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R. Jayakumar
M. Prabaharan *Editors*

Chitosan for Biomaterials III

Structure-Property Relationships

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R. Jayakumar • M. Prabaharan
Editors

Chitosan for Biomaterials III

Structure-Property Relationships

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Preface

This volume summarizes the chemical modification, structure-property relationship, biological interaction and biomedical applications of chitosan and its derivatives. It consists of twelve chapters. Chapter "Perspectives and Challenges of Using Chitosan in Various Biological Applications" describes the perceptions and challenges of using chitosan and its derivatives in different biological applications, while the chapter "Review of the Structure of Chitosan in the Context of Other Sugar-Based Polymers" focuses on the structure and properties of chitosan and its interactions with other sugar polymers. Chapter "Synthesis & Modification Strategies of Chitosan and Its interaction with Metal Ions" deals with the interactions of chitosan-metal ions and their applications in wastewater treatment. Chapter "Synthesis-Structure Relationship of Chitosan Based Hydrogels" presents various methods that are employed in the preparation of chitosan-based hydrogels and the associated structural changes as well as their uses in drug delivery, haemostasis, tissue engineering and wound healing. Chapter "Antimicrobial Properties of Chitosan and Its Derivatives" describes the antimicrobial properties of chitosan and its derivatives, while the chapter "Flavor-Related Applications of Chitin and Chitosan in Foods: Effect of Structure and Properties on the Efficacy" explains the physicochemical properties and applications of chitin and chitosan as flavoring agents in the food industry. Chapter "Modified Chitosan Films/Coatings for Active Food Packaging" contains information on chemically modified chitosan films/coatings and their structure-property relationship and uses as a green substitute for plastics. Chapter "Chitosan-Based Biosensor Fabrication and Biosensing Applications" provides different strategies of utilizing chitosan as a substrate for biosensors and the chapter "Physical and Chemical Modification of Chitin/Chitosan for Functional Wound Dressing" describes the physicochemical modification of chitin and chitosan for the effective wound healing processes. Chapter "Preparation of Chitin Nanofiber and Its Derivatives from Crab Shell and Their Efficient Biological Properties" contains information about the preparation of chitin nanofiber and its derivatives from crab shells and their biological properties, whereas the chapter "Chitosan-Platelet Interactions" describes in detail the

chitosan–platelet interactions. Finally, the chapter "Chitosan-Stem Cell Interactions" summarizes the chitosan–stem cell interactions and their importance in regenerative medicine.

This volume of chapters will be highly helpful to researchers working in the field of biomaterials, biopolymers, polymer chemistry, materials science, drug delivery, tissue engineering, wound healing, biosensors, environmental science and wastewater research. The volume also offers a platform for all researchers to perform research on chitosan-based biomaterials mainly towards their structure, properties and biomedical applications. The volume of chapters also covers recent improvements in the area as well as projections about the future research and development of chitosan-based biomaterials.

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Perspectives and Challenges of Using Chitosan in Various Biological Applications

Sivashanmugam Amirthalingam, Arun Kumar Rajendran, Prabaharan Mani, and Jayakumar Rangasamy

Contents

Abstract Chitosan is a cationic biopolymer that is derived from chitin by the alkaline deacetylation technique. It has randomly distributed D-glucosamine and N-Acetyl-D-glucosamine units in its backbone. Due to the presence of primary hydroxyl and amino groups, chitosan can be modified into various derivatives with desired functionalities. Chitosan and its derivatives are considered as potential biomaterials to be utilized in various biological applications because of their easy availability, nontoxicity, biocompatibility, biodegradability, and gelling properties. This chapter provides a detailed review on the current status and challenges of using chitosan-based materials as biosensors, food coatings, drug delivery carriers,

Sivashanmugam Amirthalingam and Arun Kumar Rajendran contributed equally to this work.

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antimicrobial agents, wound dressings, hemostatic agents, and tissue engineering scaffolds.

Keywords Antimicrobial · Biosensors · Chitosan · Hemostatic · Tissue engineering · Wound dressings

1 Introduction

Chitosan is one of the biopolymers which can be extracted in large amounts in an environmentally viable manner for biological applications [1]. Chitosan is majorly obtained by processing the shellfish wastes, such as shells of crabs, shrimp, and squids [2]. Chitosan can be further processed to obtain various derivatives such as carboxymethyl chitosan, oxidized chitosan, deaminated chitosan, and so on [3, 4]. The basic structural unit such as N-acetyl D-glucosamine present in chitosan is one of the major components of the mammalian extracellular matrix (ECM) [5, 6]. Due to this advantage chitosan and derivatives can be processed for biological and biomedical applications. Another major advantage of chitosan and its products is that they can be processed into various forms such as powders, flakes, membranes, sponges, fibers, and hydrogels (Fig. 1) [5–9]. Furthermore, chitosan and its derivatives have various useful biological properties such as bioadhesion, biocompatibility, antimicrobial effect, and enhanced wound healing ability once placed in a biological system, thus proving to be an excellent biomaterial [10–13]. This chapter describes briefly the perspectives of chitosan and its derivatives in real-world applications and the challenges that are present to overcome.

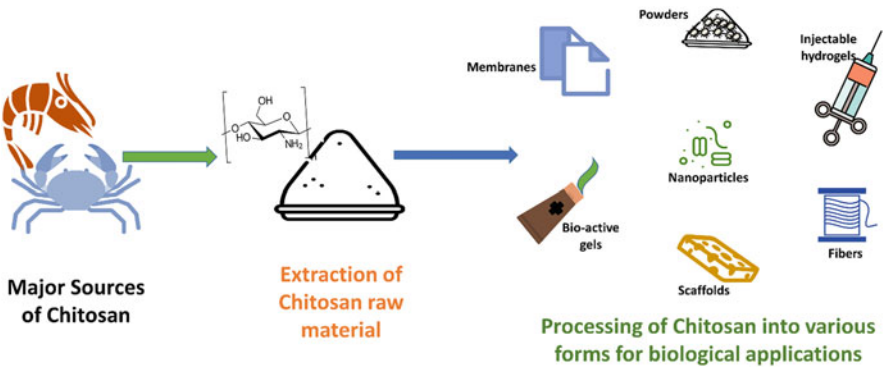

Fig. 1 Schematic showing various ways in which chitosan can be processed into various forms

2 Current Status and Challenges of Using Chitosan and Its Derivatives

2.1 Chitosan in Biosensor Applications

Recent years have seen a remarkable interest in the biosensing field with applications toward environmental monitoring, food processing, agricultural practices, and medical products [14–16]. In the preparation of an effective and reliable biosensor, immobilization of biological response elements (BRE) onto the biosensor is very critical for providing a suitable concentration of BREs immobilization and its specificity [17]. In this aspect, chitosan and its nanocomposites act as suitable immobilization surfaces. Chitosan, an aminoglucopyran, is a biocompatible, biodegradable, and gellable polymer [6, 7]. The presence of amino and hydroxyl groups in chitosan provides crosslinking moieties for immobilizing BREs onto the chitosan substrate [7, 18]. Another aspect of chitosan is that it can be easily processed into the hydrogel, providing a 3D architecture for immobilization of BREs and stability for immobilized BREs [7]. Additionally, chitosan could be utilized in a dry environment, wherein chitosan can be processed into a 3D dry scaffold, with the pore size in the range of microns. The porous structure provides an additional advantage of increasing the specific surface area of the chitosan scaffold, thereby providing more room for the BRE immobilization. Increasing the concentration of BREs per unit volume would be advantageous in the biosensor, as it would provide amplification of the output signal [5, 19] (Fig. 2).

Chitosan can be coated onto the electrode surface by simple dip-coating, as chitosan can be dissolved in acidic pH and regenerates into a hydrogel mass in pH 7–9 [7, 20]. Alternatively, spin-coating and layer-by-layer technique, thus helping to achieve a thin and dense layer of chitosan coating [21, 22]. Unfortunately, these methods are suitable for coating onto a smooth surface of the electrode, as a coating in a complex and porous electrode would result in local accumulation and uneven coating [8]. To circumvent these problems, chitosan can be deposited onto

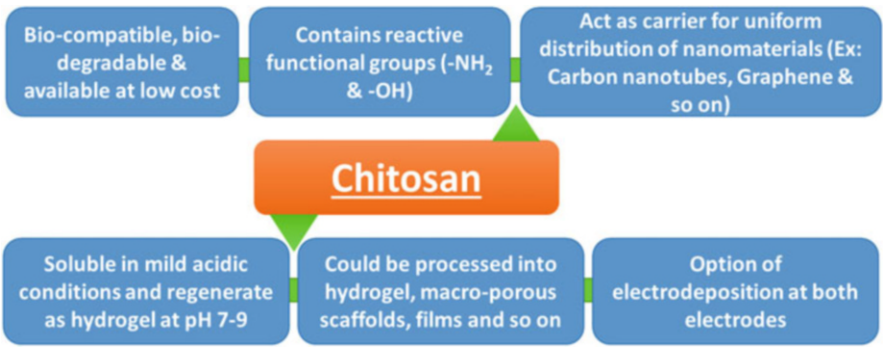

Fig. 2 Advantages of using chitosan in biosensor applications

electrode through electrodeposition. Wu et al. showed spatial-selective deposition of chitosan on to gold band electrode with a resolution of the coating 20 μm [23]. With further optimization, Buckhout-White et al. presented the resolution of electrodeposition technique could be achieved as low as 0.5–1 μm [24]. Thus, the electrodeposition technique could be utilized to coat the miniature electrodes including micro- and nano-arrayed electrodes, which are not realizable in the conventional coating techniques.

Besides utilizing pristine chitosan as the immobilization surface, nanocomposite chitosan has been utilized as it could provide higher electrical conductivity and surface uniformity than pristine chitosan [25, 26]. Here, chitosan would act as a substrate matrix and as a nanomaterial carrier. Nanomaterials such as carbon nanotubes, graphene, and carbon nanodots are hydrophobic, however, they get homogenously dispersed in the chitosan matrix, thereby proving stability to the biosensor [8, 27]. Thus, nanocomposite chitosan substrate opens the possibilities for high-performance biosensors.

Many studies have shown the use of chitosan-based biosensor with BRE as DNA [28–31], enzyme [32–34], antibody [35–37], and aptamers [38]. Recent studies have indicated that the incorporation of nanomaterials with chitosan could exhibit sensitivity and specificity in electrochemical biosensing without the use of BREs [18]. Mane et al. reported an atropine biosensor, coating made of single-walled carbon nanotube (SWCNTs) dispersed in chitosan matrix on to glassy carbon electrode [39]. Similarly, the chitosan-graphite composite showed detection specificity for dopamine [40]. These studies indicate that the nanocomposite chitosan could acquire new chemo-physical properties, which are based on the inclusion of the nanomaterial onto the chitosan. Furthermore, Al-Mokaram et al. reported non-enzymatic glucose biosensor based on chitosan-Iron oxide-polypyrrole nanocomposite coating [41]. These approaches open a new window of BRE-free biosensors.

Chitosan is bio-compatible biodegradable and cost-effective polymer. It's physical properties could be tuned based on the requirements for biosensing applications. However, more detailed studies should be focused on the stability of biosensors for a long-time application in field-conditions, which lack sophisticated facilities and have harsh environment conditions, large temperature variations, and humidity variations. Thus, the ultimate goal of developing chitosan-based biosensors should be engrossed to improve the sensitivity and specificity of competitive commercial products.

2.2 Application of Chitosan in Food Coating

Coatings are defined as coherent layers of coating material onto the substrate, either directly onto the consumable food as an edible coating or onto the surface of packaging material to functionalize them [42, 43]. In edible coating, chitosan has been widely studied as an active coating material for extending the shelf-life of food

products, especially fruits, vegetables, meat, and so on [43]. The coatings are applied directly onto the food product as a film by dip-coating, brushing, spraying, or fluidizing [44]. Chitosan coating in vegetables and fruits can reduce water loss, delay ripening, and decay [45–50]. In the case of chitosan coating in meat products, the coating can improve the meat quality by preventing moisture loss, reducing lipid oxidation and discoloration, and increasing the product appearance [51–53]. The coating could also act as a carrier for functional food additives. Additionally, chitosan coating could act as a gaseous exchange barrier [54], antimicrobial property of chitosan could hinder microbial growth and these properties could synergistically enhance the shelf-life of the coated food products [51, 53].

Current studies showed that addition of functional compounds such as antimicrobial and antioxidant agents onto chitosan coating not only helps in water loss and retard decay but also enhances the intrinsic antimicrobial property. The functional active compounds, such as a natural extract from plants, phenolic extract, or essential oils, would provide antimicrobial, antioxidant properties and enhance the food taste, thus improving shell-life for the food product and functional quality of it [47, 53]. This is attractive against conventional coating, as they would help in water loss and prevent the decaying of food products [55]. Additionally, edible chitosan coating plays a very important role in ecologically responsive packing [19]. Moreover, there is an increasing number of consumers looking for greener product coating and, in this aspect, chitosan coating is attractive (Table 1).

Chitosan as an active coating material for food products has been extensively studied for improving the shelf-life of the food products, as chitosan is regarded as a generally recognized as safe (GRAS) material by the United States Food and Drug Administration (US FDA) and its intrinsic bioactive properties. However, their performance in terms of mechanical, thermal, and water seepage properties needs to be improved for commercial viable usage. Blends and composite, including nanocomposite coatings, have been explored and need improvisation in mechanical properties [43, 56, 57]. For cost-effectiveness, chitosan with green plasticizers (glycerol, xylitol, sorbitol, and choline) [43, 58] could be explored as it is a promising technology that helps to take the materials at the industrial level and provides mechanical and thermal properties to chitosan.

2.3 Chitosan in Drug Delivery Applications

Drug delivery system is principally consisting of one or more active pharmaceutical agents with a suitable carrier. The first critical factor is to achieve the drug concentration in the therapeutic window, which could be addressed through the usage of carrier polymer. As mentioned earlier, chitosan is regarded as GRAS by US FDA. Chitosan could be easily modified, as it has hydroxyl and amino groups in its structure. Functionalization of chitosan in the –OH and $–NH_2$ groups would result in N-modified, O-modified, and N, O-modified chitosan which would have

Table 1 Chitosan edible coatings on vegetables, fruits, and meat products

Food products	Functional materials	Storage conditions (°C)	Advantages	Refs.
Tomato	Pomegranate peel extract (PPE)	23 and 4	Tomatoes coated with chitosan containing PPE improved post-harvest quality at room and cold temperature storage	[45]
Cashew nuts	Mango leaf extract (MLE)	23	Indicated higher oxidation resistance, hydrophobicity, tensile strength compared to commercial polyethylene films. The addition of MLE provided antioxidative properties	[46]
Broccoli	Essential oils and bioactive compounds	5–7	Pristine chitosan or functional active product enriched chitosan showed reduced mesophilic and psychrotrophic populations. The coating didn't affect the food taste quality	[47]
Guava	PPE	10	Coating with PPE enriched chitosan found to be most effective in maintaining the overall quality of guavas for up to 20 days at 10°C storage	[48]
Cape gooseberry	Rue essential oil	18 ± 2	Coating with rue essential oil enriched chitosan reduced the weight loss and retarded the microbial growth up to 12 days than control	[49]
Fresh-cut apples	Ascorbic acid	5	Coating with chitosan and ascorbic acid reduced browning, retained flesh firmness and held the phenolic contents	[50]
Chicken wingettes	Eugenol	4	Chitosan coating fortified with eugenol effectively reduced *C. jejuni* and aerobic bacterial contamination	[51]
Fresh chicken meat	*Artemisia fragrans* essential oil (AFEO)	4	Coated samples showed higher phenolic contents and organoleptic contents. The coating also inhibited antimicrobial growth, thiobarbituric acid reactive substances, and total volatile base nitrogen	[52]
Pork chops	Green tea aqueous extract	0	Green tea extract inclusion in chitosan reduced pH, lipid oxidation, color change, and microbial load	[53]

numerous physio-chemical properties and improved biological activities [3, 59, 60]. For example, quaternized and phosphorylated chitosan has enhanced antimicrobial and solubility properties compared to pristine chitosan, whereas thiolated chitosan has shown improved mucoadhesive properties. Sulfated chitosan has been shown to increase the growth factor binding, whereas N, O-carboxymethyl chitosan is shown to dissolve in neutral pH [3, 59]. Non-selective N, O-modification in chitosan can be achieved by an electrophilic substitution reaction. Selective O-modification in chitosan is achieved by using acids, as the acids protonate the

amino group, thereby providing the –OH group to undergo modification, whereas N-modification in chitosan is achieved by protecting the hydroxyl group [61]. Various modified chitosan and its synthesis route are depicted in Fig. 3. Chitosan NPs could be prepared in numerous methods including ionotropic gelation, microemulsion, co-precipitation, emulsion-coalescence, polyelectrolyte complexation, solvent evaporation, spraying drying, and high-pressure homogenization [59, 61]. Selection of the nanoparticle preparation method solely relies on end-product characteristics such as particle size and its size distribution range, stability, drug release kinetics, and particle shape. The ionotropic gelation method involves the usage of anionic crosslinkers such as sodium tripolyphosphate (STPP), which forms amphoteric ion-pair with chitosan [62, 63]. The molar ratio of chitosan and STPP would control the mean particle size, distribution, and drug release kinetics. One of the limitations of using this method is that chitosan NPs are relatively unstable in acidic pH and have poor drug loading capacity for large molecular weight drug payloads [64, 65]. Thus, depending on the end-product, the chitosan NPs synthesis method could be appropriately selected.

The therapeutic efficacy of the developed drug delivery system mainly relies on pharmacokinetics, tissue absorption, and metabolism. In this aspect, many studies have indicated the pharmacokinetic advantages for chitosan NPs over other polymeric NPs [66–68]. For instance, El-Shabouri found that chitosan NPs showed the highest C_{max} compared to gelatin and glycocholate NPs for cyclosporine-A delivery [68]. Recent studies have focused on stimuli-responsive drug delivery, wherein the microenvironment-triggered [69–71] or external stimuli-responsive [72, 73] delivery systems could be designed. These systems would help in achieving higher therapeutic dose at the site of requirement, thus alleviating the disease condition at the earliest [74]. Targeted drug delivery [75–78] and theragnostic [79, 80] applications using chitosan have been explored. All the studies have looked at improving the bio-distribution, drug release kinetics, and targeting. A chitosan-based nasal formulation of morphine (Rylomine™), as an absorption enhancer, is currently in clinical trials [81, 82]. We expect that Rylomine™ would go to the market soon and it would pave a path for other chitosan-based formulations to reach for clinical use, as it can help in discerning any unanticipated concerns in clinical use. We look forward to future works focusing on the long-term toxicity effects of the chitosan NPs. Most of the in vivo studies show the bioavailability and biocompatibility for 2–4 days after the administration. This is not sufficient for the long-circulating NPs to fully represent the drug distribution and the biodegradation in such a short time.

2.4 Chitosan in Antimicrobial Applications

Tackling and reducing microbial infections is one of the major treatment protocols in modern medicine. Humankind has been in continuous search of newer molecules aiding in antimicrobial activity to treat various infections, to be used as prophylaxis and to sterilize the surfaces [83]. Among various antimicrobial agents, the

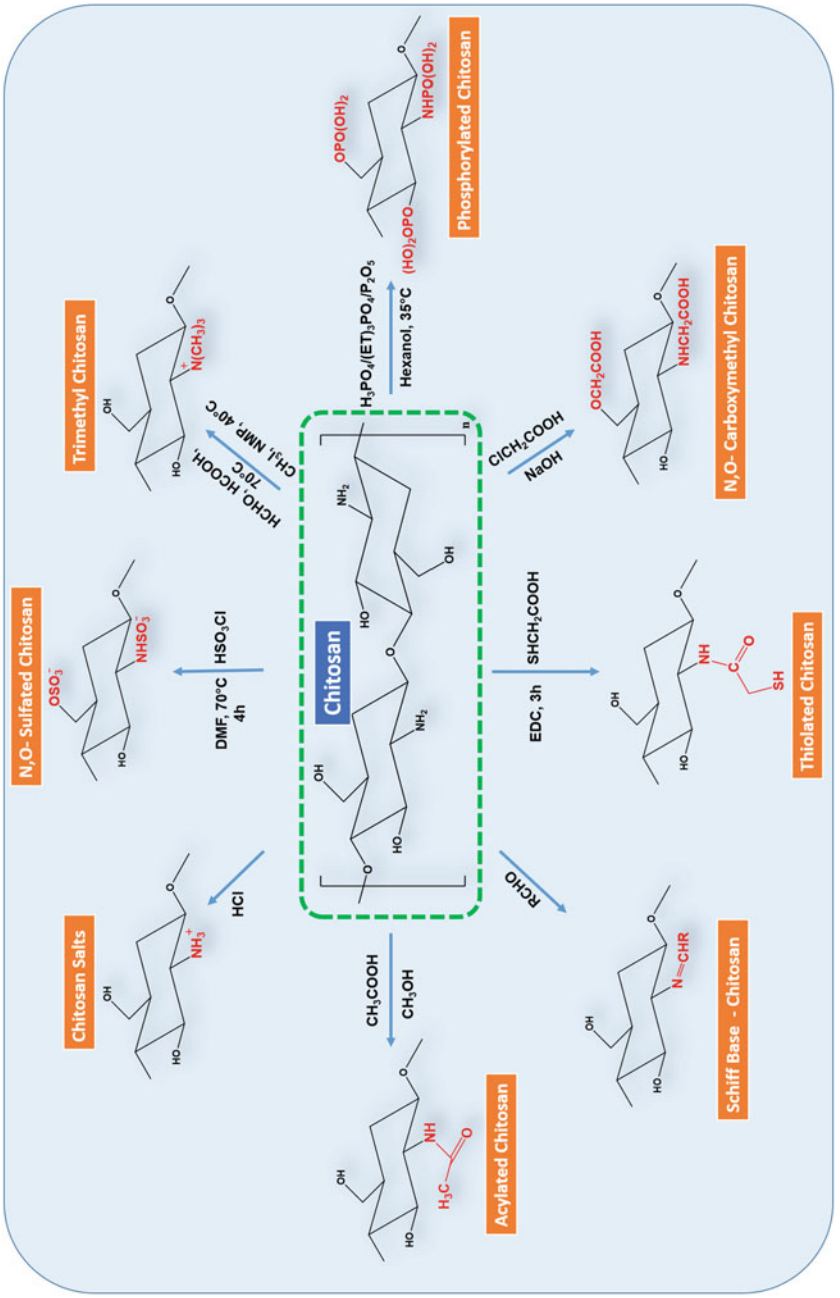

Fig. 3 Synthesis scheme of the functionalized chitosan derivatives

antimicrobial properties of chitosan have been long known and have been potentially utilized in various forms and formulations [11, 13]. Chitosan has been prepared in the form of films, sponges, nanoparticles, fiber sheets, gels, and 3D printed hydrogels [6, 84–87]. The properties such as bioadhesiveness and biodegradability further add a great advantage to chitosan-based products to be used as antimicrobials. It has been known that chitosan by itself can act as an antibacterial agent [88, 89]. This occurs mainly due to the electrostatic interaction of cations of chitosan with the anionic walls of gram-positive bacteria. This binding onto bacterial cell walls can inactivate the bacterial proteins, cause DNA damage, rupture the cell wall, and lead to cell death. In gram-negative bacterial species, chitosan can block the nutrient channels in the cell wall, thus cutting off the nutrition and leading to the death of bacterial cells [90]. Furthermore, chitosan has been formulated along with different antimicrobial agents such as tetracycline, amphotericin B, lysostaphin, silver, copper ions, zinc oxide, and iron oxide [91–96]. For example, micro and NPs of chitosan loaded with curcumin have shown good efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [97]. Chitosan has also been utilized to deliver chlorhexidine in the intracanal spaces of the tooth to successfully combat *Enterococcus faecalis* [98]. In recent years, improvements have also been made to impart antibacterial as well as antimycotic activity in chitosan formulations. For example, both ciprofloxacin and fluconazole have been loaded into chitosan bandages to provide multi-microbial efficacy [99].

Although chitosan has great potential by itself to act as an antimicrobial agent and a very good carrier to deliver various antibiotics and antimycotics, there are few bottlenecks to overcome in practical scenarios. It has been found that chitosan's antimicrobial effect is most pronounced in pH below 6 [13]. Although this could be advantageous in some of the localized infections where the microenvironmental pH is known to be acidic, chronic wounds tend to be in alkaline pH, where the real potential of chitosan could be hindered [100]. To overcome this, chemical modifications are required, so that chitosan can act at very wide ranges of pH. Also, it has been noted that the antimicrobial effect of chitosan depends on its molecular weight. Therefore, a batch-wise strict control of the molecular weight of chitosan in large-scale productions is required to achieve the maximum effect. The methods to improve the narrow molecular weight distributions during production and further processing should be taken care of. Furthermore, a small percentage of people can elicit allergic responses when in contact with chitosan due to its origin. When these bottlenecks have been overcome, chitosan-based antimicrobial medical devices would have great reach among the masses.

