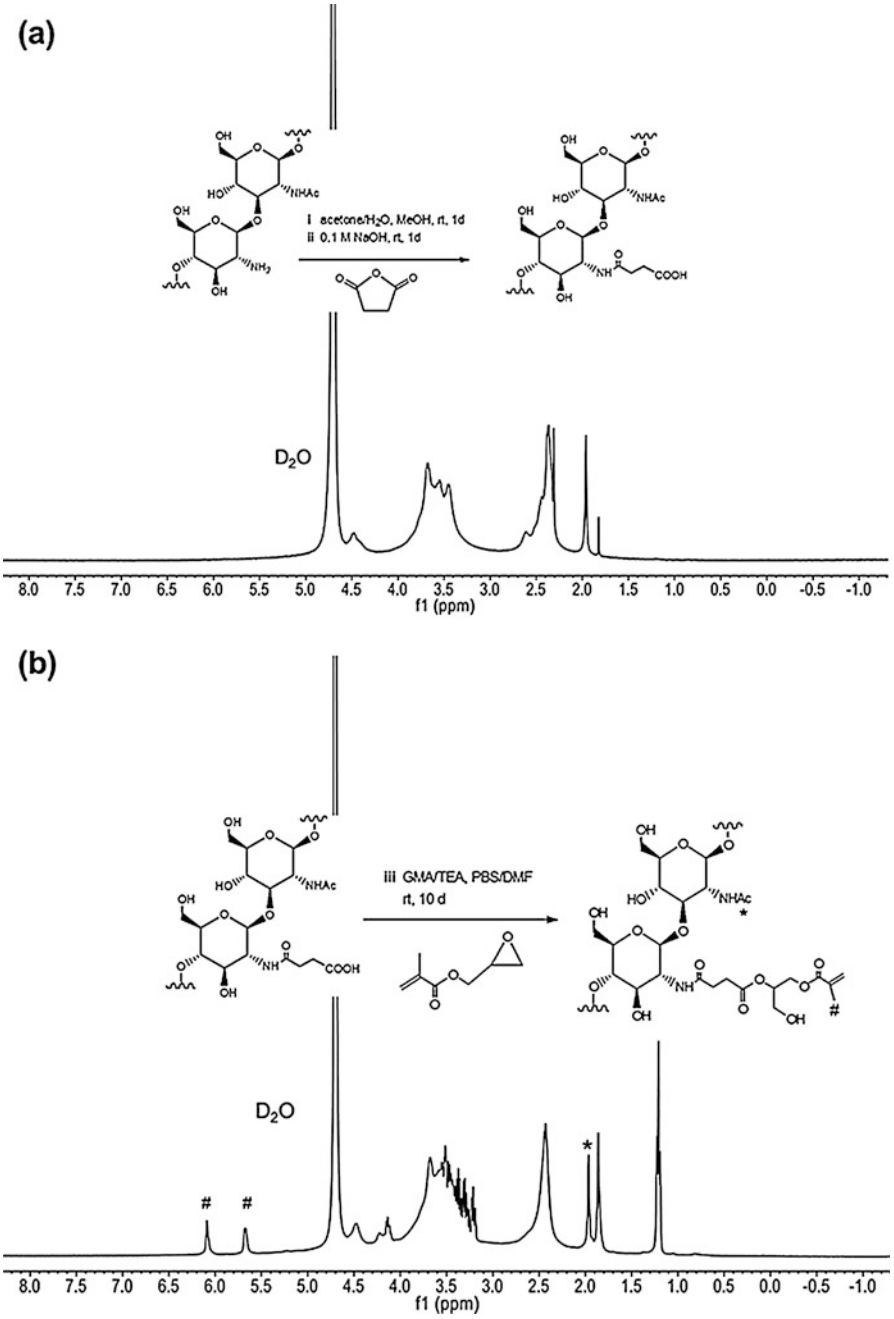

Fig. 2 1H NMR spectra in D_2O: (**a**) chitosan after N-succinylation with N-succinic anhydride (anomeric protons: d = 4.48 ppm; protons of carbohydrate backbone: 3.67–3.45 ppm; succinic $–CH_2–CH_2–$: 2.61–2.37 ppm; N-acetyl CH_3: 1.96 ppm), DS[TNBS] = 53%; (**b**) methacrylate-

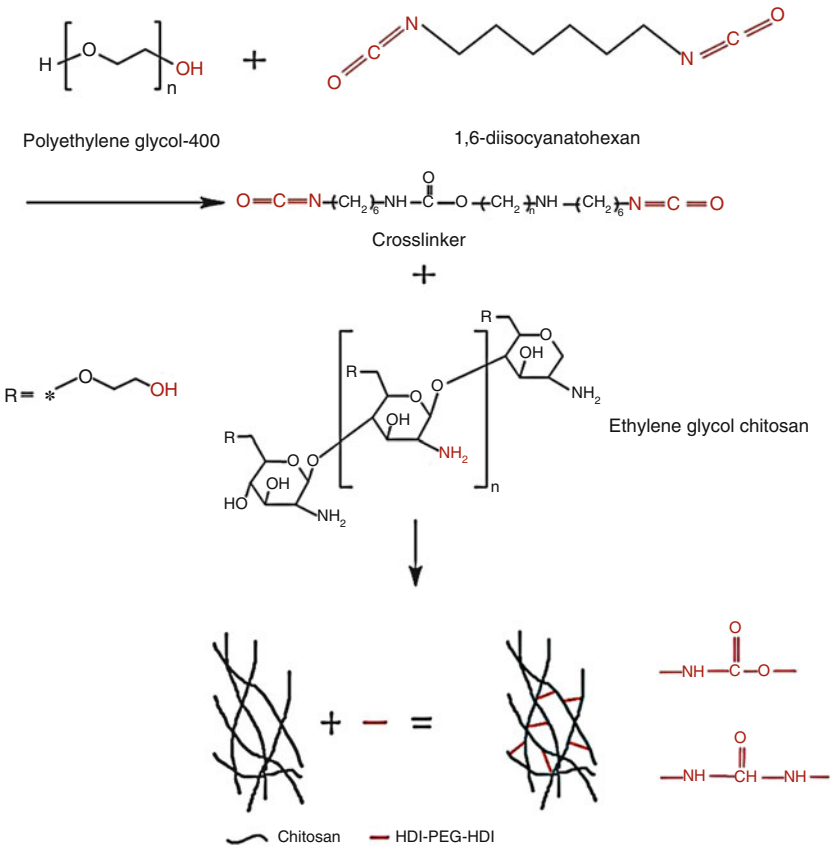

Fig. 3 A schematic drawing of the formation of the ethylene glycol chitosan hydrogel. (Reprinted with permission from [63]. Copyright © 2014, Springer Science+Business Media New York)

(PVCL) [65, 66], that both show hydrophilic to hydrophobic phase transition behavior in response to temperature increase.

Chitosan-glycerophosphate (CS-GP) system was found to be especially interesting in the biomedical field. Heat-induced chitosan gelation is triggered upon heating of the CS aqueous solution with GP added and renders physical gel around the physiological temperature. The final gelation temperature is affected by the CS degree of deacetylation and the concentration of both components. This behavior enables encapsulation of living cells or therapeutic agents within the system in its liquid state, injection in vivo and subsequent gelling at body temperature [67–69].

Fig. 2 (continued) modified chitosan (olefinic protons: d = 6.08 and 5.66; anomeric protons: 4.48 ppm, protons of carbohydrate backbone: 3.70–3.51 ppm; N-acetyl CH_3: 1.96 ppm. (Reprinted with permission from [62]. Copyright © 2015, Elsevier)

4.2 pH-Sensitive Systems

Applicable in drug delivery or bio-based adsorbents, pH-sensitive systems were obtained by graft copolymerization of N,N-diethylaminoethyl methacrylate (DEAEM) onto chitosan-tripolyphosphate (chitosan-TPP) gel beads. In acidic conditions (pH = 1), they behaved like superabsorbent gels; upon changing the pH and repeated swelling in pH = 7 and pH = 1 conditions, the chitosan-TPP-*graft*-PDEAEM beads acted as pH-responsive superabsorbent 3D matrices [34].

In another study [60], chitosan microspheres crosslinked with genipin were fabricated for removal of heparin from the bloodstream. The developed system responded to changes in pH and thus allowed to tune the rate of heparin removal. Additionally, the microspheres were cationically modified with glycidyl trimethylammonium chloride to increase heparin binding efficiency at physiological pH level (~7.4).

The pH-responsive systems can be also applied for drug delivery. Qu et al. [70] used N-caboxyethyl chitosan and dibenzaldehyde-terminated poly(ethylene glycol) to obtain hydrogels for hepatocellular carcinoma therapy. Doxorubicin release study confirmed pH-sensitivity of the developed hydrogel – due to gel degradation, the drug was released faster in the acid environment than at pH = 7.4. The higher release kinetics at lower pH (6.8 vs. 7.4) was also observed by Adeyemi et al. [71]. In their study, chitosan-based nanoparticles were loaded with bovine serum albumin, as a model protein.