2.5 Chitosan in Wound Dressing Application

Wounds are a very common issue in day-to-day life, occurring almost to everyone. Based on the origin of the wound, wounds can be classified as superficial, incised, crush, lacerated, stab, contused, and secondary wounds [101]. Generally, wounds

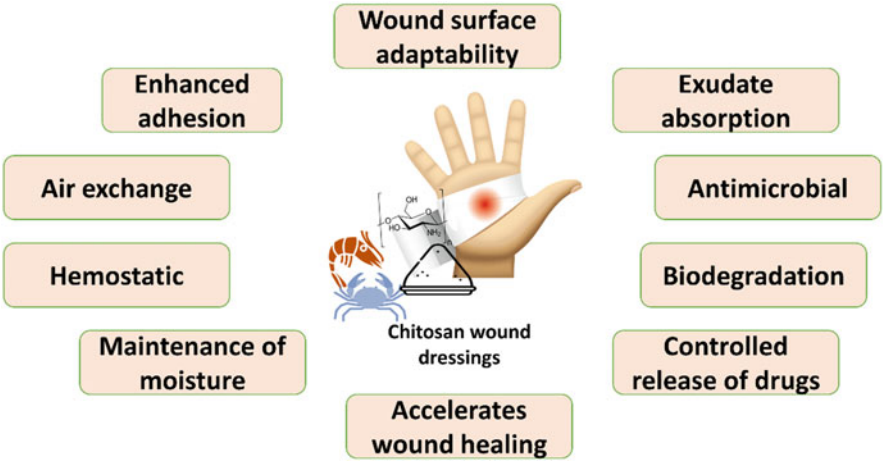

Fig. 4 Schematic describing some of the major advantages of chitosan-based wound dressing materials

can arise through either trauma or pathological conditions. Almost in every case, to have uneventful healing, a wound dressing material is required. Wound dressings can play a very important role in wound healing such as protecting the wound from further damage, preventing the microbial contamination of the wound, acting as hemostatic by stopping the bleeding thereby preventing blood loss, absorbing the wound exudates, providing a moist environment for the underlying tissues and can also deliver medicinal components to rapidly heal [6, 12, 101, 102]. The search for a versatile material that could aid in the above-mentioned functions is always on the research. One such material that could be utilized in different forms is chitosan and its derivatives. As described earlier, the biodegradable biopolymer chitosan is made up of a component of human ECM glycosaminoglycan that can be very beneficial in wound healing [9].

Chitosan and derivatives can be formed into porous sponges, membranes, or sheets in varied sizes so that they can be easily applied over the wound surface. The inherent bioadhesiveness of the chitosan, when hydrated, is very advantageous as the dressing material can attach itself to the wound surface without any external aid [10, 12] (Fig. 4). Furthermore, the sponge form of chitosan-based wound dressings can easily absorb the exudates several times their dry weight, due to the porous nature [103]. After absorbing the excess of exudates, the chitosan-based wound dressing materials can act as a hydrogel and could also maintain the moist environment for the underlying tissue, thus helping in the growth of new tissue, by preventing the drying out of the wound [104]. Chitosan-based wound dressings biodegrade into n-acetyl glucosamine, which can be utilized by the newly forming tissues to create a new ECM, thus reducing the scarring. Chitosan and its derivatives-based wound dressings have also been loaded with various antimicrobials such as ciprofloxacin, tetracycline, thymol, naproxen, silver sulfadiazine, silver

nanoparticles, copper, and glucan [91, 95, 99, 105–108]. Apart from all these advantages, chitosan is derived majorly from waste shells of crabs, shrimps, and squid, thus reducing the cost to the end-user. However, one of the serious issues that are associated with the use of chitosan and derivatives for wound dressing is that chitosan can elicit immune responses in some individuals. Being in very close contact with the open tissues where the chitosan will be in close contact with the immune cells, it should be taken care that proper sensitivity tests carried out to rule out the possible allergic reactions before applying to people. Furthermore, modifications can also be done to chitosan-based products like coating with other polymers such as hyaluronic acid, PEG, and so forth, so that the immunogenicity is reduced, thus proving a safe and wide use.

2.6 Chitosan in Hemostatic Applications

Hemostats are very vital components in first aid and also during various surgical procedures. Hemostats are agents that can arrest the bleeding in a very short period, preferable in seconds to minutes and maintaining the seal of the ruptured blood vessel, thus preventing a life-threatening condition [109]. Blood loss due to rupture of blood vessels can occur in various instances such as surgery, road traffic accidents, workplace mishaps, trauma, battle wounds, animal attacks, and natural disasters [110]. A good hemostat that could reduce the bleeding time and stabilize the blood vessel rupture even a few seconds faster could save an individual's life. A good hemostat should be easy and ready to apply, should preferably adhere to the bleeding surface, should rapidly induce the clotting of blood, should hold the blood clot together in place, thus preventing delayed bleeding, preferably should form a good seal for the ruptured blood vessel and also it will be an added advantage if it could also seal the wound, thus preventing contaminations and infections and also does not interfere in the wound healing process [111, 112].

Chitosan-based hemostats have been researched extensively so that at present there are numerous hemostats available commercially, some even recognized for military uses [110, 113]. Chitosan has an excellent blood clotting ability by itself, owing to its polycationic chemical composition. The presence of numerous amino groups, that has positive charges, can easily interact with the negatively charged blood cells such as platelets and red blood cells and initiate the blood clotting cascade (Fig. 5) [114]. The very common form of chitosan-based hemostat is the dry chitosan flakes or powders that can be rapidly applied to a variety of wounds. Such chitosan flakes or powders, due to the ability to absorb and swell up, can easily plug and seal deep wounds and form an artificial clot-like structure upon contact with blood. Various other forms such as gauzes, gels, hydrogels, sponges have also been used as hemostats [115]. Various researchers have tried to improve the blood clotting ability of chitosan-based hemostats through chemical modifications such as adjusting the degree of deacetylation, crosslinking with glutamic and aspartic acid, combining with alginic acid, zeolite, zinc ions, cellulose, kaolin, graphene oxide,

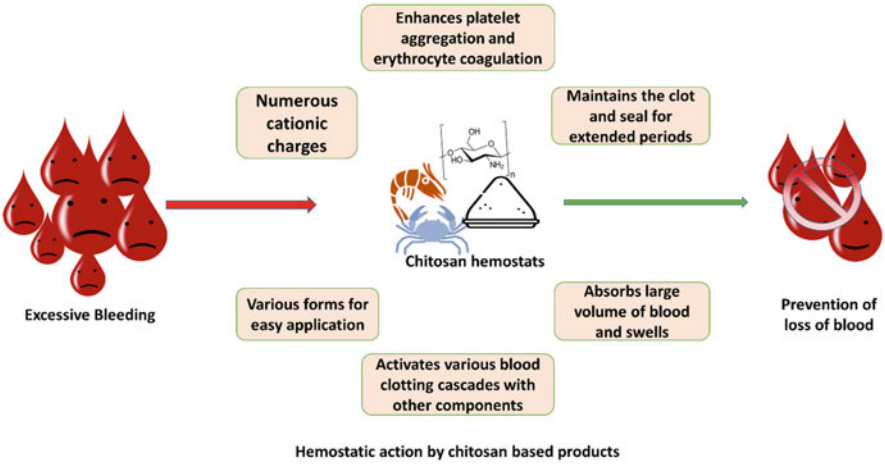

Fig. 5 Schematic showing the various modes of action of chitosan-based hemostats

and gelatin [116–122]. Recently developed chitosan-based injectable hydrogel containing potassium aluminium sulfate as a vasoconstrictor and calcium chloride as a coagulation activator was able to rapidly stop the femoral artery hemorrhage as quick as 105 ± 31 s in rat models. Furthermore, it was able to control the bleeding from the rat liver puncture model within 20 ± 10 s of application, thus proving to be a great hemostatic agent [123]. With further improvements in the chitosan hemostatic formulations such as the incorporation of molecules that aid in blood clotting, adding components that could form a very tight adhesive seal of the arteries we could expect much more rapid hemostats in the future. Further, newer formulations such as gels, injectable hydrogels, superabsorbent gellable powders would help the common people and clinicians in stopping the bleeding and saving lives.

2.7 Applications of Chitosan in Tissue Engineering and Regenerative Medicine

Tissue engineering and regenerative medicine are some of the rapidly growing fields in medicine to improve the quality of life of people suffering from loss of tissue due to various reasons such as trauma, pathological tissue removals, cancers, and developmental anomalies [124]. Biomaterials that could aid in tissue regeneration and provide rapid and uneventful healing are on continuous exploration. Among the current biodegradable biopolymeric biomaterials, chitosan and its derivatives seem to be a great option when considering various advantages such as unlimited and ecologically friendly source as it is derived from shellfish waste, large-scale production, easy processability into different forms, the ability to incorporate various chemical functionalities, the versatility to load and blend different polymers and

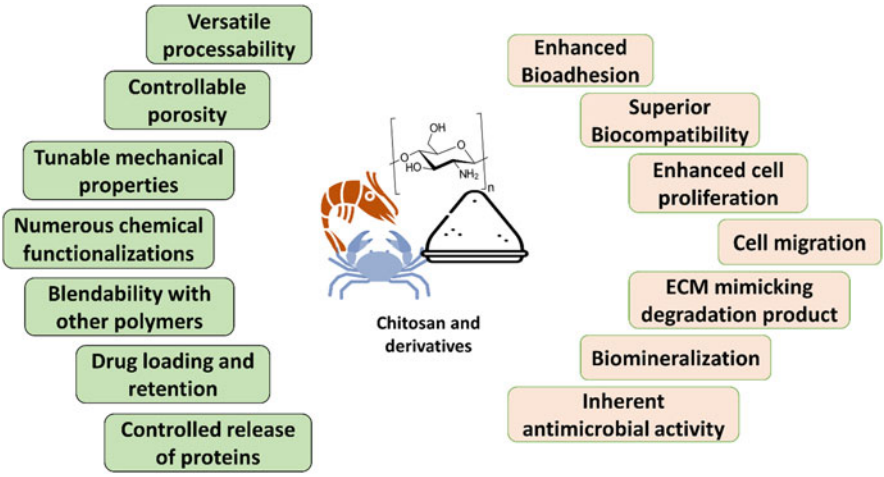

Fig. 6 Schematic listing the various advantages of utilizing chitosan and its derivatives for tissue engineering and regenerative medicine applications. Green boxes show the physical and chemical advantages and the orange boxes show the biological advantages

drugs and most of all the biodegradation product results in *N* -acetyl D-glucosamine which can be utilized by human cells to produce ECM (Fig. 6) [1, 2].

Chitosan when processed in certain ways, such as sponges, can give rise to porous structures which would help the cells to move in, attach themselves, and act as a great scaffold for new tissue to be formed [125]. Chitosan scaffolds have been used as a successful aid for almost all sorts of tissue regeneration such as skin, bone, tendon, cartilage, cardiac tissues, neurons, dental pulp, and periodontium [126–133]. The physicochemical and biological properties of chitosan and its derivatives-based scaffolds can be greatly enhanced for tissue engineering and regenerative medicine by blending with different biopolymers such as alginate, hyaluronic acid, chondroitin sulfate, collagen, gelatin, fibrin, synthetic polymers such as poly(glycolic acid), poly(lactic acid), poly(lactic-co-glycolic acid), poly (caprolactone), poly(hydroxybutyrate), poly(vinyl alcohol), and so on [125]. For utilizing the chitosan-based scaffolds in bone regeneration bioceramics such as calcium sulfate, calcium phosphate, hydroxyapatite, bioglass, and so forth have been tried upon and shown to have a good potential [134–136]. Similarly, electrically conductive components such as graphene oxide, carbon, nanotubes, gold and silver NPs, and polypyrrole have been added and utilized greatly for tissue engineering of electrically conductive tissues such as neurons, muscles, and heart [7, 137]. Researchers have also successfully loaded a variety of growth factors, peptides, and small molecules such as FGF, VEGF, BMP-2, CEMP1, NGF, RGD, PTH derived peptides, dexamethasone, and ascorbic acid for enhancing regeneration of specific tissues [6, 127, 133, 138, 139]. The ability of chitosan to be processed into injectable hydrogels, even along with the bioceramics and other components, is one of the greatest advantages so that it could be injected easily into the defects through

small surgical keyholes, having a great defect filling and adaption to the defect margins thus providing a perfect continuous bridge for the cells and nutrients to migrate into the scaffold and resulting in better healing [140]. Some researchers have even utilized chitosan to deliver DNA and RNA to enhance tissue regeneration [141, 142]. Thus, if the processing methods to reduce the batch to batch variations of chitosan, strict control of the molecular weight of chitosan, strategies to reduce the allergic responses in some people are improved, chitosan would prove as a great viable material to be utilized in the field of tissue engineering and regenerative medicine in the upcoming years.

3 Conclusion

In this chapter, we have provided a brief overview of the current status and challenges in using chitosan for biosensing, active food packaging, drug delivery antimicrobial, wound healing, hemostatic, tissue engineering, and regenerative medicine applications. In summary, the future potential of chitosan in various biomedical applications is very promising. The increased interest in chitosan has also driven the expansion of chitosan industries. Currently, many chitosan-based products are available in the market for hemostatic and wound healing applications. In the similar line, commercial utilization of chitosan in other fields would also be anticipated in near future. We believe that a serious awareness of the emergence of protocols in assessing the quality of chitosan could increase the momentum of chitosan usage in various biological applications.

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Review of the Structure of Chitosan in the Context of Other Sugar-Based Polymers

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Contents

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Abstract Chitosan is a natural amino polysaccharide composed of multiple reactive functional groups. It is a cationic polymer that is biodegradable, biocompatible and non-toxic. The structure of chitosan allows diverse modifications via its hydroxyl and amino groups, which can enhance its properties and also allow complex formation with other polysaccharides. The physicochemical properties of chitosan, such as solubility, viscosity, thermal stability, etc., majorly depend on the degree of acetylation and molecular weight. The unique physicochemical properties of chitosan enable its use in a range of biomedical applications such as tissue engineering, drug delivery and anti-microbial applications. This chapter focuses on the structure of chitosan and its interactions with other sugar polymers, highlighting its properties that have paved its way in a variety of biomedical applications.

Keywords Alginate · Biomedical applications · Cellulose · Chitosan · Functional groups · Starch · Sugar-based polymer

1 Introduction

Biologically derived polymers or biopolymers are naturally occurring polymers produced by living organisms like plants, animals, bacteria and fungi. They are older than the synthetic polymers, like plastics, and have been on the earth from billions of years. They represent one of the most abundantly available biomaterials that display a huge diversity with respect to their individual monomers and secondary structures. The main groups of biopolymers include proteins, nucleic acids, polyhydroxyalkanoates, polyphenols and polysaccharides. Polysaccharides, also known as sugar-based polymers or glycans, are linear or branched polymeric carbohydrate structures that are composed of long chains of monosaccharide units and/or oligosaccharide units, which are bound together by glycosidic linkages. Upon hydrolysis, they give rise to individual monosaccharide units or oligosaccharides. There are two types of polysaccharides, viz. the homo-polysaccharides and hetero-polysaccharides. A homo-polysaccharide contains repeating units of only one type of monosaccharide, like cellulose, starch, glycogen, chitin, etc. A hetero-polysaccharide is composed of more than one type of monosaccharide, like hyaluronic acid, peptidoglycans, etc. In both types of polysaccharide, the monosaccharides are linked either linearly or they can branch out into complex formations. The structure of monomers governs the structures and properties of the resulting polysaccharides [1]. Among the widely studied polysaccharides, cellulose,

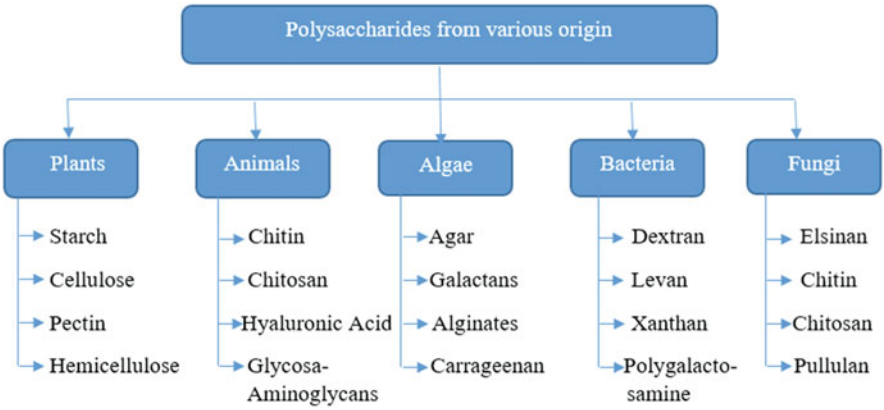

Fig. 1 Natural polysaccharides classified according to their origin

starch, chitin, glycogen and hyaluronic acids are some of the important examples. They can also be classified based on their origin, as depicted in Fig. 1.

The main functions of polysaccharides include the provision of structural support and as energy reservoirs in plants and animals. In their natural environment, they also mediate gelation, hydration and lubrication and play a role in cellular communication. Natural polysaccharides fulfil numerous functions, such as provision of energy reserves for plants (i.e. starch), as structural support to vegetal cells (i.e. cellulose in plants and chitin in crustaceans), as gelling agents to form the intercellular matrix and as reserves of several ions such as sodium, calcium and magnesium (i.e. alginate in the brown algae) [2]. They are employed for a wide range of applications due to their availability from renewable agricultural or marine food resources, versatility and biodegradability. Owing to their remarkable properties, polysaccharides are currently being used as a thickening, stabilizing, gelling, film-forming and emulsifying agents in various industrial sectors. Due to their chemical and structural diversity, biopolymers are widely used in food, pharmaceutical and biomedical industries, as sustainable, renewable, biodegradable and non-toxic raw materials. Some biopolymers like cellulose, starch, chitin and polysaccharides from seaweeds are commercially important in several fields, ranging from the food processing industry to the paper industry. A list of different biopolymers and their roles has been stated in Table 1.

Being environment-friendly and sustainable materials, development of sugar-based biopolymers has attracted increasing attention. Chitin is a structural biopolymer that plays a role analogous to that of collagen in higher animals and cellulose in terrestrial plants. It is considered to be the second plentiful biopolymer after cellulose and is of ace significance. Annually, this biomaterial is estimated to be produced in a quantity similar to cellulose as it is the major source of coastal pollution. Chitin is an amino polysaccharide polymer and is the building material that gives strength to the exoskeletons of crustaceans, insects and the cell walls of fungi [3]. Molluscs, crustaceans, insects, fungus, algae and related organisms approximately produce

Table 1 Biopolymers and their roles

Polymer	Type	Monomer units	Role
Amylose	Homo	D-Glucose	Energy storage in plants
Amylopectin	Homo	D-Glucose	Energy storage in plants
Glycogen	Homo	D-Glucose	Energy storage in bacteria and animal cells
Cellulose	Homo	D-Glucose	Structural: gives rigidity and strength to plant cell walls
Chitin	Homo	N-Acetylglucosamine	Structural: gives rigidity and strength to the exoskeleton of insects and crustaceans
Alginate	Hetero	D-Mannuronic acid and L-Guluronic acid	Structural: gives rigidity and strength to the cell wall of brown algae
Peptidoglycans	Hetero	N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM)	Structural: gives rigidity and strength to the bacterial envelope
Proteoglycans	Hetero	Glycosaminoglycans and core protein	Structural: viscosity and lubrication in joints of vertebrates
Glycosaminoglycan (hyaluronate)	Hetero	Uronic acid and GlcNAc/GalNAc	Structural: extracellular matrix in skin, connective tissue

around ten billion tons of chitin, annually. It has gained interest not only due to its high availability and limited exploration but also as a functional biomaterial due to its high potential in various fields. Chitin is a bio-renewable, environmentally friendly, bio-functional, biocompatible and biodegradable material. It owes its biodegradability to the action of chitinase enzymes that are widely distributed in nature. The application scope of chitosan, the deacetylated derivative of chitin, is multi-dimensional, as a biodegradable pressure-sensitive adhesive tape, chelating agent, water treatment additive, drug carrier, wound-healing agent and good adhesive material for sorption. Because of these advantages, this unique biopolymer has gained much attention [4]. The commercial exploitation of chitin is mainly focused on regenerating this raw material from marine waste, mainly waste constituted by shellfish, such as shrimp, crabs, lobsters and squids. Another reason for the high interest in this polymer is its wide range of physical forms and its amenability to numerous chemical modifications, which further enhance its solubility and widen its applications. The presence of multiple reactive functional groups in chitosan renders it as an ideal biomolecule for diverse manipulations, to provide a broad spectrum of derivatives for specific end-applications in different areas, as mentioned earlier. Chitosan is gaining more interest due to its strong biocompatibility and ability to promote cellular adhesion. This is majorly due to its ability to form polyelectrolyte complexes with polyanions in solutions at a low pH [5]. This chapter will focus on the structure and biological applications of chitosan and its derivatives, particularly in the context of other sugar-based biopolymers like starch, cellulose and alginate.

2 Structure and Composition

2.1 Structure of Chitin and Chitosan

Chitin $(C_8H_{13}O_5N)n$ is a linear polysaccharide, a homopolymer of N-acetyl-D-glucosamine, containing β-(1-4) glycosidic bonds, as depicted in Fig. 2. Chitin is a white, hard, nitrogenous polysaccharide, which is inelastic and present in the exoskeleton as well as in the internal structures of invertebrates. It is the second most prevalent, natural form of polymerized carbon [6]. Based on its source, chitin occurs in two main polymorphic forms, namely α and β forms. The polymorphic forms of chitin differ in the packing and polarities of near chains in successive sheets. In the β-form, all chains are arranged in parallel mode, whereas the α-chains present an anti-parallel arrangement of its individual chains. The reactivity of β-chitin isomorphs is greater than the α-isomorphs, which is important for enzymatic and chemical transformations of chitin. In these two structures, the chains are organized in sheets and held by intra-sheet hydrogen bonds. Also, in α-chitin, inter-sheet hydrogen bonds prevent diffusion of small molecules into the crystalline phase, whereas inter-sheet hydrogen bonds are absent in the crystal structure of β-chitin. This leads to swelling of β-chitin in presence of polar guest molecules, ranging from water to alcohols and amines, which penetrate the crystal lattice without disturbing the sheet organization and the crystallinity of the sample. The removal of polar molecules allows reversion to the original state of anhydrous β-chitin. The reactivity of β-chitin isomorph is larger than the α-isomorph, which is important for enzymatic and chemical transformations of chitin. Recent investigations have revealed the presence of a minor form, ϒ-chitin, which is a variant of the α-form [4, 7].

Chitosan is an unbranched cationic polysaccharide, composed of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine, precisely β-(1-4)-linked 2-amino-2-deoxy-β-D-glucopyranose and partially of β-(1-4)-linked

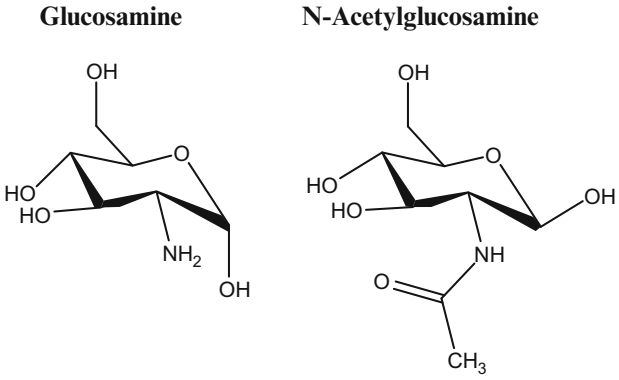

Fig. 2 Structural units of chitosan and chitin

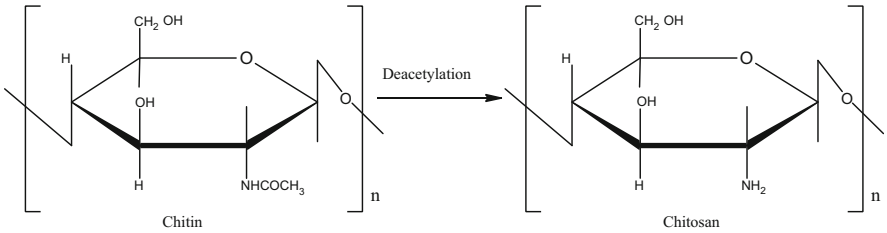

Fig. 3 Structures of chitin and chitosan

2-acetamido-2-deoxy-β-D-glucopyranose units, wherein β-linked D-glucosamine units are pre-dominant in the polymeric chains. The glycosidic bonds consist of an oxygen molecule that bridges two carbon rings and is formed when a hydroxyl group is lost from the carbon of one molecule, while hydrogen is lost from the hydroxyl group of another monosaccharide. The amine groups in chitosan become protonated at acidic pH and exhibit a strong positive electrical charge, which attracts and binds to the negatively charged molecules. Chitosan is obtained by the removal of enough number of acetyl groups from chitin, via a process known as deacetylation (depicted in Fig. 3), which is conducted under alkaline conditions or via enzymatic hydrolysis in presence of chitin deacetylase. The major difference between chitin and chitosan is their percentage of acetyl groups. In general, when the content of N-acetyl groups is >50%, the polymer is considered as chitin, while for lower values, it is considered as chitosan. The relationship between chitin and chitosan is based on the transformation degree of N-deacetylation.

2.1.1 Degree of Deacetylation of Chitosan

The degree of deacetylation (DD%) is defined as the molar fraction of glucosamine residues in chitosan, which is composed of N-acetylglucosamine and glucosamine. The DD is proportional to the degree of transformation of chitin into chitosan, which depends on several factors, including the concentration of alkali (like sodium hydroxide), the reaction temperature and time of reaction. It is one of the most important characteristics that influence the performance of chitosan in numerous applications. The higher the degree of deacetylation, the more active is the chitosan polymer. The degree of acetylation (DA) is defined as the reciprocal of the degree of deacetylation (DD). N-acylation with acetic anhydrides deposit acid amide groups within chitosan [5]. Chitosan has three types of reactive functional groups that allow chemical modifications of this biomaterial, under mild reaction conditions, to alter its properties. These functional groups include the amino or amido groups at C-2 position as well as the primary and secondary hydroxyl groups at C-6 and C-3 positions, respectively. Introduction of small functional groups on the primary amino groups and the secondary hydroxyl groups of chitosan enable its derivatization. These multiple functional groups in the chitosan chains provide versatility for

preparing molecularly imprinted polymers and for conducting structural modifications. For instance, the introduction of the carboxymethyl group in chitosan drastically increases its solubility at neutral and alkaline pH values, without affecting its cationic nature [8].

2.2 *Structural Comparison of Chitosan with Other Sugar-Based Polymers*

2.2.1 Structural Comparison of Chitosan and Cellulose

Cellulose $(C_6H_{10}O_5)n$ is a sugar-based linear chain homopolymer containing several hundred to many thousands of β(1-4)linked D-glucose units (Fig. 4b). It is a highly abundant natural polysaccharide that is considered as the backbone of the plant kingdom. It is an important structural component of the primary cell wall of green plants and makes up about half of the biomass of photosynthetic organisms [2]. The only difference between the two polysaccharides is the side chains attached to the carbon rings of the monosaccharides, i.e. the amine ($–NH_2$) groups present at the C-2 position of chitosan instead of the hydroxyl (–OH) groups found in cellulose. However, unlike the latter, chitosan possesses cationic charge, which gives it the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins and macromolecules [5]. As a result, chitin and chitosan have attracted increasing commercial attention due to their suitable characteristics, including biocompatibility, biodegradability, and ability to form films, adsorb heavy metals and chelate metal ions.

2.2.2 Structural Comparison of Chitosan and Starch

Starch $(C_6H_{10}O_5)n$ (amylum) is a semi-crystalline polymer composed of two polysaccharides, viz. amylose and amylopectin (Fig. 4c). It is used as an energy reservoir in plants. Amylose, a linear chain, typically consists of up to 3,000 glucose molecules, primarily interconnected by α-1,4 glycosidic linkages. Amylopectin is a large branched polymer, containing α-1,4 linkages, that serve as the backbone, and α-1,6 bridges at the branching points. The difference between chitosan and starch is the linear nature of chitosan, as compared to the branched structure of starch. Furthermore, there are amino ($–NH_2$) groups present at the C-2 positions of chitosan, instead of the hydroxyl (–OH) groups found in starch. Owing to hydrophilic and semi-crystalline nature of starch, it can be difficult to process this polymer since it is highly prone to absorbing moisture and is susceptible to changes in temperature. This biomaterial also has a greater structural rigidity than conventional synthetic polymers. Thus, the relatively greater hydrophobic nature of chitosan is anticipated to provide it with moisture barrier properties and water resistance. Furthermore, this

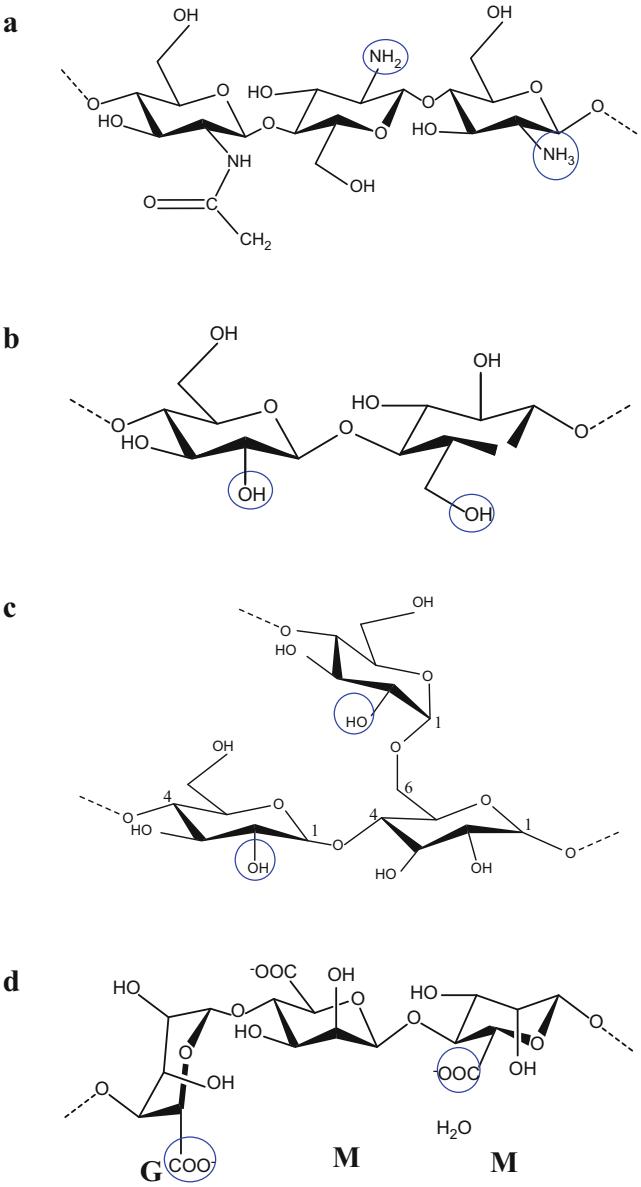

Fig. 4 Chemical structure of chitosan, cellulose, starch and alginate

biomaterial offers dimensional stability to starch films, when blended with the latter [9].

2.2.3 Structural Comparison of Chitosan and Alginate

Alginate $(C_6H_8O_6)n$ is a naturally occurring polymer found in brown algae and is anionic in nature. It is a linear, unbranched polymer containing covalently linked blocks of α(1–4)-linked L-Guluronic acid (G block) and β(1–4)-linked D-mannuronic acid (M block) residues (Fig. 4d). The alginate polymer is formed by joining of these monomers at the C-1 and C-4 positions. An ether-oxygen bridge joins the carbon at the first-position in one molecule to that at the fourth-position of another molecule. α-L-guluronate is the C-5 epimer of β-D-mannuronate. Alginate is a structural element that is located within the cell walls and the intercellular matrix, and thereby confers mechanical strength and flexibility to the marine plants to withstand the force of water in which the seaweed grows. Alginate-producing species may exhibit different compositions as the proportions of the two acids vary between species and in different parts of the same seaweed [10]. The major difference between chitosan and alginate is the presence of amino ($–NH_2$) group at the C-2 position of chitosan, instead of the hydroxyl (–OH) groups found in alginate and the presence of carboxylic groups at the C-5 position of alginate, instead of (-OH) groups in chitosan. These functional groups render alginate hydrophilic in nature. Although alginate is best suitable for designing drug carriers meant for oral drug, its hydrophilic nature makes it difficult to arrange alginate at the oil-water phase during the preparation process, resulting in unstable emulsions. Hence, alginate is mixed with chitosan to improve its hydrophilic properties, while retaining its biological compatibility and pH sensitivity [11].

These naturally occurring polysaccharides, like cellulose, alginate, starch, etc., are neutral or acidic, whereas chitosan is an example of a highly basic polysaccharide. When dissolved, the amino groups (–NH2) of glucosamine are protonated to –NH3+ and the cationic polyelectrolyte readily forms electrostatic interactions with other anionic groups. Therefore, the cationic chitosan molecules interact with the negatively charged surfaces that modify their physicochemical characteristics. Chitosan has three types of reactive functional groups. Its amino groups have both, primary and secondary hydroxyl groups at the C2-, C3- and C6-positions and these groups permit modification of chitosan [12]. The degree of deacetylation (DDA), crystallinity and molecular weight (MW) are the main aspects in which chitosan can be modified to obtain materials with different physiomechanical properties. These structural modifications of chitosan contribute to its unique functional properties. The comparisons between structural properties of chitosan and other sugar polymers are presented in Table 2.

Table 2 Comparison of structural properties of sugar-based polymers

Parameter of comparison	Chitin/chitosan	Cellulose	Starch	Alginate
Glucose unit linkages	Chitin constitutes their glucose residues as N-acetyl-D-glucosamine with β-(1-4) glycosidic bonds. Chitosan constitutes their glucose residues as β-(1-4) linked D-glucosamine and N-acetyl-D-glucosamine	Cellulose constitutes their glucose residues as β-(1-4) glycosidic bonds	Starch contains glucose residues as α-(1-4) glycosidic bonds in amylose, while amylopectin has α-(1-6) glycosidic linkages at the branching points and α(1-4) linkages, at other locations	Alginate residues contain covalently linked blocks of α(1–4)-linked L-guluronic acid (G block) and β(1–4)-linked D-mannuronic acid (M block)
Structural difference	The second carbon of glucose binds to an acetyl amine group in chitin and an amino group in chitosan	The second carbon of glucose binds to a hydroxyl group	The second carbon of glucose binds to a hydroxyl group	The fifth carbon of glucose binds to a carboxylic group
Molar mass	Chitin-203.1925 g/mol The molar mass of chitosan varies	162.1406 g/mol	359.33 g/mol	398.32 g/mol
Nitrogen	Present	Absent	Absent	Absent
Type of chain	These are long, straight and unbranched chains	These are long, straight and unbranched chains forming H-bonds with the adjacent chains	They are coiled and unbranched (amylose) or long, branched (amylopectin)	They are linear and unbranched
Function	They are structural components of exoskeletons of crustaceans, insects and the cell walls of fungi	It is a structural component of the cell wall of plants	It is the storage carbohydrate source in plants	It is a structural component of the cell wall of brown algae

3 Interaction of Chitosan with Other Sugar Polymers

The interaction of chitosan with other sugar polymers depends upon the miscibility of their components at the molecular level, which in turn depends upon the specific interactions between these components. Interaction of chitosan with other biopolymers is of great interest due to improved properties and widened application scope of these combinations. It results in blends that exhibit unique properties that neither of the parent polymers demonstrates. Chitosan contains high-polarity groups that can form hydrogen bonds with the functional groups of other sugar polymers, which

enhances their compatibility with each other. Among the natural polymers, chitosan and its blends with the other sugar polymers have always been seen with interest due to their versatility and diverse applicability. The most common interactions include formation of hydrogen bonds, ionic and dipole bonds, π-electrons and charge-transfer complexes that facilitate the absorption, distribution and controlled release of therapeutic molecules [13]. Techniques such as thermal analysis, electron microscopy, viscometric measurements and dynamic mechanical studies have been used to investigate the polymer–polymer miscibility in solution or in solid state. The interactions between chitosan and other sugar polymers are known to enhance its hydrophilicity, mechanical properties, as well as improve its blood compatibility and anti-bacterial property. The selection of polymers to be blended with the chitosan depends on the desired properties of the target materials. Chitosan has been blended with several polymers, such as gelatin, silk fibroin and cellulose, to enhance its mechanical and anti-bacterial properties [14].

3.1 Interaction Between Chitosan and Starch

Native starch exhibits limitations as a matrix material for active compounds as it has multihydroxyl structure and hydrophilic groups, which release the encapsulated molecules before they can reach the target site. The applicability of starch films is limited by their mechanical properties and efficient barrier against low polarity compounds. The semi-crystalline nature and hydrophilicity of starch can complicate its processing, especially due to its susceptibility to moisture and temperature and its greater structural rigidity than the conventional synthetic polymers. Thus, researchers have attempted to improve the properties of this biomaterial by blending it with other polymers or through chemical modifications. To overcome such shortcomings, starch-based composite films can be prepared by blending with other non-starch biopolymers, including carboxymethyl cellulose and chitosan [15]. Modifications in starch have been found to improve crystallinity, hydrophobicity and amphiphilic properties of starch. Blends of chitosan with starch have been found to improve the moisture barrier properties and water resistance, including the dimensional stability of starch films. A combination of hydrogen bonding and electrostatic interaction between cationic chitosan and anionic starch, their hydrophilicity and water compatibility enable good interaction between these polymers. The solubility of chitosan in aqueous solutions is mediated by the protonation of amine groups in the acidic environment. Chemical cross-linking was developed to prepare starch and chitosan-based hydrogels. The mixture of carboxymethyl sago or cassava starch and chitosan was used to produce bio-hydrogel through chemical cross-linking with methacrylic acid as a cross-linking agent [16].

3.2 Interaction Between Chitosan and Cellulose

Cellulose and chitosan have similar molecular structures, both containing β-glycoside linkages. The two polymers differ only in that the latter has a deoxy amino group instead of the hydroxyl group at C-2 of the pyranose ring. Cellulose and chitosan are both biocompatible, non-toxic, and naturally occurring biopolymers that have been used extensively for biomedical applications. Many researchers have reported the combined use of both these polymers. Introduction of amino groups into cellulose is important to facilitate its chemical modification. However, it is generally difficult to introduce sufficient amounts of amino groups into cellulose by simple and efficient reactions, without significant degradation of cellulose chains. Cellulose-chitosan blends have thus been used as an alternative approach to develop amino groups containing cellulosic system. Such blends are more amenable for chemical derivatization because the amino group in chitosan is much more reactive than the acetamide group in chitin. Cellulose and chitosan complexes have exhibited sufficient physical strength that can be explored in various biomedical applications [17]. Complexes of these biopolymers have been studied in the form of fibres, films and microspheres, and have been employed as a drug carrier, wound healing agents, as adsorbents, and as a range of other functional materials. Duan et al have reported that β-CD-chitosan/Fc-cellulose (beta-cyclodextrin-chitosan/ferrocene-cellulose) gel prepared at room temperature formed inclusion complexes. In this complex, cellulose and chitosan associated with each other by intermolecular inclusion interaction that resulted in self-healing properties due to host-guest interactions [18]. Roz et al reported that oxidation of cellulose films could increase adhesion between cellulose and chitosan. Oxidation produced carboxylic acid groups, that electrostatically interacted with the amino groups in chitosan, forming ion pairs. This molecular-level interaction was proved using FTIR studies. The IR spectra indicated that NH groups were involved in these molecular-level interactions, which were ultimately responsible for the enhanced interaction between chitosan and oxidized cellulose. This interaction also induced morphological changes, with increased the roughness of the film based on the combination of chitosan and cellulose. The successful adsorption of chitosan onto cellulose has paved a way for a number of applications, based on their combined properties such as biocompatibility, anti-microbial properties or bactericidal activities [19].

3.3 Interaction of Chitosan and Alginate

Polyelectrolyte complexes are useful in various applications like encapsulation of drug substances, controlled drug delivery, membranes for water treatment. These polyelectrolytes are resulted from the strong electrostatic interaction between oppositely charged polymers like chitosan (positive polyelectrolyte) and alginate (negative polyelectrolyte). As discussed earlier, chitosan is a linear polysaccharide made

up of D-glucosamine and N-acetyl glucosamine units. In addition, it consists of reactive groups: primary and secondary hydroxyl groups at C-2, C-3 and C-6 positions and amino/acetamide group. Due to multidirectional biological activities, chitosan is used in complex composition instead of other cationic natural polymers like cellulose, dextran and gelatine. Alginate is known for its ability to bind divalent metal ions like Mg2+, Mn2+ and Ca2+. The ability to bind these cations is dependent on chemical composition of alginate and binding affinity [20]. The monomers of alginate are basically epimers which are oriented in different direction, and guluronic acid is oriented in such a manner that it allows the carboxylate moieties available for ionic cross-linking [21]. Polyelectrolyte material formation with these two polysaccharides may be used to enhance specific functional properties of each component and to minimize their disadvantages. The polyelectrolyte network is formed by the interaction of a cationic amino group of chitosan and an anionic carboxyl group of alginate, intra- and inter-chain hydrogen bonding between different polysaccharide groups [20]. As both the polysaccharides are important in tissue engineering, they are often combined to improve their mechanical properties and cell interaction. Cross-linking of these materials in the hydrogel is used to provide materials with enhanced stability in physiological solutions [22].

4 Properties of Chitosan

Chitosan is the N-deacetylated product of chitin. Chitin is present in abundance and has high mechanical strength. However, due to its poor solubility, it has limited applications, which has shifted attention towards chitosan [23]. Chitosan exhibits various biological properties, such as biodegradability, biocompatibility, non-toxicity, anti-bacterial and anti-fungal effects, wound healing potential, ability to stimulate the immune system, anti-tumour activity, etc. [24, 25]. These properties make it an attractive and promising polymer for biomedical applications, such as gene therapy, tissue engineering, blood anti-coagulant, wound dressing, controlled drug/vaccine/enzyme delivery, drug dissolution, excipient for tablets, bone regeneration biomaterial, bacteriostatic, fungistatic, spermicidal agent, anti-carcinogen, anti-cholesteremic, anti-thrombogenic agent, absorption enhancer as well as suitability for biosensor/biomembrane fabrication [26–28].

In the case of chitosan, the physical properties are controlled by the molecular weight of precursor, degree of deacetylation and depolymerization, interactions with amine and hydroxyl groups present in the polymer structure [29]. The structural and biological properties of chitosan such as its cationic nature, solubility in aqueous medium, biodegradability and mucoadhesivity are results of its polysaccharide structure. It is composed of alternating repetitive units of N-acetylglucosamine and D-glucosamine linked by β-(1-4) glycosidic bonds [30]. Most of the functional properties of chitosan such as its solubility, bioactivity, biocompatibility, biodegradation depend upon its degree of acetylation [31]. Chitosan can differ in size, degree of acetylation and other physicochemical properties like crystallinity, acetylation

pattern and polydispersity depending on the source of chitin and conditions of its production [32].

Chitosan is a pH-sensitive polysaccharide, which dissolves only at a low pH. It has been used to fabricate nanoparticles, fibres, films and hydrogels [28]. It can bind with proteins, fats, cholesterol and metal ions, due to which it has also been explored as a chelating agent. Properties such as in situ gelling, hydrophilic character, mucoadhesion make it ideal for drug delivery functions [33]. As a result of its cationic nature, chitin and chitosan possess anti-bacterial properties, which are absent in cellulose [34]. The anti-microbial activity of chitosan can be further enhanced by combining it with metal nanoparticles, such as ZnO, TiO_2 and Ag. The anti-microbial property, analgesic effect and haemostatic effect of chitosan make it suitable as a wound dressing material [23].

Other polymers, such as starch, cellulose, chitin, glycogen, are also known for their biomedical applications. Starch is a biodegradable, biocompatible, non-toxic and soluble polymer. But it exhibits limitations such as poor processability, poor long-term stability, high water sensitivity and low mechanical properties, which restrict its widespread applications [35].

Glycogen is a natural, high molecular weight, biodegradable polymer. It is inert to amylases present in the bloodstream, but debranching enzymes intracellularly degrade it. Due to its high molecular weight, the kidneys without biodegradation cannot directly eliminate it. After internalization into the cells, it is rapidly degraded into D-glucose [36]. Bacterial cellulose exhibits no activity against bacterial infections, which are commonly observed in wounds and severe injuries [37]. The low solubility of cellulose in water and most organic solvents, due to its hydrogen-bonded structure, is the main limitation of cellulose-based hydrogels. The properties of cellulose can be enhanced by producing hybrids with other polymers like chitosan [29]. Collagen can be used in tissue regeneration. But its high degradation rate and poor mechanical properties restrict its biomedical applications [29].

Most of the commercial natural polysaccharides, such as cellulose, dextran, heparin, pectin, agarose and many others, are either neutral or acidic, whereas chitin and chitosan are highly basic polysaccharides. This basic nature is due to the primary amino groups present in their structures. These polymers display some specific properties, such as polyelectrolyte complexation with oppositely charged polymers, chelating action, film-forming property and polyoxy salt formation [38]. The antacid and anti-ulcer activities of chitosan are due to its gel-forming property at slightly acidic pH. The anti-tumour activity of chitin and chitosan is enhanced by the activation of macrophages [25]. This is because chitosan induces expression of macrophage activation markers, such as macrophage inflammatory protein (MIP)-2, transferrin receptor, Fas and mannose receptor [39].

Researchers have developed drug delivery systems of chitosan for the treatment of colon and hepatic diseases. Several chitosan-based hydrogels have been formulated to encapsulate radioisotope drugs which are used in site-specific cancer therapy [29]. Chitosan hydrogels can be produced in the form of micro- or nano-sized beads. These beads swell in acidic conditions and release the drug. Chitosan hydrogels are soft, flexible, non-toxic and have controlled pore size. These properties of chitosan

hydrogels are important in their biomedical applications such as drug delivery and tissue engineering [40].

Bionanocomposites are biohybrid materials having diverse morphology and structure. The properties of nanocomposites are enhanced by using a limited amount of nanofillers in the matrix. The addition of nanofillers like carbon, polysaccharides, phyllosilicates, etc. enhance the properties of bionanocomposites [41]. Chitosan is also used for the production of biocomposites. Recent studies described the production of cellulose-chitosan, gelatin-chitosan, starch-chitosan, chitosan-α chitin biocomposites, which have shown improved properties [42, 43].

Cellulose is a stable, water-insoluble, fibrous polysaccharide and plays a role in structural organization and maintenance of tensile strength. In the case of cellulose and its derivatives, their solubility acts as a major limitation in their applications. Recent studies have demonstrated that these limitations can be overcome by hydrolysis of high molecular weight cellulose into smaller fragments, which possess enhanced water solubility [44].

The most commonly used polysaccharides for drug delivery systems include chitosan, dextran, alginate, pectin and starch. Glycogen is comparatively less explored as it is degraded into D-glucose by intracellular enzymes and metabolized by physiological glycolysis [45].

4.1 Solubility of Chitosan and Other Sugar Polymers

Chitosan is insoluble in water and most organic solvents. It is soluble in acidic aqueous conditions (pH <6.3), but insoluble at higher pH, i.e. in neutral or basic conditions (pH >6.5). At lower pH, it has gel-forming property [27]. The solubility of chitosan majorly depends upon its degree of deacetylation, molecular weight, pH and protonation of free amino groups [38]. Thus, changes in molecular weight and degree of acetylation of chitosan affect its solubility [30]. The solubility of chitosan increases with a decrease in its molecular weight [46]. The solubility of chitosan also depends upon its structure; for example, the presence of protonated amino groups in the chitosan structure renders it soluble in dilute acidic solutions [24]. Highly deacetylated chitosan demonstrates higher solubility in the solutions with pH up to 6.5, but its solubility decreases with a decrease in the degree of deacetylation [25, 30]. Different types of chemical and physical interactions are involved in the solubilization of chitosan, which include hydrogen bonds, van der Waals forces, hydrophobic interactions, etc. [46].

Chitosan exhibits limited reactivity and processability. One of the major limiting factors is its poor water solubility. The poor solubility of chitosan in water and solvents of higher pH, high viscosity and its aggregation with proteins at high pH may restrict its applications [26, 34]. Due to the poor solubility of chitosan at neutral pH, its anti-bacterial activity is observed only in acidic conditions. Its solubility can be increased based on the number of unsubstituted amine groups [28]. The unmodified chitosan aggregates, at neutral pH, result in low cell penetration,

which can be overcome by modifications such as quaternization of chitosan [47]. The solubility of chitosan can be enhanced by chemical modifications such as cationization, quaternization, carboxymethylation or surface modification with metal or metal oxide nanoparticles [34]. The three reactive functional groups of chitosan, namely the amino group and the primary and secondary hydroxyl groups on C2, C3 and C6, respectively, are highly susceptible to chemical modifications [26]. Partial hydrolysation of chitosan also enhances some of its properties. The viscosity of chitosan affects its biological properties, such as wound healing, biodegradation by lysozyme. Chitosan can form gels at acidic pH due to its hydrophilic nature. These gels are used in applications such as a slow-release drug delivery system [38].

The cationic nature of chitosan is unique as compared to the other neutral or negatively charged polymers. The pH of the solution affects the charged state and properties of chitosan [25]. The solubility and ionization of polyelectrolytes are controlled by the ability of acidic media to protonate the polymer [46]. Solubility depends on the protonated amino groups in the structure and thus on the proportion of acetylated and non-acetylated D-glucosamine units [25]. The ionization of chitosan is based on the amino groups present in its structure. These amino groups can be protonated [31]. Ionization at low pH occurs due to the formation of chitosan-NH_3^+. This protonation of amines results in positive charge, which increases the solubility of chitosan. As the pH increases (neutral or basic conditions), the amines get deprotonated and the polymer loses its charge, due to which the polymer becomes insoluble. This results in precipitation or gelation of the polymer [25, 46]. The soluble-insoluble transition is observed at the pKa of chitosan (pH 6–6.5). The conformation of chitosan molecules in dilute solutions depends upon its chemical, structural characteristics (degree of deacetylation, chain length, unit distribution, etc.) and also on solution parameters including pH, ionic strength temperature, etc. [31].