4.3 Injectable Systems

Thermosensitive systems with gelation point set at a typical human body temperature are ideal candidates for application as injectable hydrogels. They can be prepared in ambient conditions and transformed into a gel after implantation. Combination of chitosan, quaternized chitosan, and α,β-glycerophosphate allowed to formulate injectable composite material (CS-HTCC/α,β-GP) that undergoes sol-gel transition at 37 °C in 3 min [68]. Additionally, CS-HTCC/α,β-GP exhibited the ability to release a drug in a controlled manner and showed antibacterial activity towards periodontal pathogens. Monette et al. [72] developed injectable hydrogels based on chitosan combined with $NaHCO_3$ and phosphate buffer. Their formulation served as an injectable scaffold for the encapsulation and localized delivery of T lymphocytes.

Wang et al. [73] prepared injectable hydrogel by introducing chitin nanowhiskers into chitosan/β-glycerophosphate disodium salt. Addition of chitin nanowhiskers significantly increased mechanical properties of the gel and reduced the gelling time at 37 °C to only 25 seconds. The nanowhiskers served as a crosslinker in the gel formation.

Application of Schiff' base reaction for the synthesis of injectable hydrogels has been reported recently [74]. Chitosan microspheres were embedded into chitosan-chondroitin sulfate hydrogel affecting properties of the composite system. Presence of solid microspheres enhanced compressive modulus of the gel, that was able to retain its structure.

5 Applications of Chitosan-Based Hydrogels

The variety of favorable properties of chitosan, such as its biocompatibility, biodegradability, antibacterial, mucoadhesive, hemostatic, analgesic, and antioxidant activity make this polysaccharide a polymer of choice in many different fields. Pharmaceutical, medical, cosmetic, or food industry – they all benefit from the use of chitosan. In the field of biomaterials, it is applied in tissue engineering, wound healing, and drug delivery systems. Due to ionic complexation described earlier, it also serves well in water purification. Some detailed examples of the application of chitosan-based hydrogels in different areas are given below.

5.1 Tissue Engineering

The rapid development of materials science and biomedicine resulted in the formation of the new field named tissue engineering (TE). Traditionally, the triad of TE consists of scaffolds, cells, and bioactive agents. The main idea behind this approach is based on regeneration of damaged tissues, rather than replacing them, by using temporary scaffold templates that degrade over time making room for a newly formed tissue. The clue here is the biocompatibility and biodegradability of the initial biomaterial support and the ability to deliver cell instructive signals. After many years of research, some basic requirements for polymeric tissue scaffolds were formulated, starting with the degradation rate matching that of regenerated tissue. TE scaffolds should be highly porous and the pore size should be fitted to a specific cell type. At the same time, this porous scaffold needs to have enough structural integrity and mechanical properties to withstand forces acting at the implantation site during the time when new tissue is formed. This includes also surgical handiness. Lastly, the scaffold has to be biocompatible and should promote cell adhesion, proliferation, migration, and differentiation, facilitating neo-tissue formation [2, 75, 76].

Chitosan, especially with high deacetylation degree, is biodegradable and biocompatible. Furthermore, chitosan hydrogels with their high water content and viscoelastic properties resemble characteristic of native tissues. Lastly, those polymer networks are easily modified to carry and release molecules influencing cell behavior. All the above features cause chitosan to be biomaterial of choice in many tissue engineering applications [77].

Grolik et al. [78] developed chitosan-based hydrogel membranes crosslinked with genipin for corneal epithelial cell cultures. The results demonstrated that especially promising properties, including favorable hydrophilicity, morphology, and mechanical characteristics, present chitosan-collagen blends that formed a homogenous mixture and both components were successfully crosslinked with genipin.

Haaparanta et al. [79] studied hybrid scaffolds in various systems: collagen/polylactide (PLA), chitosan/PLA, and collagen/chitosan/PLA for the treatment of articular cartilage defects. In another attempt [63], cartilage defect was successfully repaired with the use of ethylene glycol chitosan hydrogel. In the in vivo assay, rabbit chondrocytes cultured in the prepared hydrogel were implanted into damaged