Chitosan (CS) can be modified into various forms such as N-trimethylene chloride, ester forms like CS glutamate, CS phthalate and CS succinate. The N-trimethylene chloride is a quaternary derivative of CS that exhibits enhanced aqueous solubility, higher absorption of neutral and cationic peptide analogues at a wide pH range and intestinal permeability. The ester forms show different solubility, i.e. they are insoluble in acidic conditions and show a sustained release in basic conditions [38]. Solubility of chitosan in water can be enhanced by catechol conjugation. It makes chitosan soluble even at a neutral pH, while the conjugate is a partially soluble conjugate at a basic pH [48]. The net cationic charge of chitosan decreases at higher pH resulting in decreased oligonucleotide affinity. Microcapsules and nanoparticles of chitosan and its derivatives thus exhibit pH sensitivity and hence can control the release of encapsulated drugs [49].

Chitosan dissolves in organic acids, such as acetic, formic and lactic acids. Chitosan is most commonly dissolved in 1% acetic acid (pH 4), 1% hydrochloric acid and dilute nitric acid. But it is insoluble in sulphuric and phosphoric acids [25]. Based on its solubility, chitosan can be produced in different forms such as nanofibres, films, hydrogels, powder form [23]. The cross-linking of chitosan using

glutaraldehyde results in decreased solubility. As the concentration of cross-linking agent increases, the swelling property of chitosan decreases [38]. A weaker intermolecular hydrogen bonding results in a parallel arrangement of the main chains, which leads to the swelling of this polymer [25].

Chitin has lower applicability, as compared to chitosan, due to its insolubility and chemical non-reactivity. In case of chitin, inter- and intra-molecular hydrogen bonds are formed with acetyl, amino and hydroxyl groups in the polysaccharide chain, which aggregates chitin. Due to this, chitin is insoluble in regular solvents like water, mild acidic or basic solutions, organic solvents, etc. [46]. There is a growing interest in chemical modifications of chitin and chitosan to increase their solubility and applications [32]. The solubility of chitin is increased by converting it into chitosan, which has a favourable pKa value and degree of deacetylation [46].

Alginate is stable in acidic pH, but it swells and dissolves in alkaline pH. In chitosan-alginate biocomposites, chitosan strengthens the structure of alginate and increases its stability in the alkaline pH [50]. In alginate, the O-acetyl group affects properties such as its solubility, water capacity, polymer confirmation, etc. Due to the better interaction of chains with water molecules, acetylated alginate absorbs more water [51].

In case of starch, its applications are limited due to its water solubility, brittleness and poor mechanical properties [52]. These limitations of starch can be overcome by blending starch with other polymers such as chitosan, polyvinyl alcohol, cellulose, alginate, etc. Blending with chitosan leads to the formation of intermolecular hydrogen bonds between hydroxyl groups of starch and amino and hydroxyl groups of chitosan. This results in improved mechanical strength and lower solubility, i.e. water resistance [53].

Cellulose is insoluble in water and in many organic solvents. It is soluble in solvents such as N-methylmorpholine N-oxide and ionic liquids such as 1-butyl-3-methylimidazolium chloride (BMIMCl) and 1-allyl-3-methylimidazolium chloride (AMIMCl) [54, 55].

4.2 Thermal Properties of Chitosan and Other Sugar Polymers

The physicochemical properties of chitosan such as solubility, viscosity, thermal stability majorly depend on the degree of acetylation and molecular weight. The thermal behaviour of chitosan and its derivatives is important for specific industrial applications [56]. Characterization of the thermal properties of composites is helpful for the processing of biomaterials. Semi-crystalline polymers show non-isothermal crystallization behaviour. Most of the processing techniques are melt-based and occur under non-isothermal conditions. The resulting properties, including mechanical, physical and biodegradable behaviour, thus depend on the morphology and extent of crystallization [57]. In starch, the thermal stability increases with increase

in the content of amylopectin. The more the content of amylopectin, as compared to amylose, the more is the thermal energy required to break the molecular chains of starch [58]. Chitosan is a labile molecule, susceptible to different degradative mechanisms, including thermal degradation. It cannot withstand higher temperature (200–220°C). Its poor thermal stability can be overcome by the addition of certain stabilizing agents, such as hydroxyapatite, calcium carbonate or nanoclay, etc. These materials offer a shielding effect to chitosan, due to which it requires higher activation energy for thermal degradation of composites. Thus, stabilizing agents increase the barrier properties and insulating properties, which result in improvement in the thermal stability of chitosan [59]. Thermal stability of chitosan in chitosan-silica hybrid films is very high due to the high content of silylating agents. Also, the silica content in aerogel prevents thermal degradation of chitosan [60].

As compared to chitosan, cellulose is more stable at temperatures up to 200–300°C, possibly due to the extensive inter- and intra-molecular hydrogen bonding [61]. Studies have shown that thermal stability is enhanced in cellulose-chitosan composite materials. At high chitosan concentration (about 45%), the highest thermal stability was observed. This was due to the formation of intermolecular reaction between cellulose and chitosan, which may have improved the cohesive energy resulting in high thermal stability [62].

As a polysaccharide with high hydrogen content, chitosan degrades thermally before reaching its glass transition temperature. Therefore, chitosan solutions are used for the preparation of films, fibres, gels, particles and sponges. In the solution state, the amino and hydroxyl groups in chitosan are more susceptible for chemical modification, which enables enhancement in the functional properties of this polymer [31]. High molecular weight chitosan is considered more stable as it shows thermal stability. Various factors such as strong acids, high temperature, mechanical shearing, irradiation affect its molecular weight. The inter-chain cross-linking between free amino groups enhances the stability of polymer structure, which results in slower thermal depolymerization of chitosan [63]. Also, as discussed previously, incorporation of chitosan in composites, at higher concentrations, improves thermal stability [62]. Other polymers such as alginate exhibit various limitations, such as susceptibility to heat, poor stability, low barrier properties, incompatibility with heavy metals, etc. These properties can be enhanced by combining alginate with other biopolymers such as chitosan, cellulose [64]. In case of starch, thermal degradation and instability are important limitations with its use [65]. Thermal stability of starch is improved either by its chemical modifications, such as acetylation, or by increasing its degree of substitution (DS). The DS is the substitution of alkyl ether groups of starch by hydroxyl groups which improves the thermal stability of starch [66]. Another way of improving the thermal stability of starch is its mixing it with other polymers, such as chitosan. Studies have shown that chitosan is a good reinforcing agent and also improves the thermal stability of starch-based biodegradable films [67].

4.3 Crystallinity of Chitosan and Other Sugar Polymers

The crystallinity of a polymer defines the degree of structural order and regularity in molecular arrangements. A higher degree of crystallinity results in higher hardness and density of the polymer. In the crystalline state, the high intermolecular bonding leads to increased strength of the polymer [68]. The crystallinity of chitosan has been studied by XRD [69]. It is an important physical characteristic that controls the accessibility of amine groups of chitosan, as well as its functions such as metal sorption rate and total uptake [25]. The degree of deacetylation and crystallinity controls the sorption rate by controlling the swelling and diffusion properties of chitosan. The sorption properties can be improved by modifications such as manufacturing gel beads and decreasing crystallinity [70]. The crystalline structure of chitosan majorly depends on the deacetylation process and its chitin polymorphic form [71].

The chemical structure of chitosan, just as in case of the parent polymer – chitin, is expected to lead to the formation of a highly ordered super-molecular structure, containing a large number of intra- and inter-molecular hydrogen bonds, having a high degree of crystallinity [72]. There are three polymorphic forms of chitin, namely α, β and γ, which differ in the arrangement of chains in the crystalline region. The α-chitin contains anti-parallel chains, while β-chitin contains parallel chains, whereas γ-chitin is a combination of α- and β-chitin [71]. The crystalline form of chitosan is insoluble in aqueous solutions with a pH higher than 7. The protonated free amino groups in dilute acids facilitate solubility of this biopolymer [32]. In case of β-chitosan, a higher solubility with lower crystallinity has been reported, which results in better functional properties as compared to α-chitosan [71].

The crystallinity and polymorphism of chitosan vary depending on its origin and also the treatment conducted during the extraction process. Maximum crystallinity was observed in chitin (0% deacetylated) and fully deacetylated chitosan (100% deacetylated). In acidic conditions, chitosan acted as an excellent viscosity-enhancing agent. It also behaved as a pseudo-plastic material, leading to a decrease in the viscosity with increasing shear rate. The viscosity of the chitosan solution depended on various factors. It increased with an increase in the chitosan concentration, decrease in temperature and an increase in the degree of deacetylation [73]. The degree of deacetylation of chitosan products majorly depends on the deacetylation process parameters than on the crystalline structure [71].

In case of chitosan-alginate composites, the decreased crystallinity of the hydrogel was due to the ionic interaction between chitosan and alginate, resulting in good compatibility [74].

The crystallinity of cellulose is a major reason for its insolubility. Cellulose forms extended crystalline regions. As compared to the amorphous forms, the crystalline forms have lower free energy. As a result, crystalline cellulose is more difficult to dissolve than amorphous form of cellulose [54]. In case of chitosan-cellulose films, chitosan prevents crystallization of cellulose, resulting in decreased crystallinity of

cellulose with an increasing chitosan content [75]. In anti-bacterial cellulose-chitosan composites, the crystallinity decreased due to integration of chitosan molecules with the cellulose micro-fibrils. Here, the chitosan molecule disrupted the hydrogen bonding within cellulose chains, resulting in the lower crystallinity of the composite [76].

4.4 Non-toxicity of Chitosan and Other Sugar Polymers

The molecular weight, degree of deacetylation, source, purity and polydispersity affect the toxicity of chitosan [77]. Toxicity of chitosan depends upon the degree of deacetylation, its molecular weight and charge density. At a higher degree of deacetylation, the toxicity can be linked with its molecular weight and concentration, while at a low degree of deacetylation, toxicity is lower and less dependent on molecular weight. The toxicity of chitosan increases with increasing density [77]. Studies have clearly indicated that chitosan has low toxicity. Its reported toxicity is as low as 16 g/kg body weight, which is similar to that of sugar or table salt [31]. Chitin and chitosan are considered safe due to their low toxicity in animal investigations. They are not expected to be digested or absorbed in the human gastrointestinal tract [25].

However, modifications of chitosan such as covalent cross-linking in the formation of hydrogels, microparticles and nanoparticles production make it more toxic [77]. The most common cross-linkers used to cross-link chitosan are glyoxal and glutaraldehyde, which are effective but toxic. Glutaraldehyde is neurotoxic, while glyoxal is mutagenic. The presence of free unreacted dialdehydes, even after purification of microparticles of chitosan, can induce toxic effects. This can be overcome by using other cross-linking agents such as diethyl squarate, genipin, oxalic acid, etc. [25]. Thus, employment of chitosan-based drug delivery systems requires in vivo toxicity testing after prolonged administration [77].

Chitosan displays lower toxicity as compared to other cationic polymers such as polyarginine, polylysine, polyethyleneimine, etc. Due to this, chitosan is also used for non-viral gene delivery systems [78]. Composites of chitosan with other sugar polymers have been proven as safe for biomedical applications due to their low toxicity. For example, studies showed that alginate/chitosan/lovastatin nanoparticles (ACL) could lower serum cholesterol and were safe and non-toxic [79]. Alginate-chitosan composite microspheres were safe and effective for delivery of vancomycin and maintained effective drug concentration [80].

4.5 Mucoadhesiveness of Chitosan and Other Sugar Polymers

Mucoadhesiveness is an important property of a polymer which plays an essential role in drug delivery application. In this case, the drug delivery system (DDS) adheres to the mucous membrane, which increases the residence time of the drug and enhances concentration gradient. This leads to the controlled drug release and prolonged therapeutic effect of the drug [81]. Mucoadhesive polymers have been used for local or systemic drug delivery, via different routes such as dermal, oral, buccal, vaginal, etc. [82]. As compared to other polymers, such as starch or cellulose, chitosan presents mucoadhesive properties due to its functional amino and hydroxyl groups [83].

Cationic chitosan exhibits strong mucosal adhesion due to its electrostatic interactions with the negatively charged mucin. Along with electrostatic interactions, hydrogen bonding and hydrophobic interactions are also responsible for mucoadhesion of chitosan [84].

The mucoadhesive nature of chitosan increases with the increase in the degree of deacetylation as more positively charged amino groups become available for interaction with the negatively charged mucus. Due to this, highly deacetylated chitosan is used for drug delivery applications. Mucoadhesion results in prolonged retention at the site of action [30]. At acidic conditions, chitosan displays a cationic nature due to the protonation of amino groups. These positive charges bind with negatively charged residues (e.g. sulphated glycans and sialic acid residues) in mucin, resulting in improved mucoadhesiveness [24].

The flexibility of the molecular structure of chitosan facilitates its chemical modifications, which improve its mucoadhesion and solubility [30]. The mucoadhesive property of chitosan gels enhances the bioavailability of the loaded drugs loaded in these, which in turn leads to enhanced drug delivery [29]. The swollen form of chitosan demonstrates excellent mucoadhesiveness, due to its adherence to hard and soft tissues. A better adhesion is seen in case of epithelial tissues and mucus layer present on the tissue surfaces [73]. The mucoadhesive and absorption enhancing properties of chitosan and its derivatives result in the improvement of mucosal and trans-mucosal delivery of drugs [30].

Mucoadhesion of chitosan transiently and reversibly opens the tight junctions between the epithelial cells, resulting in enhancement of para-cellular permeability. The exposure to chitosan increases claudin-4 (CLDN-4) gene transcription in the cell. Chitosan treatment results in intracellular redistribution of CLDN-4, followed by its degradation in the lysosome. Therefore, because of its ability to bind to mucin, chitosan regulates the tight junctions by inducing changes in CLDN4 protein [85].

Alginate is an anionic polymer with carboxylic groups. It displays higher mucoadhesiveness as compared to the other polymers such as chitosan, cellulose [86]. Hence alginate-chitosan complexes can be produced and used to overcome the limitations of both these polymers. Thus, these complexes have been explored for intravaginal drug delivery, for the treatment of bacterial vaginosis [87].

4.6 Biodegradability of Chitosan and Other Sugar Polymers

Biodegradation of biopolymers is a natural process that involves living organisms or enzymes in tissues, which degrade, mineralize and redistribute biomaterials through carbon or nitrogen cycles [31]. The degradation products of chitosan are non-toxic, non-immunogenic, non-carcinogenic and do not produce any toxic or inflammatory reactions [26]. Biodegradable polymers usually degrade quickly within a few months or 2 years. The biomaterial is easily degraded and excreted from the body by the hydrolytic process. In the body, the biomaterial undergoes loss of its mechanical strength resulting in the recovery of the body system with no toxicity or any other biological effect [88]. Chemical and enzymatic degradation are the two major routes of chitosan degradation. Chemical degradation of chitosan is an acid-catalysed degradation. On the other hand, the enzymatic degradation of chitosan results in hydrolysis of glucosamine–glucosamine, glucosamine–N-acetyl-glucosamine and N-acetyl-glucosamine–N-acetyl-glucosamine linkages [77].

Chitosan is a polysaccharide that contains cleavable glycosidic bonds. It is degraded in vivo by several proteases and majorly by lysozyme. The biodegradation of chitosan results in the formation of oligosaccharides having variable lengths, which are either used in metabolic pathways such as glycosaminoglycan and glycoprotein pathways or excreted directly via the urine [24]. The non-degradable materials can accumulate intracellularly or within different organs. Hence, while considering the acute and long-term toxicity, the biodegradability of chitosan becomes crucial. Studies have shown that chitosan is highly biodegradable as a result of digestion of its molecular chains by either chitinase, that is produced by normal intestinal flora, or by lysozyme, that is present in the mucosal surfaces [30].

The most common enzymes that degrade chitosan include chitinases, chitobiases, lysozyme and other proteases that are abundantly present in nature. The enzymatic degradation of chitosan results in the release of amino sugars. Degradation of chitosan by lysozyme and several proteinases requires suitable conditions of temperature, pH and ionic strength [31]. Degradation is important for various applications of chitosan, such as the release of the small and large molecules and in functional tissue regeneration. Hence, investigating and controlling the degradation rate of chitosan-based polymers is essential [73]. The rate of degradation of chitosan is majorly related to its molecular mass, degree of deacetylation and also to the distribution of N-acetyl D-glucosamine residues in the polymer [24]. Chitosan of larger molecular size undergoes degradation by chemical or enzymatic routes to result in fragments that are suitable for renal clearance [77].

The rate and extent of biodegradation of chitosan are inversely related to its degree of deacetylation [77]. Also, the biodegradation is inversely proportional to the degree of crystallinity, which in turn is controlled by the degree of deacetylation. The biodegradability of chitosan is also affected by the distribution of acetyl groups, rearrangement of acetyl groups and their homogenous distribution, which results in lower rates of enzymatic degradation [73]. Aiba et al. suggested that the variations in the distribution of acetamide groups in chitosan result in differences in degradation

[89]. These differences occur due to variations in deacetylation, which lead to changes in inter- or intra-molecular repulsive forces and result in influencing the viscosity of chitosan [73].

The biodegradability of chitosan is an important consideration during drug delivery. In case of oral delivery, hydrolytic degradation due to acidity of the gastric medium should be considered while determining the efficacy of drug delivery [30]. Cellulose is also a biodegradable polymer, which is degraded by cellulase enzyme present in nature, but is absent in the human body. Therefore, buffers, solvents, polymers are used for its modification to enhance its degradation and bioabsorption in a living system [88].

Similar to cellulose, alginate is also non-degradable in mammals due to the lack of enzyme alginase. The ionically cross-linked alginate gels can be dissolved. But the average molecular weight of the alginate is higher than the renal clearance threshold of the kidneys and thus it cannot be removed completely from the body. Partial oxidation of alginate chains is used to make alginate degradable [90]. Starch-based biodegradable polymers are used in bone tissue engineering and also as a drug delivery system. Mostly microsphere or hydrogel forms of starch are used for drug delivery. Starch-gelatin blend microparticles are used for controlled release drug delivery of water-soluble drugs [91]. Studies have proved that chitosan with low degree of deacetylation is degraded by lysozyme. But in case of chitosan-alginate polyelectrolyte complex, the effect of lysozyme mediated degradation is very low due to strong interactions between the polymers [92]. A larger quantity of chitosan in the composite may prolong its degradation by bacteria. Chitosan coated cellulose was found to be resistant against the cellulase enzyme. Along with chitosan content, the amount of carbonyl and carboxyl groups present in the cellulose also affects the biodegradability of the composite film [93].

4.7 Biocompatibility of Chitosan and Other Sugar Polymers

Biocompatibility is the ability of a compound to get in contact with a living system without producing any harmful effect [31]. Biocompatibility of chitosan is important for its biomedical applications, such as wound dressing, sutures and artificial skins. This biomaterial has been used in the products such as HemCon bandages (manufactured by Tricol Biomedical Inc.), which have been approved for wound dressing by the US FDA in 2003 [24, 94].

The biocompatibility of chitosan varies with its physicochemical characteristics and experimental conditions [31]. It also depends upon its degree of deacetylation. The biocompatibility increases with increase in the degree of deacetylation. When the positive charge of chitosan increases, its interaction with the cells increases, which in turn results in improved biocompatibility [24]. Biocompatibility of biomaterials also depends on the protein sorption. When a material comes in contact with body fluid or blood, protein adsorption occurs. It is influenced by the surface properties of the material such as its chemistry, hydrophilicity, roughness and

charge. The matrix network in hydrogel-based materials also influences the protein sorption [28]. Various studies have proved the cytocompatibility of chitosan with different cell types, such as keratinocytes, vascular, endothelial, fibroblasts, chondrocytes, osteoblasts, neurons, hepatocytes, cancerous cells, etc. The degree of acetylation of chitosan inversely affects the cell adhesion and proliferation, i.e. the higher the degree of acetylation, the lower the cell adhesion is. The biomedical applications of chitosan, such as in drug delivery, cell scaffolds and tissue engineering, are based on its adherence to the cells and its biocompatibility [31]. The biocompatibility of chitosan, in turn, depends on several parameters such as the polymer behaviour, individual characteristics such as age, sex, health and the structure of carriers such as size, crystallinity, morphology, degradation capacity, surface characteristics and products [30]. Chitosan hydrogels exhibit high biocompatibility, due to which they are most commonly used for wound dressing and tissue engineering [28]. Biocompatibility of chitosan minimizes local inflammation. It can be moulded into porous structures, which allow the osteoconduction phenomenon [69].

Other polymers such as alginate also present good biocompatibility but are composed of inert monomers which lack bioactive ligands required for cell anchoring [95]. In case of cellulose (mostly bacterial cellulose), its biocompatibility is improved by producing cellulose composites with other polymers such as chitosan, collagen, gelatine, starch, etc. Along with biocompatibility, other properties such its water holding capacity, water release rate, thermal stability of cellulose are also improved in the cellulose-chitosan composites [96].

5 Applications of Chitosan as Compared to the Other Sugar-Based Polymers

Natural sugar-based polysaccharides like cellulose, starch, chitosan, alginate are considered to be superior to other polymers due to their biocompatibility, bioactivity, homogeneity and bio-adhesive properties. These application areas have been listed in Table 3. Also, they are renewable and can mimic the extracellular matrix (ECM) [97]. Among the plant-derived polysaccharides, the most abundant and naturally available polysaccharide is cellulose [98]. Also, it can be produced by certain bacteria like *Acetobacter, Sarcina ventriculi* and *Agrobacterium,* etc. [99]. Starch is another biopolymer, which is found in plants, green and red algae. Commercially available chitosan is mainly from animal origin [100]. Chitosan can be obtained from shells of crustaceans like crabs, shrimps, lobsters, as well as fish scales and many other organisms like insects and fungi, etc. [12]. Alginate is another important polysaccharide typically obtained from brown seaweed. Commercially available alginate is normally extracted from brown algae (*Phaeophyceae*) [90].

These materials have been engineered into biologically superior variants by numerous physical and chemical methods like copolymer grafting, atom transfer

Table 3 Polysaccharides, advantages, disadvantages and main application areas

Polysaccharide	Advantages	Disadvantages	Applications	References
Chitosan	Biocompatibility, anti-microbial, innocuous, easily degradable, adsorbability, film formation	Low water solubility, poor spinnability, poor strength	Drug delivery carrier, gene transfer, haemostasis material, medical dressing, hydrogels, tissue engineering	[12, 97, 98, 99]
Alginate	Low toxicity, biocompatibility, tumour suppressor, immunity booster	Bad biodegradability, cell attachment poor	Pharmaceutical excipient, medical dressing	[90, 100, 101]
Starch	Abundant sources, low price, degradation products safe and non-toxic, non-antigenic	Poor mechanical strength, more hydrophilic, poor blocking performance	Haemostasis material, tissue-engineered scaffold, drug delivery carrier, bone repair material	[102, 103, 104, 105]
Cellulose	Abundant sources, low-cost material	Rare adverse reactions	Pharmaceutical adjuvant	[102, 106, 107]

radical polymerization, etc. to make them suitable materials for biomedical applications [101].

Among these polymers, chitosan is significantly more versatile and is hence employed for numerous applications, as discussed subsequently.

5.1 Drug Delivery

Delivering drugs at different target sites, along with controlling the concentration delivered over a specific period, is a challenging task. The uptake of drug by the organs surrounding the intended site of action can lead to side effects. If the drug is delivered in exceptionally higher amounts, they can cause adverse reactions or toxicity. Hence, targeted drug delivery systems that are able to control the rate of drug release are being developed [102]. Starch has been employed in conventional as well as specialized drug delivery systems [103, 104].

Starch nanoparticles and matrix systems have been fabricated for targeted drug therapy. Starch-based delivery systems, such as polymeric micelles, polymeric capsules, grafts and hydrogels, etc., have been explored for controlled release of anti-cancer drugs. Similarly, these systems have also been investigated for drug delivery to the lungs via the nasal pathway [105]. Starch-polyethylene glycol copolymer micelles have been designed by grafting this biopolymer with carboxyl-terminal polyethylene glycol. This copolymer was cross-linked with lipoic acid via disulphide bonds and the resulting glutathione responsive system was employed for intracellular delivery of doxorubicin [106].

Although a versatile drug carrier, certain properties make starch less efficient in conventional and innovative formulations. The absorption of water by starch varies with the relative atmospheric humidity, the presence of numerous hydroxyl groups in starch and its derivatives, which can modify its interaction with drug molecules and the morphology of starch. This is also known to facilitate the breakdown of these carriers, leading to the release of active substances contained within [107]. This hydrophilic nature and the intrinsic moisture content of starch are among the important factors that will affect the particular formulation by affecting properties like flow, compaction and tensile strength in drug delivery platforms like granules and tablets. These problems associated with native starch are being overcome through the development of modified starch such as pregelatinized starch, cyclodextrin, carboxymethyl starch, etc. [103, 107].