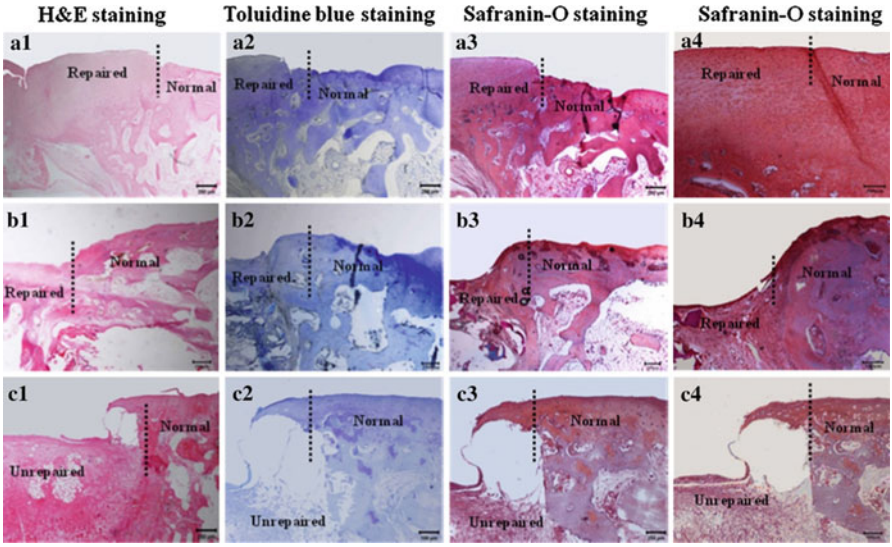

Fig. 4 Repair of cartilage defects in vivo. Ethylene glycol chitosan-chondrocytes repair rabbit knee joint defects at 12 weeks. (**a**) Experiment group; (**b**) Blank group (ethylene glycol chitosan hydrogel scaffold without chondrocytes); (**c**) Control group; 1–3 scale bar 250 μm, 4 scale bar 100 μm. (Reprinted with permission from [63]. Copyright © 2014, Springer Science+Business Media New York)

knee joint. Over the period of 12 weeks, chitosan hydrogel degraded and the defect was filled with cartilage-like tissue (Fig. 4).

Similarly to other polymers, chitosan has relatively weak mechanical properties, hence for successful application in bone TE, it needs to be combined with other materials, usually with bioactive ceramics, for improved mechanical and biological performance. Dan et al. [80] fabricated chitosan-gelatin-nanohydroxyapatite (nHAp) composite scaffold with a mean pore size of 100–180 μm, showing improved attachment and growth of the MC3T3-E1 cells due to the presence of nHAp. Qiao et al. [81] reported research toward developing self-sterilized bone TE scaffold in three steps: crosslinking of chitosan with genipin, freeze-drying of the construct, and biomineralization of nHAp layer. To improve antibacterial properties silver ions were introduced via in situ synthesis of silver phosphates in the HAp nanostructure by substitution of calcium ions. Using the technique of high compression, Kucharska et al. [82] fabricated microsphere-based scaffolds composed of chitosan and tri-calcium phosphate (TCP) (Fig. 5).

5.1.1 Design and Construction of Three-Dimensional Chitosan Scaffolds

All native tissues are three-dimensional (3D) complex structures, hence cells prefer 3D scaffolds over 2D surfaces. 3D scaffolds resemble the architecture of the native extracellular matrix, providing an appropriate environment for cell growth and

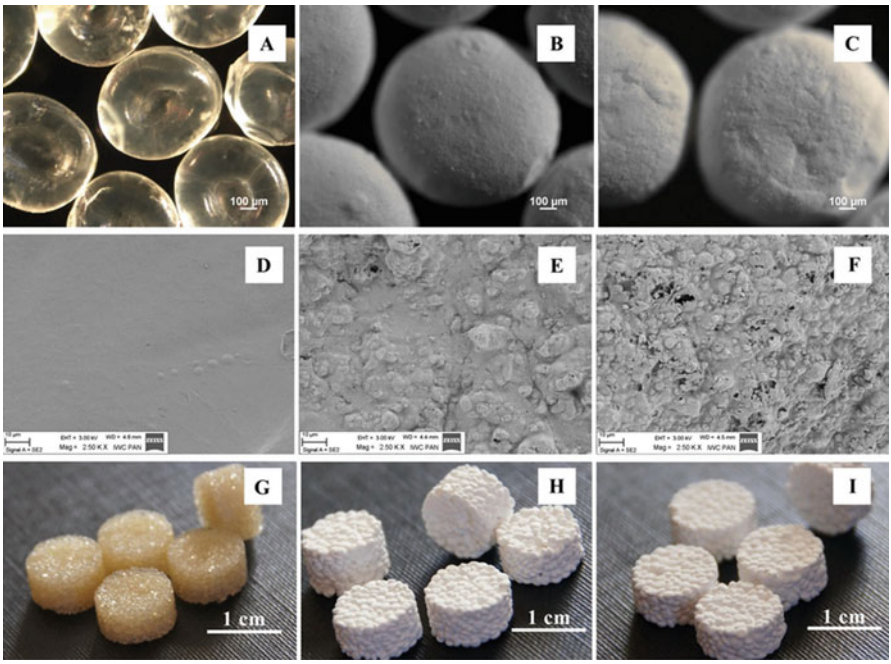

Fig. 5 Photographs of CH, CH_5TCP, and CH_10TCP microspheres, respectively, from the left-hand side. (**a**–**c**) Optical microscope, ×5 magnification; (**d**–**f**) SEM analysis of granules microstructure, magnification ×2500; (**g**–**i**) The digital photographs of formed CH and CH/TCP scaffolds. (Reprinted with permission from [82]. Copyright © 2015, Springer Science+Business Media New York)

proliferation. There is a growing interest in various fabrication methods that allow obtaining 3D morphologies. Those methods include electrospinning, phase-separation, self-assembly, freeze-drying, and 3D printing [83].

Electrospinning technique allows forming continuous nanoscale or microscale fibers from the polymer solution, in the presence of the electric field [84, 85]. The polyelectrolyte nature of the chitosan dissolved in acidic media complicates the process, as protonated amine groups of the chitosan backbone start to repulse each other when the high voltage is applied. One of the first successful preparations of electrospun chitosan mats using trifluoroacetic acid (TFA) as a solvent was reported by Ohkawa and coworkers [86]. Chitosan amino groups formed salts with TFA, thus eliminating the problem of ionic interactions and facilitating the spinning process. Chitosan nanofibrous mats were also fabricated using TFA-dichloromethane and concentrated acetic acid.

Freeze-dried scaffolds are formed by freezing of the polymer solution and then removal of the frozen solvent crystals by direct sublimation under vacuum, leaving empty spaces – pores. In the freeze-drying process, scaffold architecture may be tuned by changing the solvent and polymer type, the polymer solution concentration, and the freezing conditions [84, 87]. The morphology and the pore size of chitosan

scaffold are affected by the freezing temperature. When samples were frozen at lower temperatures, the cooling rate was faster and the ice crystals had less time to grow – resulting pores were smaller, but their amount was higher. On the other hand, when the cooling rate was slower (lower freezing temperature), crystals had time to grow yielding a lower number of bigger pores [87]. In the work of Kavitha Sankar, Rajmohan, and Rosemary [88], freeze-drying was used to obtain chitosan sponge with blood absorbing capacity suitable for hemostasis. Freeze-drying can be also applied to the fabrication of chitosan-based composite scaffolds. Mohammadi et al. [89] prepared freeze-dried chitosan scaffolds reinforced with multiphasic calcium phosphate short fibers that exhibited enhanced mechanical and biological properties.

One of the latest technologies in scaffold fabrication is 3D printing [90]. However, when it comes to hydrogels, it is also especially challenging. Precise, layer-by-layer deposition of hydrogels is difficult due to their usually weak mechanical properties causing deformation of the scaffold architecture as the hydrogel struts tend to collapse under the weight of subsequent layers [91]. Many attempts were made to overcome this problem, mainly by using in situ gelling method. This way, the 3D printed shape was retained, but the chemicals used for gelation increased the risk of cytotoxicity. An alternative approach was proposed by Wu et al. [92]. The chitosan-based printable ink was prepared in a mixture of acetic, lactic, and citric acid and printed directly in air, in ambient conditions. Relatively, high chitosan concentration combined with the printing of very thin struts enabled partial hardening of the struts by solvent evaporation. Then, the scaffolds were physically crosslinked by neutralization in sodium hydroxide solution. As a result, the authors obtained highly flexible chitosan scaffolds with precisely controlled architecture for guided cell growth (Fig. 6).

In the 3D bioprinting technology, living cells can be incorporated into hydrogel matrix and fabricated into bioscaffold with precisely controlled architecture. In one of the recent studies [93], gelatin/sodium alginate/carboxymethyl chitosan (Gel/SA/CMCS) was combined with bone mesenchymal stem cells (BMSC). Resulting 3D hydrogel scaffold exhibited homogenous cell distribution, good mechanical properties, antibacterial activity, and favorable degradation rate. BMSCs loaded into this complex polymer matrix showed high viability.

5.2 Wound Healing

Each year, many people suffer from skin wounds, e.g., skin lesions or burns. This common health problem can have serious consequences as skin serves as a barrier protecting the organism from many harmful factors, like, e.g., bacteria. Hence, it is important to provide appropriate conditions for wound healing and thus recreating damaged protective layer – the skin. Wound healing process consists of four phases, i.e. hemostasis, inflammation, proliferation, and remodeling [94]. Chitosans with their high swelling ability, wound healing, antibacterial, hemostatic, and analgesic properties earned considerable interest as a wound management material.