The drug loading capacity of natural cellulose is significantly low due to the extensive intra- and inter-molecular hydrogen bonding. Thus, modified cellulose is being used as a carrier for drug delivery applications [108]. Due to its extensive intramolecular bonding and low solubility in comparison with starch, the use of cellulose is limited for drug delivery [109]. In recent years, efforts have been made to overcome this limitation through hydrolysis of high molecular weight cellulose into smaller fragments, which can increase its solubility. Commercially available cellulose derivatives such as ethylcellulose, carboxymethyl cellulose are excellent starting materials having superior functions in drug delivery, formulations, wound dressing [97]. Nanocellulose, derived from native cellulose, can result in drug release within several minutes to several days. The release time primarily depends on the formation of nanocellulose and the active pharmaceutical ingredient encapsulated in it. Thus, nanocellulose can be used as a versatile carrier via different delivery routes [110]. In 2015, Hosseinidoust et al. suggested that nanocellulose could stress cells due to its high charge density. In 2017, Seabra et al. reviewed the in vivo and in vitro investigations related to nanocellulose. It was observed that nanocellulose resulted in very low toxicity in several models. However, due to the occasional severe toxicity reported, further investigations with nanocellulose were recommended by researchers [111, 112].

Alginate is known for the delivery of variety of low molecular weight drugs. It is most useful when a primary or secondary bond between drug and alginate is to be broken to regulate the kinetics of drug release leading to rapid release of drug molecule. Alginate has been widely studied in many drug delivery applications, in combination with chitosan, as their combination forms ionic complex, which is useful in drug release [90]. Multiparticulate system of alginate and chitosan containing triamcinolone were prepared by ionotropic gelation method for colonic drug delivery [113].

Chitosan and cellulose have an overall similar structure, the only difference being the presence of the amino group at the C-2 position in chitosan, while cellulose having a hydroxyl group at this position. Therefore, these two materials are miscible with each other and the resulting composite material has improved mechanical properties (due to cellulose) and anti-bacterial properties (due to chitosan) [114].

Among the aforementioned natural sugar-based polymers, chitosan is widely used in drug delivery systems [115]. Chitosan has gained enormous importance in the pharmaceutical industry because of its wide availability, non-toxic nature, biocompatibility and biodegradability, which make it an ideal carrier for drug therapy. Some other biological activities of chitosan include its haemostatic, bacteriostatic, anticholesteremic, anti-carcinogenic, fungistatic actions, etc. [116]. Also, it has distinct structural similarity to the mammalian glycosaminoglycans (GAGs), which are a family of hetero-polysaccharides in mammals that are primarily located on the surface of cells and in the extracellular matrix (ECM) [117].

The polycationic property of chitosan gives it remarkable mucoadhesive property due to its positive charge. Because of this, it has better and longer adhesion to mucous, which in turn can enhance the efficiency of drug delivery by providing enhanced contact time for drug penetration [78]. However, the lower solubility of chitosan is the major drawback. Thus, this material has been modified into different formulations like microspheres, tablets, nanoparticles, nanofibres, beads, films, hydrogels, conjugates, etc. for diverse applications [118]. These systems have overcome the issues associated due to low solubility, poor stability, toxicity, various side effects, low selectivity of diverse drug molecules. Chitosan nanoparticles are a good example of a drug carrier that has displayed controlled drug release, along with improvising drug solubility and stability, enhancing efficacy and reducing toxicity. Their small size has enabled them to traverse various cell barriers, like the blood-brain barrier, etc., thus allowing them to deliver drugs at various sites within the body [78]. Existing work in the area of chitosan and its combinations with other sugar polymers being utilized for drug delivery has been summarized in Table 4.

5.2 Vaccine Delivery

Vaccines have significantly improved human health by offering protection against many life-threatening diseases. Traditional vaccines are composed of either whole pathogen or its parts to induce a robust immune response. In case of infections caused by the human immunodeficiency virus, mycobacterium tuberculosis, hepatitis C virus, etc., the traditional vaccines have limited efficacy or cause severe adverse effects. Thus, vaccine makers shifted their focus on subunit vaccine, nucleic acid vaccines, peptide vaccines, which were safe as well as efficacious and employed additional immune-stimulating materials, known as adjuvants. Numerous compounds like lecithin, saponin, mineral oil, agar have been studied for their ability to enhance the effectiveness of antigens, most of which are toxic and unsuitable for human use. At present, aluminium-based compounds are largely used in the human vaccine. Recently, biopolymer-based vaccine delivery systems and adjuvants have emerged as alternatives for aluminium-based compounds [141]. We will focus mainly on the role of sugar-based polymers in vaccine delivery.

Table 4 Combination of drugs and polysaccharides, route of administration, work done and its results

Drug	Polysaccharide and their combination used	Delivery route	Summary of work done	Results	references
Insulin	*Chitosan*: Carboxylated chitosan/ poly (methyl methacrylate) nanoparticles	Oral	A system based on carboxylated chitosan grafted poly(methyl methacrylate) nanoparticles was prepared and loaded with insulin and tested on normal and diabetic rats to examine its drug release properties	The results indicated that the prepared system can be used as a carrier for protein drugs like insulin	[119]
	Alginate and chitosan: Alginate-chitosan nanoparticles	Oral	Nanoparticles were prepared by ionotropic pre-gelation of an alginate core followed by chitosan polyelectrolyte complexation and insulin encapsulated into them	Orally delivered nanoparticles lowered basal serum glucose levels by more than 40% with 50 and 100 IU/kg doses sustaining hypoglycaemia for over 18 h	[120]
	Starch: Starch and polyethylene glycol	Oral	Polyethylene glycol(PEG) was conjugated with starch acetate. After its incubation with insulin solution, self-aggregated nanoparticles with mean particle size of 32 nm are formed	PEGylated starch acetate nanoparticles are highly bio-adhesive and can be utilized as a carrier system for controlled delivery of insulin	[121]
	Cellulose: Carboxymethyl cellulose/poly(acrylic acid) hybrid hydrogels	Oral	Biodegradable and pH-responsive carboxymethyl cellulose/poly (acrylic acid) hybrid hydrogels are synthesized. The hydrogels de-swell in acidic artificial gastric fluid but rapidly swell in neutral artificial intestinal fluid (AIF), rendering selective enzymatic degradation of the gels as well as accelerated drug release from insulin-loaded hydrogels in AIF	Oral administration of insulin-loaded hydrogels to streptozotocin-induced diabetic rats leads to continuous decline in the fasting blood glucose level within 6 h post-administration, and the relative pharmacological availability increased more than ten times compared to free insulin solution.	[122]

Procaine hydrochloride and imipramine hydrochloride	*Chitosan*: Procaine hydrochloride and imipramine hydrochloride	–	A system of chitosan nanocrystals (CNC) modified by chitosan oligosaccharide was prepared and compared with native CNC and oxidized CNC systems in terms of drug release properties for potential use as carriers for model drugs	CNC modified chitosan oligosaccharide resulted in the best sustained release profile and can be used for drug delivery for local anaesthetics and periodontal cavities	[123]
Methotrexate	*Chitosan*: Trimethyl chitosan modified by poloxamer	–	Trimethyl chitosan was modified by poloxamer and loaded with methotrexate to prepare a carrier system. To test the system, analysis and assays of morphology, drug release and cytotoxicity were carried out	The results showed that cellular uptake and apoptosis were enhanced indicating that the prepared system could be used for drug delivery in cancer therapy	[124]
	Cellulose: Hydroxyethyl cellulose	Intravenous (IV)	Methotrexate was linked to cellulose by methyl ester bonds. A conjugate containing on average 9.5 molecules of MTX per molecule of cellulose was developed. Gel filtration HPLC analysis showed that the conjugate contained approximately 2% free drug	The conjugate showed lower cytotoxicity in tumour cell line than the free drug. In the in vivo experiment to treat orthotopically implanted mammary tumours, the conjugate applied intravenously showed more inhibition of tumour growth than free drug	[125]
	Alginate: Sodium alginate nanospheres	–	Sodium alginate nanospheres containing methotrexate were prepared by controlled gelling method	The drug bound to nanospheres produced a comparatively better cytotoxic effects at all concentration than the free drug	[126]
Carbamazepine	*Starch*: Porous starch	–	Carbamazepine (CBZ) was used as a drug candidate to exhibit solubility enhancement potential of porous starch (PS). PS and CBZ-loaded PS systems were	In vivo studies conducted in mice displayed a superior performance of CBZ-PS as compared to neat CBZ	[127]

(continued)

Table 4 (continued)

Drug	Polysaccharide and their combination used	Delivery route	Summary of work done	Results	references
			characterized with respect to IR, DSC, XRD, SEM and dissolution kinetic studies		
	Chitosan: Carboxymethyl chitosan nanoparticles	Nasal	With the purpose of increasing the concentration of carbamazepine for the treatment of epilepsy, it was loaded on carboxymethyl chitosan nanoparticles to be used nasally in mice models	The system was capable of enhancing drug bioavailability and its delivery to brain	[128]
	Chitosan- alginate: Chitosan-sodium alginate carbamazepine microspheres	–	Chitosan-sodium alginate microspheres loaded with carbamazepine and studied for its controlled release	The formulation provided a desired carbamazepine loaded sodium alginate microspheres with the controlled release drug delivery and can be used in treatment of epilepsy	[129]
Progesterone	*Chitosan*: Glycol chitosan/ superparamagnetic iron oxide nanoparticles	–	A polymeric-metallic hybrid nanoparticles of Fe3O4 was prepared and coated with glycol chitosan. To test the capability of system for drug release, it was loaded with progesterone and was studied for characterization	Satisfying biocompatibility and suitable controlled drug release of the model drug were observed	[130]
	Chitosan and alginate: Chitosan-sodium alginate nanoparticles	Nasal	Progesterone-loaded hydrogel nanoparticle formulations have been prepared by polyelectrolyte complex formation between trimethyl chitosan and sodium alginate	In vivo studies on male rats demonstrated five-fold increase in brain progesterone concentrations compared to basal progesterone level after 30 min of hydrogel nanoparticle inhalation	[36]

Bovine serum albumin	*Chitosan*: Chitosan nanoparticles grafted by carboxymethyl-cyclodextrin	Oral	Carboxymethyl-β-cyclodextrin grafted chitosan nanoparticles were synthesized and tested for oral delivery of bovine serum albumin in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and simulated colonic fluid (SCF) to have a system with ability of efficient oral drug delivery of protein drugs	Fabricated system was more efficient in releasing drug in SIF and SCF than in SGF. Overall results indicated that protein drugs can be delivered orally by the prepared system	[131]
	Cellulose: Cellulose-g-poly (acrylic acid) hydrogels	Oral	Bovine serum albumin (BSA), a model protein drug, was loaded into the hydrogels, and the release profile in simulated gastrointestinal fluids was investigated	Increased steady release of less than 10% in simulated gastric fluid demonstrated the potential of these hydrogels to protect BSA from the acidic environment of the stomach	[132]
Paclitaxel	*Chitosan*: Chitosan/ linoleic acid/ poly (L-malic acid)	Intravenous (IV)	Chitosan was modified by linoleic acid and poly(L-malic acid) to form double grafted chitosan nanoparticles which were later loaded by paclitaxel and tested in vitro and in vivo for drug loading and releasing capacity and antitumour activity	The developed system exhibited sustained release and tumour inhibition effect, suggesting that it can be utilized as drug carrier for IV administration of hydrophobic, anti-cancer drugs	[133]
	Cellulose: Cellulose nanomaterial	Oral	Paclitaxel encapsulated in cellulose nanomaterial, as naïve paclitaxel degrades faster during its delivery	High release rate was observed on first day, also paclitaxel accumulation in tumour tissues increased	[134]
	Starch: Porous starch	–	Paclitaxel was loaded into porous starch in the form of nanoparticles (PNPS), and the properties of PNPS were investigated by using raw paclitaxel and the system of paclitaxel directly loaded into	Compared with raw paclitaxel and PPS, PNPS exhibited the more prominent dissolution rate and bioavailability, in which the bioavailability of PPS and PNPS were	[135]

(continued)

Table 4 (continued)

Drug	Polysaccharide and their combination used	Delivery route	Summary of work done	Results	references
			porous starch (PPS) as control groups	2.94 and 5.42 times of that of raw paclitaxel, respectively	
Lidocaine HCl, Ketoprofen, Dexamethasone	*Chitosan*: Chitosan/catechol	Buccal	A mucoadhesive hydrogel composed of chitosan and catechol was fabricated and cross-linked by genipin. Prepared hydrogel was loaded with drugs and was tested for the capability for buccal delivery of the drug in rabbit models	Sustained release of drug was observed in vitro. Mucoadhesion and biocompatibility of the hydrogel were also favourable. In vivo study confirmed the same. So, the fabricated hydrogel has potential for buccal drug delivery	[136]
Lidocaine	*Cellulose and alginate*: Alginate/carboxymethyl cellulose/polyethylene oxide nanofibre films	Buccal	The drug release behaviour was controlled by cross-linking lidocaine-loaded alginate/carboxymethyl cellulose/polyethylene oxide nanofibre films prepared by electrospinning	An excellent anti-adhesion barrier was developed that can be applied to treat patients in the medical field	[137]
Dexamethasone	*Starch*: Starch and polycaprolactone	–	A polymeric blend of starch with polycaprolactone was used to produce a microparticle carrier for the controlled release of dexamethasone	The in vitro release studies showed a sustained release pattern with 48% of the encapsulated drug being released for a period of 30 days	[138]
Tamoxifen	*Chitosan*: Lecithin and chitosan	Oral	Tamoxifen loaded on phospholipid, chitosan nanoformulation, tamoxifen released from lecithin/chitosan nanoparticles across excised rat intestinal wall mounted in an Ussing chamber was investigated	Compared to tamoxifen citrate suspension, the amount of the drug permeated in intestinal wall using the nanoformulation was increased from 1.5 to 90 times	[139]
Morphine	*Chitosan*	Nasal			[140]

			The chitosan-morphine nasal formulations tested in healthy volunteers in comparison with a slow intravenous infusion (over 30 min) of morphine	Results showed that the nasal formulation was rapidly absorbed within 15 min or less and bioavailability of nearly 60%	

5.2.1 Starch-Based Vaccine Delivery Systems

Earlier studies have shown that the starch alone has low immunogenicity, but starch derivatives can be used as adjuvants for intranasal vaccination. Recently it was found that raw starch microparticles have immunostimulant properties in mice vaccinated with BCG and challenged with *Mycobacterium tuberculae* [142]. These microparticles conjugated with human serum albumin have resulted in robust antibody response in mice, when administered intraperitoneally, intramuscularly, intravenously [143]. However, the physical properties of starch such as its hydrophilic nature and low mechanical strength may be disadvantageous for vaccine preparations.

5.2.2 Cellulose-Based Vaccine Delivery Systems

Cellulose derivatives such as hydroxyethyl cellulose, carboxymethyl cellulose (CMC), hydroxypropyl cellulose and hydroxypropyl methylcellulose have been routinely used for controlled delivery of vaccines. In one experiment, Yongbo et al. prepared novel cellulose nanoparticles using charged CMC and quaternized cellulose (QC) and evaluated their potential for the in vitro delivery of DNA vaccine. At higher concentration of CMC (0.1 mg mL^{-1}), DNA dissociated too early so resulted in low transfection efficiency. However, a lower concentration of CMC (0.05 mg mL^{-1}) effectively reduced the binding between QC and DNA and therefore provided an efficient transfer, which was comparable to Lipofectamine. The improvement in transfection efficiency, while maintaining low toxicity, indicated suitability of CMC–QC nanoparticles for DNA vaccine delivery [144].

Wooyoung et al. developed a vaccine using microcrystalline cellulose (Avicel PH-101) as a delivery carrier of recombinant protein-based antigen against erysipelas. For studying its immunogenicity, groups of mice were immunized with different constructs, like soluble cellulose-binding domain (CBD)-surface protective protein (SpaA), Avicel coated with CBD SpaA, whole bacterin of *E. rhusiopathiae* (positive control), and PBS (negative control). After 2 weeks the mice were challenged with 1×10^7 CFU of *E. rhusiopathiae.* It was observed that the 100% survival rate could be achieved using the Avicel coated with antigen in *E. rhusiopathiae*-challenged mice [145].

5.2.3 Alginate-Based Vaccine Delivery System

Alginate nanoparticles are hydrophilic carriers of antigen that prolong the antigen release and due to their adjuvant property enhance the immunogenicity than the traditional vaccines. Also, in nasal and oral administration of the vaccine, because of mucoadhesive properties and increasing permeability, they reduce the degradation of antigen in an acidic environment. Diphtheria toxoid loaded alginate nanoparticles

were prepared by ionic gelation method and characterized. The loading of toxoid on the nanoparticles resulted in high loading capacities and subsequent release studies showed long release profile. When guinea pigs were immunized with diphtheria toxoid loaded alginate nanoparticles, they showed the highest humoral immune response than conventional vaccine [146]. To prevent immediate desorption of antigens from chitosan carriers in the gastrointestinal tract, bovine serum albumin (BSA) loaded chitosan microparticles were coated with sodium alginate by layer-by-layer technology. The alginate coating on chitosan microparticles could result in the protection of BSA, in in vitro acidic conditions, for 2 h and thus was deemed to be effective for oral administration of vaccine [147].

The live Bacille Calmette–Guérin (BCG) and *Mycobacterium indicus pranii* (MIP) were encapsulated in 2–4 μm alginate particles and mice were immunized with liquid aerosol and dry powder aerosol containing alginate encapsulated mycobacterium particles. It was found that the dry powder aerosol of alginate encapsulated mycobacterium particles resulted in superior immune response than the liquid aerosol [148].

5.2.4 Chitosan-Based Vaccine Delivery Systems

Among the sugar polymers, chitosan has been known as the most suitable material for vaccine delivery. Chitosan has displayed adjuvant properties, which are important for vaccine delivery [149]. In the formulation of vaccines, chitosan has been used to adsorb or encapsulate the antigen for enhancing the efficiency of administration [150].

In case of mucosal vaccination by nasal route, chitosan has been reported to increase the residence time of antigen and hence result in a higher antigen uptake due to its mucoadhesive properties [151]. Achievement of prolonged local delivery of immunomodulatory cytokines to improve immune response is one of the goals in the vaccine delivery system. Zaharoff et al. reported that subcutaneous injection of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) formulated in chitosan solution resulted in longer retention of rGM-CSF at the injection site. This caused a significant enhancement in antigen presentation and overall protective response, as compared to the other lipid-based adjuvants [152].

Most of the studies that have employed chitosan as an adjuvant or carrier for protein and subunit vaccines exhibited the potential effect of chitosan on the immunogenicity of vaccine administered. Chitosan in soluble form as well as in particulate system can improve antigen uptake at mucosal epithelia, which allows vaccine access to sub-epithelial antigen-presenting cells (APCs) and results in increase in local immune response. In some cases, improvement of immunogenicity of DNA vaccine was achieved through the use of cationic liposomes and chitosan-based particulate delivery systems. These carrier systems were able to enhance the uptake of antigens by APCs and could induce T-cell response, which resulted in improved immunogenic response, especially by the mucosal route [153]. Due to the cationic nature of chitosan in weak acidic conditions, highly negative charged DNA

forms strong electrostatic interaction with it, which enables efficient vaccine delivery [154]. Illum et al. formulated a flu vaccine as chitosan-DNA nanoparticle complex. Upon intranasal and intramuscular administration of these complexes in BALB/c mice, elevated antibody titres were observed as compared to the administration of naked DNA [155]. Cui and Mumper evaluated the topical application of DNA nanoparticles. DNA chitosan nanoparticles and DNA coated on chitosan/carboxymethyl cellulose nanoparticles were applied topically in mice. Both the systems showed increased titres of IgG over a prolonged period, showing that topical application of chitosan-DNA vaccine could stimulate the immune system [156].

Oral vaccination is better than systemic administration of vaccine as it avoids pain and risk of infection associated with the injection. It is also cost-effective and makes vaccination easy for the large population. Oral vaccination can induce both, mucosal and systemic immune response [157]. An effective T-cell immunity can be achieved at a lower dose of plasmid DNA if the delivery system can be targeted to the APCs and induce endolysosomal DNA vaccine release [158]. Mannose bearing chitosan was found to target APCs and provide sustained release of DNA vaccine [159]. Murthy et al. demonstrated that rapid degradation of chitosan particles in the lysosomal compartment after cellular uptake can result in dissociation of DNA from the polymer. The DNA vaccine is then expressed and processed, which results in MHC-I restricted antigen presentation and successive higher immune response than the administration of DNA alone [160].

5.3 Tissue Engineering

Tissue engineering is a field of biomaterial development which involves combining cells, scaffolds, biologically active molecules into a functional tissue. The objective of tissue engineering is to prepare a functional construct that can restore, maintain or improve damaged tissue or organ [161]. For use in tissue engineering, the polymer must be biocompatible and should possess specific mechanical, biochemical and physical properties [162]. Various natural sugar-based polymers like cellulose, starch and chitosan, alginate have been investigated for their use in repair and regeneration of compromised tissues [62, 163, 164].

Alginate exhibits excellent biocompatibility and biodegradability and thus has been employed for tissue engineering and drug delivery. It can be readily processed for constructing three-dimensional scaffolds, such as hydrogels, microspheres, sponges, etc. [165]. Alginate gels have been widely explored as vehicles to deliver proteins or cell populations that can direct the regeneration or engineering of various tissues and organs in the body [90].

Cellulose, being polymer of glucose, is an ideal candidate for biomaterial manufacturing because of its chemical, physical and mechanical properties. Cellulose is the most abundant polymer in nature and also presents a low-cost platform for tissue engineering. Cellulose-based biomaterials satisfy the criteria of biocompatibility, bioactivity required for tissue engineering [166].

Also, due to tunable mechanical properties, drug-releasing capabilities and moisture maintenance, cellulose is an interesting candidate for preparing artificial skin and wound dressings. However, cellulose does not exhibit anti-microbial properties, which are important for wound healing. Therefore, anti-microbial agents can be combined with cellulose biomaterials [162]. Nanofibrillar cellulose dressings have been used to heal and regenerate the skin of burn victims. Hakkarainen et al. demonstrated that functionalized cellulose dressing could be superior to the existing commercially available products, like Suprathel®. Epithelial skin regeneration and lack of inflammation for cellulose dressing were the major benefits observed. The dressing attached easily on the wound and detached on their own after skin regeneration [167]. Moreover, anti-microbial properties were conferred by synthesizing composite materials containing chitosan due to its efficacy against a wide range of microorganisms [168]. Tran et al. developed composite material using cellulose and chitosan. The composite material displayed combined advantages of both components, namely chemical and mechanical stability due to cellulose and excellent anti-microbial due to chitosan. This composite material could inhibit the growth of a wide range of microbes, such as *methicillin-resistant Staphylococcus aureus* (MRSA), *vancomycin-resistant Enterococcus* (VRE), *Staphylococcus aureus* and *Escherichia coli* [169].

Various scientists have shown that chitosan and its derivatives can be used to repair or replace a part or whole tissues like skin, cartilage, bone, ligaments, tendon, blood vessels, intervertebral disc, etc. Here, chitosan is often co-grafted or combined with natural/semisynthetic/synthetic polymers, such as polyvinyl alcohol, polyacrylic acid and polyethylene glycol, etc., to increase its strength. Cross-linked silk fibroin/chitosan sponges have better thermal stability than sponge made up of pure silk fibroin or pure chitosan and it can be a potential scaffold material for the development of new tissues and organs [170]. There are many applications of chitosan in tissue culture as discussed in the following sections.

5.3.1 Skin Tissue Engineering

Skin healing is a complicated process consisting of four overlapping stages – haemostasis, inflammation, proliferation and tissue remodelling. It involves blood elements, extracellular components, soluble factors and cells. Therefore there is a requirement of treating skin lesions with dressings that can ensure physical protection, along with the enhancement of healing and provide anti-microbial protection and reduce scar formation [164]. Chitosan is a promising skin substitute material due to its biocompatibility, biodegradability, haemostatic nature, enhanced fibroblast synthesis, scar-free healing and accelerated tissue regeneration [170].

The cationic nature and presence of C-2 amino and C-3, C-6 hydroxyl group affords important biological properties to chitosan. The cationic chitosan and negatively charged blood cells interact with each other and result in the haemostatic activity [171].

Table 5 Commercial haemostatic products containing sugar polymers

Polysaccharide	Commercial haemostatic products	References
Chitosan	Axiostat®, Chitoflex®, Chitoseal®, Celox®	[177]
Starch	PerClot®, Instantaneous®	[178]
Cellulose	Surgicel®, Traumastem®, Haemostem®	[180, 181, 182]
Alginate	*Algisite M, Sorbsan, Cutimed®* Sorbact®	[101, 183, 184]

Fei Han et al. developed novel gelatin-chitosan sponges. Biocompatibility and in vivo studies showed that the sponges could improve cell adhesion, proliferation and provided effective support to promote wound healing. The developed scaffold was proposed as a potential skin tissue engineering material, exhibiting good biocompatibility and physical properties [172]. Chitosan has very strong haemostatic activity, which is primarily dependent on its molecular weight, irrespective of the host coagulation pathway [173]. Chitosan with higher molecular weight can increase pro-coagulation effect due to higher interaction with polyelectrolytes [174]. The wound-healing effect of chitosan is dependent on its molecular weight, degree of deacetylation and also on its physical states like microspheres, fibres, hydrogels, films, membranes, etc. [175]. The moderate deacetylation caused by the formation of mesh-like structure within chitosan enhances its interaction with the blood components. With higher deacetylation, chitosan forms hydrogen bond within itself, leading to the formation of a crystalline structure that exhibits limited interaction with the blood cells [176]. The commercial haemostatic products based on sugar polymers have been stated in Table 5.

5.3.2 Bone Tissue Engineering

In bone tissue engineering, biodegradable substitute acts as a temporary skeleton to support the bone tissue regeneration by gradual self-degradation and is eventually replaced by new bone tissue. In the case of treating a vertebral fracture, bone cement must possess proper injectability, bioactivity, rapid setting time, stiffness, low setting temperature [179].

Alginate, an anionic polymer, has gained more importance in bone tissue engineering due to its biocompatibility and gel-forming activity. Many composite materials such as alginate-chitosan, alginate-gelatin, alginate biosilica, and alginate ceramic have been investigated till date. These materials show enhanced biochemical suitability in terms of porosity, mechanical strength, biocompatibility, cell proliferation and osteogenic differentiation [180]. However, one critical drawback of ionically cross-linked alginate gel is limited long-term stability in physiological conditions, because these gels can dissolve after the release of divalent ions into surrounding [90]. This drawback can be minimized by combining alginate with chitosan, these composites are more stable as electrostatic interactions as well as hydrogen bonding between these two play important role in the increased stability of the composite [20].

Polymers like starch, chitosan exhibit many advantageous properties for the aforementioned orthopaedic applications [181, 182]. Starch-based scaffolds support attachment, proliferation and differentiation of bone marrow stromal cells. Also, scaffolds based on starch and gelatin exhibit high porosity, up to 52.51%, which is suitable for cell interaction, cell growth and vascularization [183]. Bone cement based on corn starch blended with cellulose acetate was prepared by polymerization, via the free radical technique. This construct promoted the healing process at the implantation site [184]. However, these polymers present several drawbacks such as difficulty in processing, very high water uptake, low mechanical strength and instability for long-term application. Also, the chemical modifications can give rise to toxic by-products and reduce the degradation rate of starch-based biomaterials [185, 186].

Chitosan can be alternative for above polysaccharides as along with its other biological properties, it is capable of supporting matrix deposition by osteoblast, osteoconduction and local inflammatory action, which are important in bone tissue engineering [182]. The mechanical properties of chitosan were improved by the addition of the hydroxyapatite due to its similarity with bone inorganic components [187]. Addition of hydroxyapatite to the matrix resulted in compressive strength increase compared to chitosan alone [164]. Also, other composites such as nano-zirconia/chitosan, nano calcium-zirconate/chitosan, strontium-modified montmorillonite composite with comparable mechanical properties were developed [188]. The polycationic nature of chitosan was used to produce complexes with polyanionic polymers to improve the mechanical properties of the resulting composite scaffolds [189]. The layering of chitosan on top of metal implants (e.g., titanium) increased osteointegration [190]. Nowadays, chitosan is being used in 3D printing for various tissue engineering applications. Chitosan hydroxyapatite hydrogel was prepared by the thermal cross-linking reaction, using glycerol phosphate disodium salt and was printed successfully. The cells seeded on the printed scaffold were found with enhanced osteogenic marker expression in comparison with 3D printed alginate and alginate-hydroxyapatite scaffolds [191].

Similarly, chitosan silk fibroin blend is useful in cartilage regeneration. Chitosan has structural similarity to sulphated glycosaminoglycans, which provided a compatible microenvironment for chondrocyte proliferation, extracellular matrix synthesis and chondrogenesis [192]. Chitosan could interact with collagen via electrostatic interaction between abundant amino and sulfo groups [193].

5.4 Anti-microbial Applications

Polysaccharides like cellulose, alginate, starch are not intrinsically anti-microbial and they require chemical incorporation of an antibiotic compound. Chitin and chitosan have been investigated as anti-microbial materials against a wide range of target organisms like algae, bacteria, yeasts and fungi in in vivo and in vitro experiments involving different forms of chitosan, such as solutions, films and

composites. Although the exact mechanism is not known, three anti-bacterial mechanisms of chitosan have been reported: (1) the ionic surface interaction leading to cell wall leakage, (2) the inhibition of mRNA and protein synthesis via penetration of chitosan in the microbial cell, (3) the formation of an external barrier, chelating metals and provoking the suppression of essential nutrients for microbial growth [168].

The anti-fungal mechanism of chitosan interferes with cell wall morphogenesis and affect fungal growth [194]. The microscopic observation showed that the chitosan oligomers diffuse inside hyphae, interfering enzyme activity responsible for fungal growth [195].

Several studies have shown that the anti-microbial property of chitosan depends on the molecular weight and degree of deacetylation, the lower the molecular weight and degree of deacetylation, the higher the effect on reducing microbial growth [168]. Studies carried out on *Bacillus cereus, E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enterica, B. subtilis, Listeria monocytogenes* and *Klebsiella pneumoniae* proved that lower the molecular weight of chitosan, the greater was the effect on reduction in the microbial growth [196].

Similarly, some studies proved that the anti-microbial effectiveness of chitosan increased as the degree of deacetylation was lowered. Experiments on chitosan having different degrees of deacetylation were carried out against fungi such as *Aspergillus fumigatus, Aspergillus parasiticus, Candida albicans, Fusarium oxysporum;* gram-positive organisms like *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Staphylococcus saprophyticus* and gram-negative bacteria such as *Escherichia coli, Enterococcus faecalis, Salmonella typhimurium, Pseudomonas aeruginosa, Aeromonas hydrophila, Shigella dysenteriae, Vibrio cholerae, Vibrio parahaemolyticus.* Results showed that the anti-microbial activity increased with decreasing deacetylation degree [197]. Combination of silver nanoparticles (AgNPs) and chitosan nanoparticles was more efficient in inhibiting growth of *E. coli*, compared with either AgNPs or chitosan alone. The positively charged chitosan enhances the binding of AgNPs to the negatively charged surface of cells. It is believed that it leads to leakage of intracellular constituents and other proteins, therefore inactivating the bacteria. The addition of AgNPs to chitosan prevented formation of biofilm specific to E. coli thus helping purification of water [198].

6 Summary

Chitosan, the deacetylated form of chitin, has both, amino and hydroxyl groups, which can form stable covalent bonds with other naturally occurring sugar complexes. The protonated amino group becomes a polycation that can form ionic complexes with a variety of natural or synthetic anionic species. The amino groups in chitosan structure can be protonated, providing solubility in diluted acidic aqueous solutions, which confers several remarkable properties to chitosan and offers it unique opportunities in the biomedical field. The versatility and unique

characteristics, such as hydrophilicity, film-forming ability, biodegradability, biocompatibility, anti-bacterial activity and non-toxicity of chitosan, have all contributed to the successful development of various blends for medical applications. Chitosan is one of the most commonly used biopolymers in the biomedical field. It exhibits various biological properties, such as biocompatibility, biodegradability, non-toxicity, anti-bacterial, anti-fungal, anti-tumour activity, mucoadhesiveness, etc. The structural and functional properties of chitosan such as its cationic nature, solubility, bioactivity depend upon its molecular weight and degree of deacetylation. The insolubility of chitosan in water and most organic solvents and poor thermal stability can act as limiting factors for its applications. These limitations of insolubility and thermal instability can be overcome by chemical modifications such as cationization, quaternization, partial hydrolysation and by addition of stabilizing agents, such as hydroxyapatite, calcium carbonate or nanoclay, etc. Other properties of chitosan such as mucoadhesiveness and biodegradability play an essential role in drug delivery applications. Biocompatibility of chitosan is essential for its use in wound dressing, sutures, etc. Chitosan can be used as a chelating agent due to its binding property with proteins, fats, cholesterol and metal ions. Chitosan can also be blended with some other sugar polymers such as alginate, cellulose, starch, etc., to form biocomposites, films, beads, nanoparticles, etc. These combinations have improved biophysical and mechanical properties and overcome their limitations, such as poor long-term stability, low solubility, poor mechanical properties, etc.

Chitosan, alginate, cellulose and starch are useful polymers due to their biocompatibility, bioactivity, homogeneity and bio-adhesive properties. These polymers are derived from natural sources and have various applications in the biomedical field. Their properties can be enhanced by various chemical and physical modifications so that they can be used in drug delivery, tissue engineering and other pharmaceutical applications. In the drug delivery applications, these polysaccharides play important role in carrying various drugs at the target site. Starch, alginate, cellulose have been employed for various drug delivery applications, but are met with drawbacks such as the poor mechanical properties and hydrophilic nature of starch, poor cell interaction of alginate, low drug loading capacity of cellulose. These drawbacks can be minimized by use of chitosan and its derivatives, due to their non-toxic nature, biocompatibility and biodegradability and also the haemostatic, bacteriostatic, anticholesteremic, anti-carcinogenic, fungistatic actions of chitosan. Also, the polycationic property of chitosan gives mucoadhesive property, which helps in enhanced contact time for drug delivery. Raw starch microparticles can be used in vaccine preparation against *Mycobacterium tuberculae* due to its immunostimulant property. Cellulose derivatives, like CMC and QC, are useful in DNA vaccine delivery. Alginate can prolong the antigen release and can result in better immunity than the traditional vaccines. Chitosan has been used as a carrier for various antigens, as well as DNA molecules. It has also been used to encapsulate or adsorb the antigen molecules on its surface, enhance its efficacy during administration and enhance the antigen uptake by APCs. Due to the cationic charge of chitosan, it can bind efficiently with negatively charged DNA and result in efficient transfer of DNA vaccines.

In the field of tissue engineering, alginate, cellulose, starch can be used for preparing hydrogels and scaffolds due to its biocompatibility, bioactivity and tuneable mechanical properties. Also, various skin dressing materials, based on these polysaccharides, are commercially available. But due to lack of anti-microbial property, there is often a requirement of adding anti-microbial agents, while using the above polymers for tissue engineering applications. The composite materials made from cellulose and chitosan have suitable mechanical strength, as well as anti-microbial activity, which can be useful against many types of microorganisms. The cationic chitosan and negatively charged blood cells interact with each other and result in haemostatic activity, which is helpful in skin tissue engineering applications. Various haemostatic products are commercially available in market. Alginate, starch, cellulose exhibit many advantageous properties for the orthopaedic applications, such as suitability for osteoblast attachment, proliferation and differentiation. The limitations of difficulty in processing, very high water uptake, low mechanical strength and instability for long-term make them less applicable in bone tissue engineering. Chitosan can be an alternative for the above-mentioned polysaccharides as along with its other biological properties it is capable of supporting matrix deposition by osteoblast, osteoconduction, and local inflammatory action, which are important in bone tissue engineering. Among these polymers, chitosan has displayed innate anti-microbial properties against a wide range of organisms like algae, bacteria, yeasts and fungi, in in vivo and in vitro experiments involving different forms of chitosan, such as solutions, films and composites. Cellulose, alginate and starch have their own unique properties and limitations as discussed earlier. The structural and chemical nature of chitosan makes them better candidates, as compared with native polysaccharides, for their use in the biomedical field.

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Synthesis and Modification Strategies of Chitosan and Its Interaction with Metal Ions

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Contents

Abstract Chitosan is one of the most important and widely available biomaterials produced by nature for remediation of its resources. It is obtained by deacetylation of chitin extracted from marine waste materials and highly useful to adsorb the toxic ions existing in the water body as it has a large number of active functionalities of hydroxyl and amine groups. The chitosan has several advantages including low cost, bio-degradable, and bio-compatibility over other chemically synthesized materials in the laboratory. Different chitosan-based materials have been identified and tested in the removal of toxic anions, especially, metals, metalloids, dyes, hydrocarbons,

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organic pesticides, etc. In this review, we focused on the functionalized forms of chitosan with metal ions and their adsorption on toxic ions, such as fluoride, phosphate, nitrate, and arsenic from waters using batch experiments. Also, it intensively focused on the synthetic methods, feasibility, and the regeneration/reusability of the materials. The results of this review indicated that the metal-loaded chitosan-based composite materials have shown much higher efficiency than the raw forms of chitosan and metal/metal oxides or hydroxides due to the synergistic interaction and the affinity of the materials. In particular, the mechanism of interactions of toxic ions and the metal ions present in the composites was thoroughly reviewed using analytical and the experimental data that existed in the literature. The reported adsorption densities, collected by recently reported materials, and the selectivity test results were much higher than those of other bio-based materials and inorganic nanocomposites. This review critically discussed the real-water analysis, cost-effectiveness of the reported materials. Importantly, the clean-up and the disposal methods for the pollutant sorbed materials and other areas requiring further research were addressed.

Keywords Adsorption · Arsenic · Chitosan · Fluoride · Nitrate · Phosphate

1 Introduction

A large amount of biomass waste is discarded into the environment without pretreatment. For example, shrimp shell waste is generated in huge quantities from the shrimp industries throughout the world, and it is primarily disposed of into the sea, resulting in intense environmental pollution [1]. India is a major producer of shrimp, which is more than 1,00,000 tons of shrimp bio-waste generated annually and only an insignificant amount of that bio-waste is utilized for the extraction of chitin while the rest is discarded. According to the report in 2012, a total of 250 chitinolytic bacteria from 68 different marine samples were screened by employment enrichment method which utilized native chitin as the sole carbon source. The shrimp shell waste is utilized for the production of natural biopolymers like chitin, chitosan, N-acetyl glucosamine, chitooligosaccharide, other essential amino acids, etc. [2]. Chitin was prepared by a two-step extraction method from shrimp shells using citric acids and deep eutectic solvents. The acids could effectively remove some minerals and proteins followed by deep eutectic solvents with the assistance of microwave and the deproteinization efficiency was more than 88% [3]. It is a linear amino polysaccharide comprised of β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose structure and the deacetylated form of chitin is known as chitosan, i.e., β-(1,4)-2-deoxy-2-amino-D-glucopyranose using NaOH and the chitin with a degree of deacetylation of above 70% is considered as chitosan. Chitosan is insoluble in water but soluble in acetic acid solvents below pH 6. Monoprotic acids such as formic, acetic, hydrochloric, and lactic acids are used for dissolving chitosan, and mostly 1% acetic acid solution used to dissolve chitosan. But it does not dissolve in

diprotic and polyprotic acids such as sulfuric and phosphoric acids and it has poor stability over pH 7 due to precipitation/gelation that takes place in the alkali pH range. Chitosan solution forms a poly–ion complex with anionic hydrocolloid to produce a gel.

2 Chemistry of Chitosan

2.1 Covalent Functionalization of Chitosan Without Metal Ions

Chitosan is used to modify chemically to change its structure and properties to meet the requirements of specific applications. The free amine groups are strongly reactive and can be easily modified chemically or physically. One simple modification of chitosan is protonation. Besides, the protonation of amine groups in acidic solutions gives the chitosan a cationic behavior and consequently the potential for attracting the negatively charged species [4]. The protonated form of chitosan is useful to attract negatively charged species like fluoride, chromate ions, and anionic dyes from water through electrostatic interactions [5, 6]. Initially, Viswanathan and Meenaksi have developed protonated chitosan beads (PCB) for fluoride removal from water, in which they found a maximum uptake of fluoride ions of 1664 mg/kg [5], then for copper uptake densities on chemically functionalized chitosan beads were 52, 86, and 126 mg/g for protonated chitosan beads, carboxylated chitosan beads, and grafted chitosan beads, respectively [7] using the same adsorbent was Pokhrel et al. have reported a review article on the chemical functionalization of chitosan and their applications, in which the chemical and physical functionalization of chitosan without altering the degrees of solubilization [8]. Amine groups were responsible to modify chemically with other functional groups rather than hydroxyl groups due to their basic nature for various applications. In 2015, Macquarrie and Hardy have reviewed the chemically bonded groups on chitosan without metal ions for catalysis inorganic transformations [9]. Recently, Mincke et al. [10] have developed chemically functionalized chitosan adsorbents for selective recovery of palladium and platinum groups from acidic aqueous solutions. In this article, the authors have used various functionalities, viz. 1,10-phenanthroline-2,9-dicarbaldehyde (Ch-PDC), [2,2′-bipyridine]-5,5′-dicarbaldehyde (Ch-BPDC), and glutaraldehyde cross-linked chitosan grafted with 8-hydroxyquinoline-2-carbaldehyde (Ch-GA-HQC) for selective recovery of Pd(II) and Pt(IV) metal ions from waters. The obtained maximum adsorption densities based on Langmuir isotherm were 340.3 and 203.9 mg/g for Pb(II) and Pt(IV), respectively, on (Ch-GA-HQC). The sorption mechanism established by external mass transfer is followed by the intra-particle diffusion process and chemisorption [10]. Likewise, a 3D sponge of 11-mercaptoundecanoic acid-functionalized chitosan (MUA) as an effective adsorbent for methyl orange adsorption was also used for antibiotic-free antibacterial activity. The maximum uptake of methyl orange was 388 mg/g, which is much higher

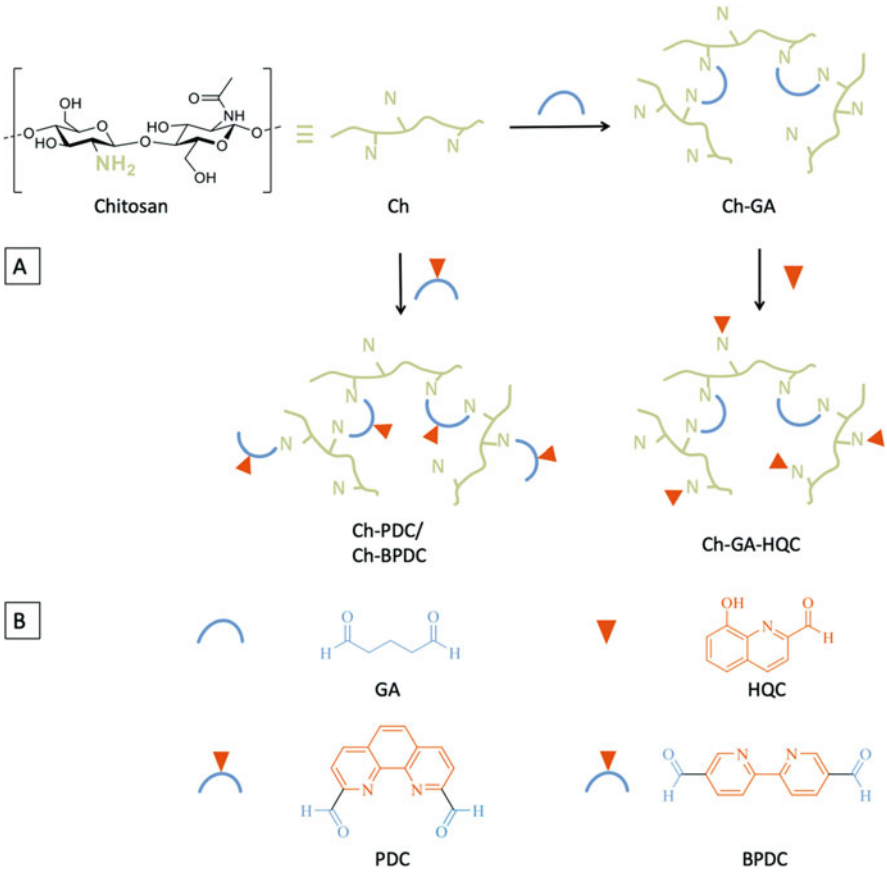

Fig. 1 Schematic overview of the (**a**) synthesis of Ch-PDC, Ch-BPDC, and Ch-GA-HQC and (**b**) the chelating and cross-linking compounds. The figure was adapted with permission from Ref. [10]

adsorption densities compared to many chitosan-based adsorbents in the literature [11]. Interestingly, to improve the adsorption efficiencies of the metals from water, the chitosan moiety was functionalized with more amine groups which tend to be complexed with metal ions easily. Zarghami et al. have developed polyamidoamine (PAMAM)-grafted dendrimer through a divergent growth approach, in which the Pb (II) ions were successful [12]. Moreover, the chemical functionalization of chitosan using different strategies was proposed by Ji et al. for drug delivery systems in detail [13]. Padmaja group has delivered a superabsorbent, namely chitosan–thiobarbituric acid showed maximum uptake capacities of 1,357.69, 2,504.86, and 2,475.38 mg/g for respective Hg°, Hg^{2+}, and $CH_3Hg^{+\cdot}$ and the adsorbent could be reused several times using 0.01 N thiourea, perchloric acid, and 0.2 N NaCl solutions [14]. However, this review aims to present the metal ion interaction on the raw and functionalized forms of chitosan (Fig. 1).

2.2 Functionalization of Chitosan with Metal Ions

Functionalization of chitosan in its amine and hydroxyl groups with various toxic-free metal ions is used for adsorption of toxic ions removal from water, which provides higher adsorption densities, hydrophilic character, and faster adsorption kinetics. Different types of metal ions were immobilized on the surface functionalities of chitosan through in situ and ex situ synthetic methods. These reactive functional groups may interact with metal ions through different mechanisms depending on the metal, the pH, and the matrix of the solution. The free-electron doublet on nitrogen may bind metal cations at pH close to neutrality (or weak acidity). The donation of lone paired electrons plays an important role in the amine–metal interaction. The interaction of metals on amine moiety present in the chitosan was studied using Raman spectroscopy and density functional theory (DFT). The most obvious change of the surface Raman spectra pattern arises from the amino group. It's interesting to note that NH_2 vibration modes show a significant frequency shift and an abnormal Raman enhancement in the measurements. Zhao et al. [15] have explained well about the aniline adsorption on the metal centers via Raman spectroscopy with the substantial aid of DFT calculations. In this paper, the amine–metal interaction mainly arises from the donation of amino lone pair orbital to metal unoccupied s band as described well [15]. According to Pearson's hard and soft acid-based (HSAB) concept, the hard acid metals tend to bind with hard base moieties to form a stable adduct and the same phenomenon for soft-based metals. Based on this concept, the NH_2 groups of chitosan considered as hard base the loading of metal ions should be hard acid behavior which leads to forming a stable interaction between them (Fig. 2).