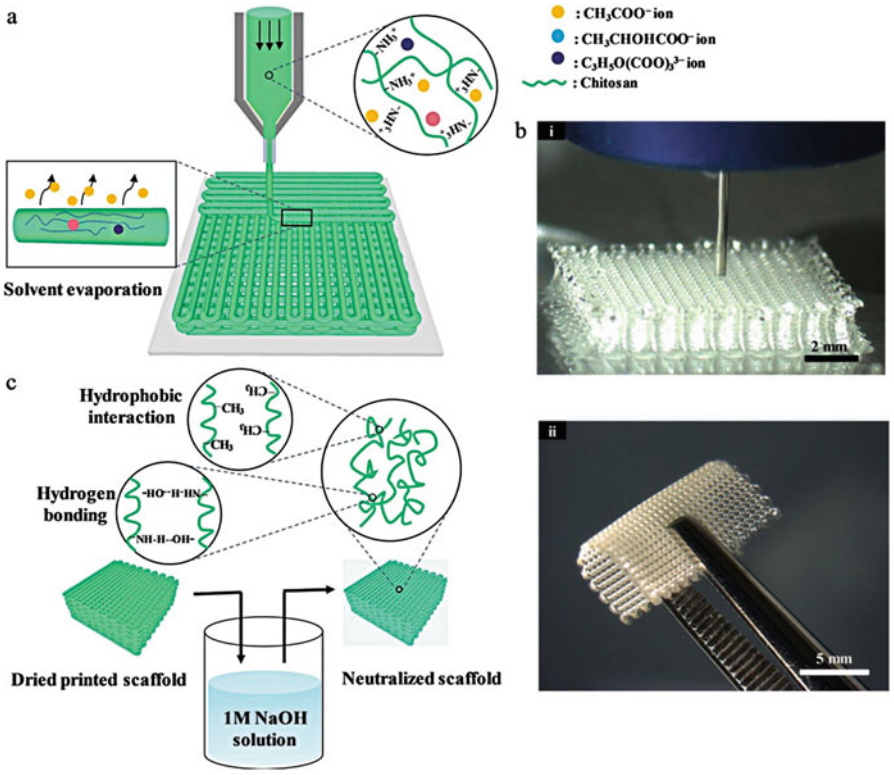

Fig. 6 (**a**) Schematic representation of 3D printing of a chitosan ink prepared using an acidic mixture and partially hardened via solvent evaporation. (**b**) (i) Optical image of the printing of a 30-layer 3D chitosan scaffold through a 100 μm micronozzle and (ii) optical image of a 10-layer chitosan scaffold fabricated with a 100 μm micronozzle and folded using a tweezer. (**c**) Schematic illustration of the neutralization step for yielding physical gelation with hydrophobic interaction and hydrogen bonds to form a chitosan hydrogel scaffold. (Reprinted with permission from [92]. Copyright © 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

Huang et al. [95] used scalded rats model to investigate analgesic and wound healing effect of chitosan and carboxymethyl chitosan. The results confirmed that both, CS and CMCS, promote wound healing; however, analgesis was only observed in the case of CMCS. In another study [96], low molar mass O-CMCS was reacted with furfuryl glycidyl ether, creating the furan-coupled O-CMCS (VLC-chitosan). Next, bovine serum albumin (BSA) was encapsulated in the polymer matrix by crosslinking of VLC-chitosan in the presence of Rose Bengal under visible light irradiation. In vivo animal assay demonstrated that VLC-chitosan containing murine epidermal growth factor (mEGF) reduced inflammation and sustained epithelial regeneration and fibrosis.

Chitosan oligosaccharides have anti-inflammatory, immunostimulating, and antioxidant properties. Thus, they are able to accelerate wound healing, what was

recently confirmed by Sandri et al. [97]. In the tested system, chitosan oligosaccharide was combined with a clay mineral, halloysite nanotubes (HNTs). Resulting nanocomposite material proved to be biocompatible with human dermal fibroblasts. Moreover, after 18 days of in vivo assay using murine model, full recovery of skin structure was observed.