3 Applications of Chitosan for Environmental Remediation

3.1 Fluoride Retention Using Metal–Chitosan Based Composites

Fluoride is one of the essential microelements required in trace amounts for the normal growth and development of various organs in our body more prominently bone and teeth. Fluoride is thus considered beneficial in drinking water at levels of about 0.5 mg/L but it is harmful once it exceeds 1.5 mg/L which is the World Health Organization (WHO) limit being followed in most of the nations. The Bureau of Indian standards has fixed a permissible limit of fluoride in drinking water as 1 mg/L. The excess of fluoride content in the groundwater sources is one of the major environmental health issues with the continuing growth of population and human intervention in alteration of water quality being one of the prominent causes. In India, 230 districts of 20 states are at risk of high levels of fluoride in drinking water. There are two types of fluorides: (1) ionizable/non-ionizable and (2) organic/

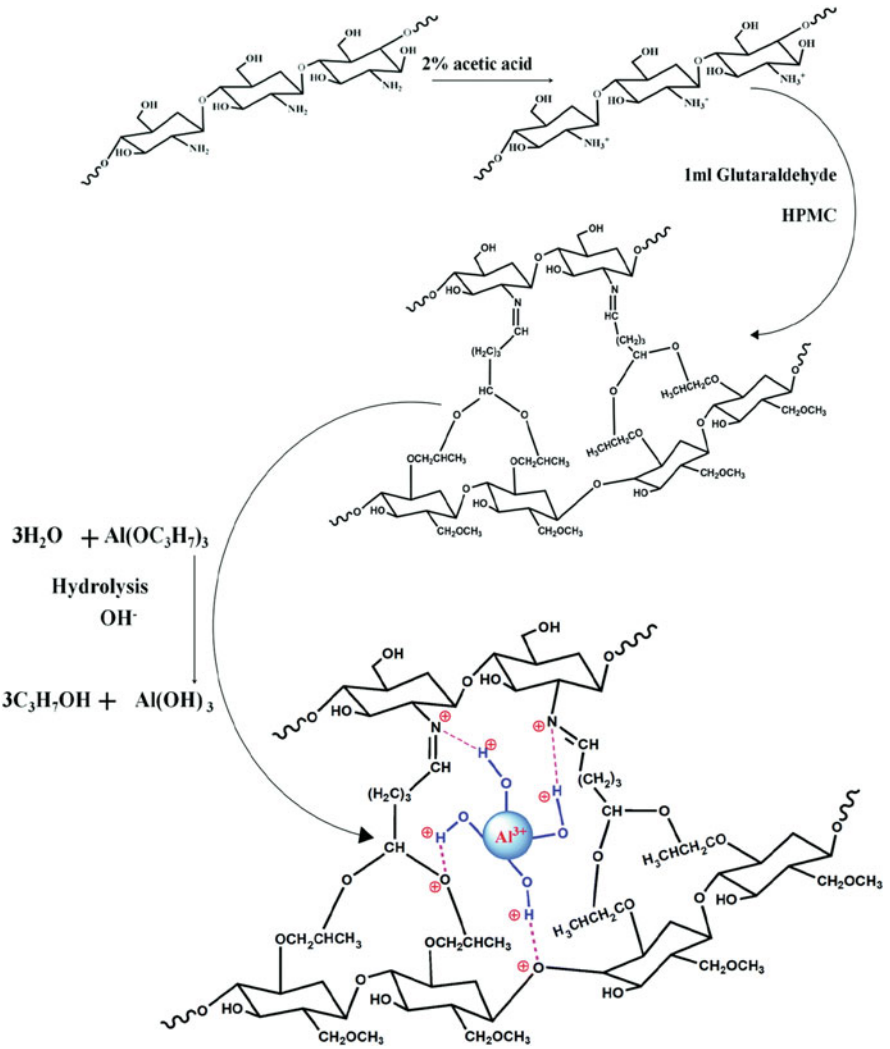

Fig. 2 Alumina cross-linked chitosan with HPMC (hydroxypropyl methylcellulose). The figure was adapted from Ref. [16]

inorganic, in which organic fluoride does not dissolve rapidly in water until fluoride ion is released via any chemical reaction. Mostly the inorganic fluoride ions come from rock phosphates, minerals, and earth crust (950 mg/L) in its ionic form. Excessive intake of fluoride contained water may pose serious health concerns like dental, skeletal, and non-skeletal forms of fluorosis depending upon the concentration of the fluoride contents in water [17]. There are several technologies used in the defluoridation of water: reverse osmosis, nanofiltration, dialysis and electro-dialysis, and adsorption. Among them, adsorption appears to be the most appropriate solution

because it is cost-effective, simple to operate, and produces high-quality treated water [18]. Recently, chitosan-based composites using metals, metal oxides, and bimetals have been receiving large attention as alternative sorbents in water treatment processes as these kinds of materials yield high adsorption densities. To date, several successful attempts made toward the development of new materials with the maximum adsorption densities have been undertaken by employing the following strategies: (1) metal ions encapsulations, (2) magnetic nanoparticle coatings, and (3) combination of both (1) and (2) in the chitosan matrix. Viswanathan and Meenakshi have started working on chitosan and modified forms of the chitosan for fluoride removal from waters. The chemically modified forms of the chitosan, i.e., protonated and carboxylated forms beads showed excellent fluoride adsorption densities were 1.66 and 1.33 mg/L at the initial concentration of 10 mg/L [5, 19]. Then, to enhance the adsorption performance of fluoride, metal-loaded chitosan beads were prepared which showed higher adsorption densities than the protonated and carbolated forms of beads. The fluoride adsorption capacities on La- and Fe-loaded chitosan beads were 4.71 and 4.23, respectively. To continue this, La–Zr bimetallic chitosan beads were prepared by Prabhu and Meenakshi later [20], where the adsorption capacities were ~3.8 times higher than the protonated forms. Also, the material was regenerated with 0.1 M NaOH about 6 times for maximum reuse of the materials. Further, the authors have synthesized metals (Al^{3+}, Ce^{3+}, La^{3+} and Zr^{4+}) incorporated chitosan grafted polyamidoamine dendrimers for the adsorption of fluoride from water. The results showed that Zr-loaded PAAGCB was more selective with the maximum adsorption densities of 17.47 mg/g than the other metal ions loaded PAAGCB. The thermodynamic study results indicated that the adsorption process was spontaneous and endothermic. The Zr-PAAGCB material could be effectively utilized as an adsorbent for fluoride adsorption [21]. Similar adsorption densities were obtained for magnetic materials on the chitosan matrix. The order of the magnetic materials prepared by several authors and the capacities was arranged in decreasing order as Fe_3O_4/chitosan/$Al(OH)_3$ beads [22] > magnetic hydrocalumite–chitosan [23] > magnetic hydrotalcite–chitosan [24] > magnetic hydroxyapatite [25]. The main advantages of these magnetic materials are easy separation of materials from solution and it suppresses separation process time and the cost of the process. Chitosan modified with highly electropositive metals and a supported material delivered high adsorption capacities. For example, Ce–chitosan (153 mg/g), *Aloe vera* supported Nano Zr chitosan [26] and Al–chitosan–hydroxypropyl methylcellulose [27]. The *Aloe vera* supported Zr nanoparticles were prepared by a simple method. The extract of *Aloe vera* was used as a reducing agent to develop Zr nanoparticles and further the nanoparticles were embedded in the chitosan matrix. Recently, a record high adsorption densities of fluoride were obtained by Affonso et al. [28] using carbon nanotube functionalized with chitosan sponge from effluent of fertilizer production industry. These adsorbents have shown a record high maximum adsorption density of 975.4 mg/g based on the kinetic and freundlich isotherm analysis results. The high adsorption caapacity was attributed to the chitosan functional groups and the high interaction area promoted by sponge form and the carbon nanotube. Additionaly, the material was reused for about

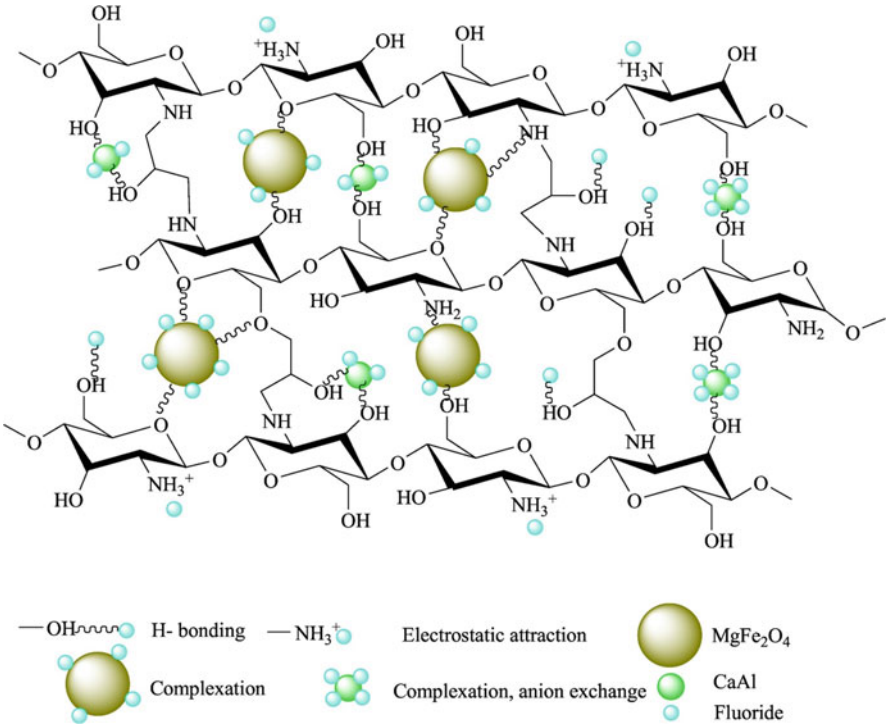

Fig. 3 A schematic illustration of fluoride adsorption mechanism onto the magnetic MgFe2O4–chitosan–CaAl composite. The figure was adapted with permission from Ref. [27]

5 times with the aid of 0.1 M NaOH solution as an eluent [28]. Mostly the developed materials for fluoride adsorption were regenerated with NaOH with the cycles range between 5 and 10 (Fig. 3). As a result, the metal-incorporated chitosan composites proved to be more efficient adsorbents than their chemically modified counterparts. The other chitosan-based materials reported for fluoride sorption are displayed in Table 1.

3.2 Newly Developed Chitosan-Derivatives for Arsenic Adsorption

Arsenic exists as oxides in soil, sediments, and water in many parts of the world and originates from both natural and anthropogenic processes. Arsenic existed in four different types of states as −3, +3, 0, and +5, in which +3 form, called arsenite (H_3ASO_3, As(III)) and arsenate ($HAsO_4^{2-}$, As(V)) occurs naturally in predominant forms. Arsenite is more toxic (50 times) than arsenate due to the less mobility in the

Table 1 Functionalized forms of chitosan for fluoride removal from water

S. No.	Adsorbent material	Fluoride adsorption capacity (mg/g)	Regeneration eluent	Regeneration cycles	Isotherm model	Kinetics	Ref.
1	Protonated chitosan beads	1.66	0.1 M HCl	–	Langmuir–Freundlich	Pseudo-second-order	[5]
2	Carboxylated chitosan beads	1.39	0.1 M HCl	–	Langmuir–Freundlich	Pseudo-second-order	[19]
3	La–chitosan beads	4.71	–	–	Freundlich	Pseudo-seond-order	[29]
4	Fe–chitosan beads	4.23	–	–	Freundlich	Pseudo-second-order	[30]
5	La–Zr mixed oxide–chitosan beads	6.41	0.1 M NaOH	06	Langmuir	Pseudo-second-order	[20]
6	Zr–chitosan dendrimer beads	17.47	–	–	Freundlich	Pseudo-second-order	[21]
7	La–rare earth metal oxide/chitosan	22.35	0.5 M NaOH	07	Freundlich	Pseudo-second-order	[31]
8	Chitosan–Fe(III) beads	45.45	5% NaCl	14	Freundlich	Pseudo-second-order	[32]
9	Fe_3O_4/chitosan/$Al(OH)_3$ beads	76.63	–	–	Langmuir	Pseudo-second-order	[22]
10	Magnetic nanohydroxyapatite–chitosan	4.77	0.1 M NaOH	04	Langmuir	Pseudo-second-order	[25]
11	Magnetic hydrotalcite–chitosan	5.03	0.1 M NaOH	05	Langmuir	Pseudo-second-order	[24]
12	La–silica gel/chitosan	4.90	–	–	Langmuir	Pseudo-second-order	[33]
13	Magnetic hydrocalumite–chitosan	6.80	–	–	Langmuir	Pseudo-second-order	[23]
14	Bentonite clay/chitosan	9.80	0.1 M NaOH	05	Freundlich		[34]

(continued)

Table 1 (continued)

S. No.	Adsorbent material	Fluoride adsorption capacity (mg/g)	Regeneration eluent	Regeneration cycles	Isotherm model	Kinetics	Ref.
						Pseudo-second-order	
15	Chitosan–polyaniline/zirconium	8.71	0.1 M NaOH	05	Freundlich	Pseudo-second-order	[35]
16	Zr^{4+}-chitosan/gelatin composite	12.13	0.05 M NaOH	05	Langmuir	Pseudo-second-order	[36]
17	La–synthetic resin@chitosan	17.50	0.01 M NaOH	06	Freundlich	Pseudo-second-order	[37]
18	Al–La oxyhydroxides–chitosan	49.54	0.1 M NaOH	05	Freundlich	Pseudo-second-order	[38]
19	Fe–Al–Mn oxyhydroxide–chitosan	55 ± 0.5	–	–	Freundlich	Pseudo-second-order	[39]
20	Silica/chitosan	58.80	1 M NaOH	10	Langmuir	Pseudo-second-order	[40]
21	*Aloe vera* supported Nano Zr chitosan	96.58	0.1 M NaOH	10	Langmuir	Pseudo-second-order	[26]
22	Al–chitosan–hydroxypropyl methyl cellulose	125.1	Methanol	04	Langmuir	Pseudo-second-order	[16]
23	Ce–chitosan composite	153.3	2 M NaOH	03	Langmuir	Pseudo-second-order	[41]
24	$MgFe_2O_4$–chitosan–CaAl composite	263.15	0.1 M NaOH	05	Langmuir	Pseudo-second-order	[27]
25	Carbon nanotube/chitosan sponge	975.4	0.1 M NaOH	05	Freundlich	Pseudo-first-order	[28]

water. The chemistry of arsenic and its toxicity was well explained in the previous review article by Hao et al. [42]. The maximum permissible limit of arsenic in groundwater is 10 μg/L, described by WHO. The removal mechanisms of the toxic inorganic pollutants on chitosan adsorbents are controversial with theories including chemisorption and complexation. It is reported that the uptake of arsenic on amine moiety in chitosan through coordination with unprotonated amine groups and the protonated form of amine groups would tend to adsorb arsenic via an electrostatic interaction at different pH conditions [43]. Chitosan in its unmodified form had lower adsorption capacities than the metal incorporated chitosan moiety due to its lack of active sites for adsorption as listed out in Table 2. Kwok et al. have reported the mechanisms of arsenic removal on chitosan and nano chitosan in detail at different pH conditions and the adsorption–desorption behavior for arsenic at various eluents [43]. Further, the unmodified chitosan was used for the removal of arsenite and arsenate ions from water at pH 5 and found that the maximum adsorption densities were 1.83 and 1.94 mg/g, respectively. The chitosan was regenerated with 0.5–0.1 M H_2SO_4 for about 15 cycles, which is far preferable to the mere three cycles afforded by coconut husk carbon in arsenic recovery [44]. To improve its adsorption capacities, the chitosan was modified with chelating resins, and the densities were improved drastically to 62.42 mg/g with pseudo-second-order kinetic models for arsenate removal, and the materials were regenerated using 0.2 M NaOH and utilized materials for further five times with maximum adsorption densities. Metal impregnation is proposed to increase arsenic uptake capacity and favor chitosan selectivity over other metal ions and co-ions. The metals such as lanthanum, zirconium, iron, molybdenum, aluminum, and manganese are known to form complexes with arsenic ions and for their strong affinity for chitosan [45]. Salih et al. [54] have recently reported chitosan/diatomaceous composite beads as an effective adsorbent for the removal of toxic ions from water. The binary maximal adsorption densities of As(III) and As(V), as determined from the Langmuir isotherms were 87.81 and 44.07 at a pH 6, respectively. The presence of phosphate as co-existing anions has an obvious impact on the adsorption of arsenic species due to isomeric forms of phosphate and arsenic. In the desorption experiments, the chitosan/diatomaceous composite beads were examined using 0.2 M NaOH as an eluent and the adsorbents were reused for five consecutive cycles and the losses in bead mass after 5 regeneration cycles were measured as approximately 8% and 11% for As(III) and As(V) loaded beads, respectively. Likewise, a microsphere using iron-loaded chitosan was prepared by Lin et al. (2019) for the removal of toxic As(III) ions from water [46]. The As(III) ions tend to bind with the Fe-rich surface of chitosan through electrostatic interaction at acidic pH conditions and over pH 8, the result was contrary, the excess of –OH ions would compete with arsenite ions and the densities were decreased. The mechanisms of interaction was confirmed by FTIR and XPS studies, in which the FTIR studies confirmed that the presence of NH stretching vibration mode was shifted to 1,371 cm^{-1} from 1,387 cm^{-1} and a new peak was observed at 819 cm^{-1} corresponded to Fe-O-As after adsorption. The magnetic chitosan bead and composie were made by two different groups and found that the adsorption densities were higher at compoiste form rather the beads, due to higher

Table 2 Adsorbents used for the removal of arsenite and arsenate from waters

S. No.	Adsorbent material	Adsorption capacity (mg/g)		Regeneration eluent	Regeneration Cycles	Isotherm model	Kinetics	Ref.
		Arsenite	Arsenate					
1	Chitosan	1.83	1.94	0.5–0.1 M H_2SO_4	15	–	First-order	[44]
2	Chitosan-chelating resin	–	62.42	0.2 M NaOH	05	Langmuir	Pseudo-second-order	[49]
3	α-Fe2O3 impregnated chitosan beads	9.355	–	0.5 M HCl	10	Langmuir	Pseudo-second-order	[50]
4	Chitosan/PVA/Fe^0	142.9	200.0	0.01 M NaOH	05	Langmuir	–	[51]
5	Magnetic chitosan beads	35.7	35.3	0.1 M NaOH	05	Langmuir	Pseudo-second-order	[52]
6	Zr–chitosan composite	–	190.0	0.5 M NaOH	06	Langmuir	Pseudo-second-order	[53]
7	Fe–chitosan microsphere	95.97	–	1.0 M NaOH	03	Freundlich	Pseudo-first-order	[46]
8	Chitosan/diatomaceous	87.81	44.07	0.2 M NaOH	05	Langmuir	Pseudo-second-order	[54]
9	Fe–chitosan/ZnO@ alginate microsphere	–	63.69		05	Langmuir	Pseudo-second-order	[55]
10	Fe_3O_4–chitosan composite	73.69	79.49	0.1 M NaOH	05	Langmuir	Pseudo-second-order	[56]
11	Magnetic chitosan bead	–	65.5	Na_2EDTA	05	Langmuir	Pseudo-second-order	[57]
12	Chitosan-iron oxide films	–	33.87	–	–	Freundlich	Pseudo-first-order	[58]
13	Ionotropic chitosan microspheres	–	120.77	–	–	Langmuir	Pseudo-second-order	[59]
14	Chitosan–Fe–Al LDH@rGO	–	167.79	1.0 M NaOH	04	Langmuir	Pseudo-second-order	[47]

15	Goethite/GO/chitosan composite	289.42	–	0.5 M NaOH	05	Freundlich and Sips	Pseudo-second-order	[48]
16	Molybdate loaded chitosan beads	–	75.0	0.1 M phosphoric acid	03	Langmuir	Pseudo-second-order	[60]
17	Lanthanum–chitosan nanofiber	–	83.6	8% in mass NaOH	02	Langmuir	Pseudo-seond-order	[61]
18	Chitosan–diatomite	–	11.95	0.1 M NaOH	06	Langmuir	Pseudo-second-order	[62]
19	Fe–Mn@ chitosan/alginate	24.06	–	NaOH	04	Freundlich	Pseudo-second-order	[63]
20	Fe–chitosan	–	14.95	1% NaOH	04	Langmuir	Pseudo-second-order	[64]
21	Fe–chitosan nanosheets	108.6	–	0.5 M NaOH	04	Langmuir	Pseudo-second-order	[65]
22	Chitosan–$Zr_xAl_{1-x}OOH$ composite	69.90	–	0.1 M NaOH	04	Langmuir	Pseudo-second-order	[66]
23	Chitosan–iron sand filters	26.0	56.0	–	–	Langmuir	Pseudo-first-order	
24	Chitosan–montmorillonite composites	48.7	–	0.1 M H_2PO_4	03	Freundlich	Pseudo-second-order	
25	Chitosan electrospun nanofibers	–	11.2	0.003 M NaOH	10	Freundlich	Pseudo-second-order	

surface area and more active sites existed in the composite form. The respective adsorption densities of arsenite and arsenate were 73.69 and 79.49 mg/g on magnetic chitosan composite which is higher adsorption densities than the magnetic chitosan beads (65.5 mg/g). Lanthanum and molybdenum ions are considered as hard acids which tend to bind with arsenate, a hard base, by well understood HSAB principle.

Bimellaic assembled chitosan materials were utilized to maximize the adsorption efficiencies of both As(III) and As(V) from waters. Recently, Priya et al. [47] have developed a material consisting of Fe–Al layered double hydroxides on chitosan with graphene oxide materials. The nanocomposite was successfully prepared by hydrothermal and ex situ polymerization process with an enhanced surface area which provides more number of active sites. The LDH moieties were attached with GO groups through electrostatic interaction and the negatively charged arsenic species interacted with this nanocomposite through coordination, ligand exchange, and electrostatic interaction mechanisms. The adsorption capacities of the materials were found to be 167.80 mg/g, which is higher than the pristine forms of the materials [47]. Another group from China has recently developed goethite/graphene oxide/chitosan nanocomposite materials for the removal of As(III) from water. They found that the multifunctional groups such as NHCO, C-O, O-H, and Fe-O were responsible for adsorption of arsenite ions from water. The maximum adsorption density of 289.42 mg/g was calculated using the Langmuir isotherm model and the material was used five consecutive times with the minimal loss of its efficiencies [48].

Chitosan-supported mixed metal oxyhydroxides ($Zr_xAl_{1-x}OOH$) were prepared by Prabhu and Sasaki for the simultaneous removal of toxic fluoride and arsenite ions from single and binary solutions. A higher amount of arsenite was adsorbed in binary solutions than the single solutions, through electrostatic interaction and ligand exchange mechanisms at different pH conditions. Further, the material was supplied to remove the three-component system of natural co-existing anions and found no significant decrement in the adsorption densities. The material could be reused about four times using 0.1 M NaOH as an eluent. Again bimetallic rich chitosan–alginate biopolymeric nanocomposite was developed by Zeng et al. (2020) that materials showed a surface area of 128.16 m^2/g and showed maximum efficiencies of 24.06 mg/g [63]. The mechanism of attraction of arsenic by complexation and the desorption by NaOH could be used for four adsorption and desorption cycles (Fig. 4). The other materials used for the removal of toxic arsenite and arsenate using chitosan-based materials are listed in Table 2.

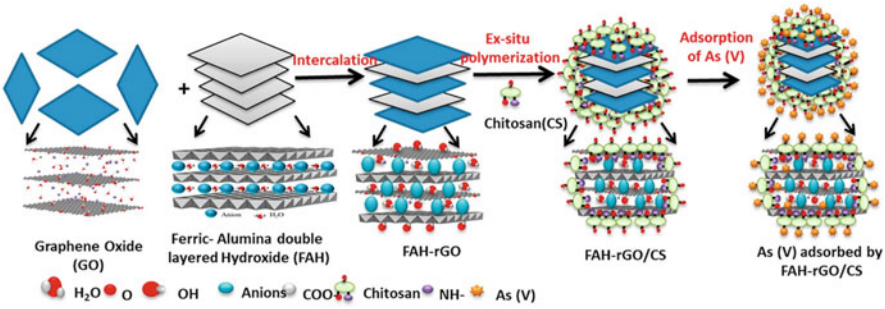

Fig. 4 A schematic view of mechanism of intercalation and ex situ polymerization of chitosan and its adsorption of arsenate from water. The figure was adapted with permission from Ref. [47]

3.3 Metal and Metal-Free Chitosan Materials for Phosphate and Nitrate Adsorption

The nutrients pollution in a water body such as nitrogen and phosphorus are serious environmental issues worldwide. The dissolved nutrients species ammonium, nitrate, and phosphate are particularly important pollutants, resulting in aquatic eutrophication, which is an abundance of aquatic plants, growth of algae, and depletion of dissolved oxygen [67]. The nitrate ion in drinking water is 40 mg/L set by WHO, when it exceeds causes public health hazards including infant methemoglobinemia called Blue baby syndrome and the other forms of carcinogenic nitrosamines and nitrosamides. Agricultural fertilizers, aquaculture, agri-food industries, municipal wastewaters, and detergent manufacturing industries are the main sources of nitrate and phosphate release in the waterbody. Also, thermal power plants discharge their wastes into water that contains large amounts of nitrate ions [68]. A lot of materials were tried to remove the maximum amount of nitrate and phosphate ions through adsorption method. A few review articles explained well the materials used for the removal of those ions from water. This review is currently updating the chitosan-based materials used for the removal of phosphate and nitrate ions from water. Kumar et al. [69] investigated the NO_3^- and PO_4^{3-} adsorption properties of chitosan encapsulated lanthanum oxide admixed *Aegle marmelos* (La_2O_3AM@CS) composite beads. The hydrothermal supported La_2O_3AM@CS composite beads possess the NO_3^- and PO_4^{3-} adsorption capacity of 27.84 and 34.91 mg/g, respectively [69]. The pH influences the NO_3^- and PO_4^{3-} adsorption capacity of La_2O_3AM@CS composite beads, and SO_4^{2-} ion mainly competes with NO_3^- and PO_4^{3-} adsorption due to charge and size effects. They also reported that the chitosan-supported metal/bentonite composite beads viz. Zr@CSBent, La@CSBent, and Ce@CSBent for NO_3^- adsorption from water. Among the studied materials, Zr@CSBent composite beads showed a higher adsorption capacity of 23.89 mg/g [70]. Banu et al. reported the Zr^{4+} ions implanted chitosan–soybean husk activated bio-char composite beads (Zr-CS-SAC) for NO_3^- and PO_4^{3-} adsorption and possesses the maximum adsorption capacity of 90.09 and 131.29 mg/g at 30°C