5.3 Drug Delivery

Controlled drug delivery systems (DDSs) allow to overcome drawbacks of traditional, systemic administration of various therapeutics, i.e., low bioavailability of the active agent, and the unnecessary exposure of the whole organism to the drug, while its activity is usually needed only locally. DDSs have the ability not only to deliver therapeutic molecules to the specific location but also to release them in a controlled manner within the predefined time frame. Different types of materials were tested as possible drug delivery systems, with special attention paid to hydrogels, and among them chitosan-based ones.

As it was mentioned previously, crosslinking affects the swelling behavior of the hydrogels and consequently alters the drug diffusion characteristic. Slower drug release was observed in the case of chitosan-laponite (CS-LP) system when compared to pure CS due to strong ionic interactions between positively charged NH_3^+ groups of the chitosan chain and negatively charged laponite particles [54]. Dense, crosslinked polymer network is less prone to water absorption and swelling, preventing rapid dissolution of the drug entrapped within the network.

Sustained drug release system for wound dressing material was developed by Murthy et al. [98] by impregnating ciprofloxacin-loaded chitosan microparticles (CSMP) into the chitosan (CS-CSMP) or poly(vinyl alcohol) (PVA-CSMP) scaffolds. The high swelling ability of CS-CSMP scaffolds allowed to reach 82% of drug release. Both in vitro studies with NIH 3 T3 fibroblasts and HaCaT human keratinocytes confirmed biocompatibility of the scaffolds.

Cihan et al. [99] developed well-defined, spherical, hollow chitosan nanoparticles (size range: 30–300 nm) for the controlled delivery of highly lipophilic drug (Probucol). It was possible by first dissolving the drug in the hydrophobic core of the micelles of Pluronic copolymer through ionic gelation and crosslinking of chitosan around the micelles. Results showed very high drug uptake (up to 93.4%) and pH-sensitive behavior of the nanoparticles – significant increase of the drug release was observed at the slightly acidic pH (~6.8) when compared to pH 7.4. Ionic gelation (with TPP) was also incorporated for preparation of DDS based on N-succinyl-chitosan (SCS), N-glutaryl-chitosan (GCS), and taxanes [100]. Drug-loaded SCS and NCS spherical particles had a diameter of 300–350 nm and more than 50% of the drug releasing efficiency. Cytotoxicity of the taxane-containing nanoparticles against cancer cells was confirmed.

More complex, smart system was developed by Cui et al. [101]. Folic acid (FA) functionalized thiolated chitosan was used to fabricate multistimuli responsive microcapsules (MCRS-CS-MCs) via the sonochemical method. The obtained MCR-

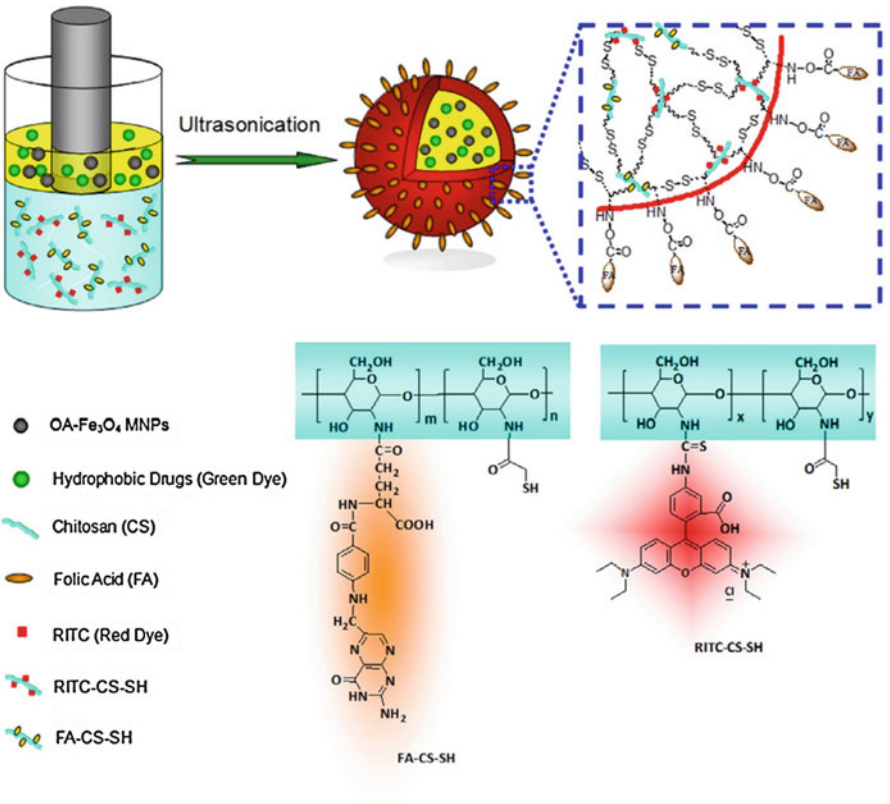

Fig. 7 The sonochemical synthesis schematic of multistimuli responsive smart chitosan-based microcapsules (MSRS-CS-MCs). (Reprinted with permission from [101]. Copyright © 2017, Elsevier)

CS-MCs spherical particles were loaded with oleic acid (OA) modified Fe_3O_4 magnetic nanoparticles and a green fluorescent dye, while FA and red fluorescent dye were immobilized onto the microcapsule shell (Fig. 7).

5.4 Water Purification

Water purification is currently an emerging problem worldwide. Industrial wastewaters are usually contaminated with organic and inorganic substances, such as heavy metal ions, pesticides, dyes, various microorganisms, and solid particles. Many of those can be removed using natural adsorbent systems. Chitosan, thanks to the presence of free amino groups, can serve as an effective adsorbent and coagulant, reducing the amount of suspended solids, chelating metal ions, or binding dyes.

Superhydrophobic and superoleophylic chitosan sponge was developed via TPP/citral crosslinking and octadecanethiol modification for the removal of oils from water in environmental remediation [102]. The CS sponge was able to absorb the amount of oil equal up to 60 times of its own weight and remained high absorptive capacity after many cycles. It was proposed that the oil or organic solvents were absorbed by the sponge due to hydrophobic interactions between them and the sponge and the capillary pore effect. Tested absorptive capacity included, e.g., hexadecane, toluene, chloroform, hexane, dichloromethane, pump oil, soybean oil and silicone oil. Combined with a peristaltic pump, the sponge allowed for continuous oil-water separation.

Chitosan may be also applied as a carrier for photocatalyst and photosensitizers immobilization for the visible-light-driven photodegradation of aqueous pollutants. Gmurek et al. [103] investigated the application of photosensitive phthalocyanine-immobilized chitosan as a structure generating singlet oxygen ($^1O_2$). The results confirmed its applicability with natural sunlight and effective water pollutant removal. Moreover, photoactive chitosan did not lose their photosensitizing properties even after several cycles.

Ampholytic chitosan/carrageenan (CS/CRG) microspheres exhibited high adsorption capacity towards cationic and anionic dyes and heavy metal ions. The microspheres adsorbed water contaminants efficiently due to their strong electrostatic and chelating affinity. Additionally, they were modified with Fe_3O_4 nanoparticles to allow magnetic separation. Developed microspheres are environmentally friendly since they can be easily recycled, reused and biodegraded in soil [104].

5.5 Other Applications

Chitosan is often applied as a coating material to improve cell compatibility of the substrate material and add functional groups on the surface that enable further modifications. In a recent study by Douglas et al. [105], chitosan was deposited on the titanium surface and subsequently modified with polyphenol-rich plant extracts. Chitosan coating improved cell adhesion, enhancing biological properties of the Ti. In another study [106], chitosan was covalently immobilized onto Ti surface to reduce the risk of implant-related infection by preventing bacterial colonization and enhance implant osseointegration.

6 Conclusions

Chitosan, a derivative of one of the most widespread natural polymer – chitin, gets an increasing attention both in academic and applied research due to its favorable properties. Chemical structure of chitosan allows various controlled specific modifications that is one of the most important advantages of this polymer over other polysaccharides like starch or galactomannans. Moreover, it is nontoxic, antibacterial, biodegradable, and biocompatible that makes chitosan very attractive

material in the biomedical field. Chitosan, with its outstanding properties, is one of the most promising and recently studied bio-based polymers for drug delivery, tissue engineering, gene therapy, and other medical applications. Hence, in this chapter, we present the state of art in the area of chitosan-based hydrogels – their preparation, properties, and application directions.

Chitosan is also an attractive material to further develop "green" chemistry strategies, including liquid solvents, wastewater treatment, heavy metal chelation, and biosensing. The results obtained so far show that there is still room for structural and morphological modification of chitosan to design novel chitosan-based hydrogels with enhanced properties. For instance, one of the latest technologies in scaffold fabrication is 3D printing, which for hydrogels is quite challenging; precise, layer-by-layer deposition performed easily for thermoplastic matrices is hard to achieve for hydrogels because of deformations occurring due to their weak mechanical properties. Nanostructural modifications may help to offer better solutions toward innovative hydrogel 3D printing approaches in biomedicine.

Acknowledgments The National Center for Research and Development (NCBiR), Poland, is gratefully acknowledged for support under STRATEGMED grant No STRATEGMED3/303570/7/NCBR/2017.

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