[71]. Jiang et al. [72] synthesized the Fe_3O_4/ZrO_2/chitosan composite using a low-cost method at the mild condition. The developed Fe_3O_4/ZrO_2/chitosan composite has an adsorption capacity of 89.3 mg/g and 26.5 mg P/g for NO_3^- and PO_4^{3-}, respectively [72]. Karthikeyan et al. developed the lanthanum-loaded chitosan membrane (La@CS) by casting technique, and the La@CS membrane showed the PO_4^{3-} and NO_3^- adsorption capacity of 76.6 and 62.6 mg/g, respectively [73]. The same authors also developed Fe^{3+} cross-linked chitosan/alginate (Fe-CS-Alg) hybrid beads for PO_4^{3-} and NO_3^- from an aqueous solution and having the maximum adsorption capacity of 84.74 and 69.10 mg/g, respectively [74]. Further, they moved to prepare the cerium loaded chitosan-β-cyclodextrin (Ce-CS-β-CD) microspheres and applied for the removal of PO_4^{3-} and NO_3^-, which exhibited the excellent adsorption capacity of 88.54 and 72.12 mg/g for PO_4^{3-} and NO_3^-, respectively [75]. They also prepared zirconium fixed chitosan–starch membrane (Zr-CS-ST) for PO_4^{3-} and NO_3^- removal, which possesses the adsorption capacities of 86.28 and 70.88 mg/g, respectively [76]. An environmentally friendly Al^{3+} incorporated chitosan and gelatin (Al@CS-Ge) microspheres were fabricated by Karthikeyan et al. [77] and utilized as an effective adsorbent for the removal of PO_4^{3-} and NO_3^-. The main interaction between the two biopolymeric materials entrapped with metal ions would tend to adsorb more nitrate and phosphate ions due to the availability of the active sites for adsorption. The electrostatic interaction, complexation, and ion-exchange mechanisms were the mainly governed factors for adsorption of nitrate and phosphate ions from waters [77]. Kim et al. [78] have applied iron oxide nanoparticle–chitosan (ION-chitosan) composites for PO_4^{3-} removal from the natural water. The ION-chitosan composite is not pH sensitive between the pH values of 5.0 to 9.0 and has the PO_4^{3-} removal capacity of 0.059 mg P/g [78]. Kumar and Viswanathan prepared multivalent metal ions like Zr^{4+}, Fe^{3+}, and Ca^{2+} cross-linked chitosan bentonite composites Zr@CSBent, Fe@CSBent, and Ca@CSBent, respectively. The developed composites possess the efficient PO_4^{3-} adsorption capacities of 40.86, 22.15, and 13.44 mg/g than the specific CSBent composite [79]. The Zr^{4+}-imprinted chitosan (CCP-Zr) was prepared by forming-membrane and cross-linking method by Liu et al. [80]. The CCP-Zr have the maximum monolayer adsorption capacity of 71.68 mg/g at pH 3 and 30°C [80]. The chitosan beads modified with zirconium (ZCB) were developed by Liu and Zhang for PO_4^{3-} removal from an aqueous medium, which possesses the maximum monolayer adsorption capacity of 60.6 mg/g [81]. Mahaninia and Wilson cross-linked the chitosan beads with glutaraldehyde and epichlorohydrin and used for the removal of phosphate dianion (HPO_4^{2-}) from an aqueous solution at pH 8.5 and 22°C and possesses adsorption capacity of 22.4–52.1 mg/g [82]. Nthumbi et al. [83] prepared chitosan/polyacrylamide nanofibers and used them for the removal of PO_4^{3-}. The nanofibers had an adsorption capacity of 392 mg/g and removal efficiencies of 97.4% in synthetic and field water samples [83]. Sowmya and Meenakshi have developed various types of chitosan-based adsorbent materials

including quaternized chitosan beads (QCB) [84], hydrotalcite/chitosan (HT/CS) composite, quaternized chitosan–melamine–glutaraldehyde resin (QCMGR) [85], protonated a carboxylated cross-linked chitosan bead (PCB), (CCB) [86], La(III)-loaded silica-chitosan composite (LSCC) [87], Zr(IV) cross-linked chitosan beads (ZrCB) [88], zinc-loaded cross-linked chitosan beads (ZnCB) and zinc-loaded carboxylated cross-linked chitosan beads (ZnCCB) for the removal of both PO_4^{3-} and NO_3^- from water [89]. The reported the adsorbent materials possess very good adsorption capacity for the removal of PO_4^{3-} and NO_3^- from synthetic and real-water samples. The LSCC material exhibited the highest affinity for phosphate removal and nitrate removal from water out of all the developed materials. Based on this scenerio, Banu et al. (2018) [90] synthesized the La^{3+}-incorporated chitosan–montmorillonite composite (La-CS-MMT) for the effective removal of PO_4^{3-} from aqueous solution, which successfully removed 92% of the PO_4^{3-} within 30 min. By electrostatic attraction, the phosphate ions were adsorbed on La-CS-MMT to form an outer-sphere complex, as confirmed by the D-R isotherm, and the adsorbed material was then desorbed with 0.1 M NaOH. In regard to MMT and CS, hydrogen bonding is a common mechanism for their interaction with phosphate and nitrate. Zavareh et al. [91] reported the magnetic Cu–chitosan/Fe_3O_4 nanocomposite for selective and effective removal of PO_4^{3-}. The synthesized magnetic adsorbent has the maximum adsorption capacity of 88 mg P_2O_5/g, which is higher than the neat chitosan and chitosan/Fe_3O_4 according to the Langmuir isotherm [91]. Kumar and Viswanathan have prepared the tetra amine copper(II) salt grafted chitosan (TAC@CS) composite beads for PO_4^{3-} adsorption, and the TAC@CS composite beads posses the enhanced adsorption capacity of 41.42 ± 0.071 mg/g compared to other prepared adsorbent materials [92]. Kumar and Viswanathan have developed magnetic adsorbents like amine-grafted magnetic gelatin (AGMGel) composite, magnetic-chitosan-assisted hydrotalcite (MCSHT), and magnetic-alginate-assisted hydrotalcite (MAlgHT) composite beads for the adsorption of NO_3^- and PO_4^{3-} from water. The researchers also synthesized zirconium oxyhydroxide (Zr@CSKN) composite by hydrothermal method for enhanced nitrate and phosphate adsorption [93–95].

Zhao and Feng reported the modified chitosan beads for PO_4^{3-} and NO_3^- adsorption with different pH, the initial concentration, contact time, and adsorbent dosage. The results displayed that modified chitosan beads are an excellent adsorbent for the removal of NO_3^- and PO_4^{3-} with an adsorption capacity of 32.15 and 33.90 mg/g, respectively [96]. Golie and Upadhyayula have synthesized chitosan/bentonite, chitosan/titanium oxide, and chitosan/alumina (ChBT, ChTi, and ChAl, respectively) for NO_3^- adsorption from aqueous solution by batch biosorption experiments, and the composites possess the adsorption capacity of 35.68 and 43.62 and 45.38 mg/g, respectively [97]. Banu and Meenakshi reported the one-pot synthesis of chitosan grafted quaternized resin for the removal of NO_3^- and PO_4^{3-} from water. The removal efficiency of NO_3^- and PO_4^{3-} on chitosan quaternized resin was 78% and 90%, respectively, with 0.1 g of adsorbent and the initial concentration as 100 mg/L [98]. A granular chitosan–Fe^{3+} complex with high chemical stability and good environmental adaptation was developed by Hu et al.

and used for the adsorption of NO_3^- and possesses the adsorption capacity of 8.35 mg NO_3–N/g based on Langmuir–Freundlich model [99]. Zhang et al. (2018) and Zhao et al. (2020) have developed the composites like Fe(III)-doped chitosan (CTS-Fe) [100] and cross-linked Fe(III)-chitosan (CTS-Fe-CL) [101] for the removal of phosphorus. The maximum phosphate adsorption capacity for CTS-Fe and CTS-Fe-CL was 15.7 and 10.2 mg P/g at 30°C, respectively. Lanthanum-chitosan hydrogel coated with polydopamine (La-CS@PDA) was synthesized with abundant amino groups, and the composite followed the Langmuir isotherm model with an adsorption capacity of 195.3 mg/g. Chitosan/Zeolite Y/Nano ZrO_2 nanocomposite adsorbent was synthesized by Teimouri et al. [102] and applied for the removal of NO_3^- from the aqueous solution. The NO_3^- adsorption process was described by Langmuir isotherm model with the adsorption capacity of 23.58 mg/g [102].

The nanochitosan-graphene oxide composite (NCS@GO) was prepared by Salehi and Hosseinifard, which is examined for adsorption of PO_4^{3-} and NO_3^- from aqueous solutions. The zirconium loading was optimized in NCT@GO composite to make it selective for the adsorbate anions, and NCS@GO/H-Zr established a very good PO_4^{3-} and NO_3^- uptake of 172.41 mg P/g and 138.88 mg N/g, respectively. The removal capacity of P and N anions was also assessed in bi-component systems and the Freundlich isotherm fitted well, suggesting the multilayer formation. Real samples analysis indicated that the prepared material works well for the removal of P and N anions from contaminated waters [103]. Yazdani et al. [104] reported the zinc (II)$^-$chitosan complexes as a bio-sorbent for PO_4^{3-} removal from aqueous solutions. The zinc(II) ions into chitosan expand its performance towards PO_4^{3-} removal from 1.45 to 6.55 mg/g. Using functionalized nanochitosan/clinoptilolite (Nano-CS/Clino) composites, nitrate ions were removed from water. The Nano-CS/Clino@PEHA composite was found to have a higher nitrate adsorption capacity of 277.77 mg/g than Nano-CS/Clino@H (227.27 mg/g) and Nano-CS/Clino (185.18) [104, 105]. More recently, Fu et al. [106] developed the polyethylenimine-grafted chitosan core-shell (Fe_3O_4/CS/PEI) magnetic nanoparticles and utilized them for adsorbing PO_4^{3-} in water. The adsorption isotherms of PO_4^{3-} on Fe_3O_4/CS/PEI particles were well fit by the Langmuir equation, and its maximum adsorption was 48.2 mg/g at an equilibrium pH of 3.0 and 25°C [106]. Non-cross-linked lanthanum-chitosan (La-CTS-0X) and cross-linked lanthanum-chitosan (La-CTS-1X/2X) composites were prepared as new complex biosorbents for effective PO_4^{3-} removal from wastewater by Liu et al. [107]. The maximum PO_4^{3-} adsorption capacity for La-CTS-0X/1X/2X was 47.28, 57.84, and 31.01 mg/g at pH 6, respectively [107].

The materials used for the removal of phosphate and nitrate were mostly regenerated with NaCl and NaOH at different concentrations depending upon the metal concentrations of composites/hybrids and the maximum re-useable cycles are between 5 and 10. The metal-loaded chitosan-based composites showed extraordinary performance than the pristine forms due to the hydroxide ions and the metal affinity towards the nitrate and phosphate ions from water. The quarternized forms of

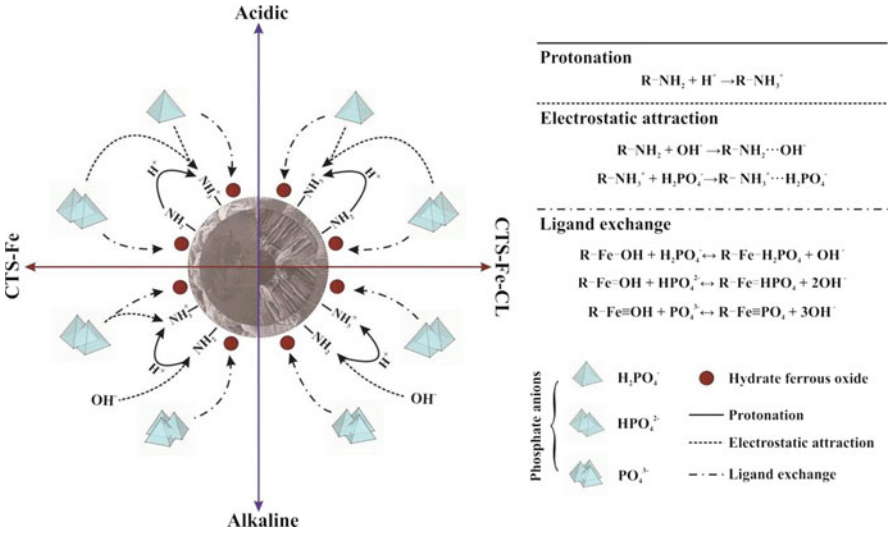

Fig. 5 Mechanism of phosphate adsorption using Fe(III)-doped chitosan and cross-linked Fe(III)-doped chitosan. The figure was adapted with permission from Ref. [100]

chitosan materials attract phosphate and nitrate ions through a ligand-exchange mechanism which can be confirmed by an ion-chromatogram, where the Cl^- ions concentrations can measure periodically and the phosphate and nitrate ions concentration can be measured simultaneously (Fig. 5 and Table 3).

4 Summary and Outlooks

Recently, studies have proposed the synthesis of novel, new, and eco-friendly nanomaterials that use adsorbent technologies to remove toxic ions from waters and wastewaters. Overall, the reported materials with the background of chitosan showed ultra-high adsorption densities toward the removal of fluoride, arsenic, phosphate, and nitrate ions from water under batch experiments. Mostly, the materials used for this review were prepared by conventional method and a few of them were synthesized by in situ and ex situ synthetic methods which significantly improved the stability of the materials. Besides, the surface area of the materials was also enhanced due to the synergistic interaction of the active material with the chitosan background. At different pH levels, the mechanisms of interactions with toxic ions include electrostatic interactions, inner-and outer-sphere complexation, and ligand exchange. Despite the wide applications were adopted for new generation material for the removal of fluoride, arsenic, phosphate, and nitrate from water, some drawbacks still exist.

Table 3 Removal of phosphate and nitrate ions from water using chitosan-based sorbents

S. No.	Adsorbent material	Adsorption capacity		Regeneration eluent	Regeneration cycles	Isotherm	Kinetics	Ref.
		Phosphate (mg/g)	Nitrate (mg/g)					
1	Chitosan Encapsulated lanthanum oxide	34.91	27.84	NaOH	6	Freundlich	–	[69]
2	Zr^{4+}, La^{3+}, and Ce^{3+} cross-linked chitosan assisted bentonite composite	–	23.89, 19.54 and 16.63	0.01 M NaOH	6	Freundlich	Pseudo-first-order	[70]
3	Zr^{4+} ions embedded chitosan–soybean husk activated bio-char composite beads	131.29	90.09	0.1 M NaCl	5	Freundlich	Pseudo-first-order	[71]
4	Fe_3O_4/ZrO_2/chitosan composite	26.5	89.3	–	–	Langmuir	Pseudo-first-order	[72]
5	Lanthanum incorporated chitosan membrane	76.6	62.6	0.1 M NaOH	5	Freundlich	Pseudo-first-order	[73]
6	Fe^{3+} loaded chitosan and alginate biopolymeric hybrid beads	84.74	69.10	0.1 M NaOH	5	Freundlich	Pseudo-first-order	[74]
7	Cerium incorporated chitosan-β-cyclodextrin	88.54	72.12	0.1 M NaOH	5	Freundlich	Pseudo-first-order	[75]
8	Zirconium entrenched chitosan-starch membrane	86.28	70.88	0.1 M NaOH	5	Freundlich	Pseudo-first-order	[76]
9	Al^{3+} incorporated chitosan and gelatin microspheres	90.14	74.15	0.1 M NaOH	5	Freundlich	Pseudo-first-order	[77]
10	Iron oxide nanoparticle-loaded chitosan composites	0.059	–	0.001 M NaOH	6	Freundlich	Pseudo-second-order	[78]
11	Zr^{4+}, Fe^{3+}, and Ca^{2+} chitosan-supported bentonite composite	40.86, 22.15, and 13.44	–	–	–	Freundlich	Pseudo-first-order	[79]

12	Zirconium (IV) loaded cross-linked chitosan	71.68	–	–	–	Langmuir	Pseudo-first-order	[80]
13	Zirconium(IV) modifying chitosan biocomposite	61.7	–	0.5 M NaOH	5	Langmuir	Pseudo-first-order	[81]
14	Cross-linked Chitosan beads	52.1	–	0.05 M NaCl	4	Sips	–	[82]
15	Chitosan/polyacrylamide nanofibers	392.0	–	2.0 M HCl	–	Freundlich	Pseudo-second-order	[83]
16	Quaternized chitosan beads	59.0	67.5	0.025 M NaCl	10	Freundlich	Pseudo-second-order	[84]
17	Hydrotalcite/chitosan composite	57.87	–	–	–	Freundlich	Pseudo-second-order	
18	Chitosan–melamine–glutaraldehyde resin	112.5	97.5	0.025 M NaCl	10	Freundlich	Pseudo-second-order	[85]
19	Protonated cross-linked chitosan beads (PCB) and carboxylated cross-linked chitosan beads	58.5 & 48.8	113.1 & 90.6	0.1 M NaOH	5	Freundlich	Pseudo-second-order	[86]
20	Lanthanum-loaded chitosan-silica and lanthanum-loaded chitosan materials	155.4 84.2	–	0.25 M NaOH	5	Freundlich	Pseudo-second-order	[87]
21	Zr(IV) was loaded in the cross-linked chitosan beads	69.54	250.63	0.025 M NaCl	10	Freundlich	Pseudo-second-order	[88]
22	Zn(II) loaded chitosan beads and Zn(II) loaded carboxylated chitosan beads	59.00 and 67.50	27.56 and 31.45	0.025 M NaCl	5	Freundlich	Pseudo-second-order	[89]

(continued)

Table 3 (continued)

S. No.	Adsorbent material	Adsorption capacity		Regeneration eluent	Regeneration cycles	Isotherm	Kinetics	Ref.
		Phosphate (mg/g)	Nitrate (mg/g)					
23	Lanthanum (III) encapsulated chitosan-montmorillonite composite	128.5	–	0.1 M NaOH	5	Freundlich	Pseudo-second-order	[90]
24	Cu–chitosan/Fe_3O_4 Nanocomposite	88.0	–	$CuSO_4$	5	Langmuir and Freundlich	Pseudo-second-order	[91]
25	Tetra-amine copper(II) chitosan beads	41.42	–	0.25 M NaOH	4	Freundlich	Pseudo-second-order	[92]
26	Modified chitosan microspheres	33.90	32.15	0.1 M NaOH	4	Langmuir	Pseudo-second-order	[96]
27	Chitosan/bentonite, chitosan/titanium oxide, and chitosan/ alumina composite	–	45.38	NaCl	7	Freundlich	Pseudo-second-order	[97]
28	Chitosan quaternized resin	30.4	23.7	0.1 M NaCl	7	Freundlich	Pseudo-second-order	[98]
29	Amine-grafted chitosan hybrid beads	42.95	38.40	0.1 M NaOH	8	Freundlich	Pseudo-second-order	[93]
30	Zirconium oxyhydroxide capped chitosan/kaolin framework	38.34	31.83	0.025 M NaOH	6	Freundlich	Pseudo-second-order	[94]
31	Magnetic-chitosan-assisted hydrotalcite beads	35.98	–	0.1 M NaOH	6	Freundlich	Pseudo-second-order	[95]

32	Chitosan–Fe^{3+} complex	–	8.35	0.1 M NaCl	–	Langmuir–Freundlich	Pseudo-second-order	[99]
33	Fe(III)-doped chitosan and cross-linked Fe(III)-chitosan composites	–	15.7 and 10.2	0.5 M NaOH	5	Freundlich	Pseudo-second-order	[100]
34	Chitosan/zeolite Y/Nano zirconium oxide nanocomposites	–	23.58	–	–	Langmuir	Pseudo-second-order	[102]
35	Zirconium functionalized nanochitosan-graphene oxide composite	172.41	138.88	0.5 M Na_2CO_3	10	Freundlich	Pseudo-second-order	[103]
36	Zinc(II)–chitosan complexes	7.37	–	–	–	Sips	Pseudo-second-order	[104]
37	Nanochitosan/clinoptilolite pentaethylene–hexamine composite	–	277.77	1 M NaOH	3	Freundlich	Pseudo-second-order	[105]
38	Polyethylenimine-grafted Fe_3O_4@ chitosan core-shell magnetic nanoparticles	50.8	–	0.05 M NaOH	5	Langmuir	Elovich model	[106]
39	Polydopamine on lanthanum–chitosan hydrogel	195.3	–	0.3 NaCl and 0.1 M NaOH mixture	5	Freundlich	Pseudo-second-order	[101]
40	Cross-linked lanthanum–chitosan	57.84	–	0.1 M NaOH	3	Langmuir	Pseudo-second-order	[107]

1. The chitosan-based materials can be easily dissolved in 1–2% acetic acid and they can be modified in various forms by adding the metal ions in it. But the adsorption takes place at acidic conditions and most of the metal ions may undergo dissolution which leads to taking the secondary treatment to recover the metal nanoparticles from water. Also, the excess loading of metal ions might block the active sites and the metal hydroxide is the only one responsible for the removal of toxic ions from water. The water quality parameters before and after the treatment were analyzed using standard methods for the determination of residual toxicity.
2. The regeneration of the chitosan-based materials is mandatory to reduce the cost factor of the process and the feasibility of the studies. Most of the studies were focused on batch adsorption and only a few were reported with column experiments. Regeneration studies of the materials were rarely reported in this study. In order to develop the model for domestic and community models, the column study can be applied to promote the next step of development. Only a few of the reported studies were focused on the recovery of adsorbed material and desorbed metals. Newly developed desorption techniques for metallic ions using recyclable chemicals reduce the consumption of concentrated acids and alkalis.
3. Real-water analysis is the fundamental factor in deciding the applicability of the adsorbent. Naturally, the groundwater contains several anions, cations that interfere with the adsorption of toxic ions from water. It is important to choose adsorbents that are able to withstand even higher concentrations of interference without affecting their adsorption capacities. After the ions were adsorbed, the adsorbents were disposed in landfills or ponds, easily contaminating water, soil, and the air is referred to as secondary pollution. The arsenic and fluoride-sorbed materials were carefully stabilized before disposed of. There are few reports about the stabilization of Cs^{+}, Sr^{2+} using fly-ash-based zeolite materials, which helps to avoid releasing them into the environment again.
4. Adsorbents cost factors are the important parameter for practical feasibility. This will be done by comparing the cost of the method developed in the laboratory and that of other commercially available models.

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Synthesis-Structure Relationship of Chitosan Based Hydrogels

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Contents

Abstract Chitosan is a biopolymer which is non-toxic, biocompatible and biodegradable and it is harvested from chitin by deacetylation process in the presence of a strong base. When it exists in the solution form, mucoadhesive property is observed due to the cationic nature of the polymer. The favourable characteristics and the flexibility in development process of chitosan, enables it to find a wide range of application in the biomedical field by existing in various forms such as hydrogels, scaffolds, membranes, sponges, fibres, nanoparticles, microparticles, beads, etc. Over the past decade, chitosan based hydrogels have gained much attention owing

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to the similarity with that of the native extra-cellular matrix. However, modifying these hydrogels using different methods is also of utmost importance, so as to tailor their structural property which satisfies the needs of various biomedical applications. This review contributes in giving a brief insight about the various methods that are employed in preparation of these hydrogels and the associated structural changes as well as the use of these chitosan hydrogel in drug delivery, haemostasis, tissue engineering and wound healing area.

Keywords Chitosan · Chitosan derivatives · Chitosan hydrogel · Cross-linking · Drug delivery · Haemostasis · Porous hydrogel · Tissue engineering · Wound healing

1 Introduction

Chitosan, a cationic polysaccharide, usually exists in the nature as chitin and is obtained by the process of alkaline deacetylation and consists of glycoside linkages that link D-glucosamine and N-acetyl-D-glucosamine units. Molecular weight and degree of acetylation are main parameters which have influence on the acid/base characteristics, sorption and solubility property [1]. So, chitosan with over 60% of deacetylation degree (DD, the free content of amino acids in chitosan chains) are the ones that are dissolvable in dilute acid solutions. Properties such as biological and physicochemical are dependent on the presence of functional groups such as hydroxyl and amine groups in its backbone, wherein if other suitable functional groups are introduced it provides an advantage in improving the properties of chitosan [2]. Interesting properties like mucoadhesivity, biocompatibility and biodegradability are displayed by chitosan and the degraded products from chitosan are also non-immunogenic, non-carcinogenic and non-toxic [3]. The additional advantages of chitosan compared to other polymers are that it can be prepared into different types, dosage forms, microspheres/particles [4], nanoparticles [5, 6], beads [7], fibres [8], hydrogels [9], bandages [10], sponges [11, 12] matrix or scaffolds [13]. These different forms of chitosan have potential applications in tissue engineering and regenerative medicine area. Among the different forms, chitosan hydrogels have garnered attention and accelerated research in the biomedical area. The chitosan hydrogel has shear thinning, swelling and biodegradable property. These properties can be altered by associating the hydrogels with physical or chemical modifications [14].

Chitosan based hydrogels are regarded to be smart or intelligent, in a sense that they perceive the stimuli and respond by exhibiting changes in their behaviour either physically or chemically, i.e., dramatic changes in their network structure, mechanical strength, permeability or swelling behaviours in response to internal and external stimuli of the body [15]. The above-mentioned properties can be easily obtained using appropriate preparation methods. The preparation methods employed in the processing of chitosan hydrogel bring out important changes in physicochemical

properties such as swelling, degradation, porosity, solubility and drug release behaviour. It is very important to know that the different type of methods have been used for the preparation of chitosan and porous chitosan hydrogels by the researchers. So in this review, we overview the various preparation methods of different types of chitosan based hydrogels reported so far and its applications in the field of drug delivery, haemostasis, tissue engineering and wound healing.

2 Preparation Methods of Chitosan Hydrogels

2.1 Neutralization

This method enables obtaining chitosan hydrogel without incorporating molecules such as polyions or complexation agents [16]. When exposed to alkali solvents like NaOH, urea, gaseous ammonia, $NaHCO_3$, the protonated amine groups and charge density in the chitosan solution will induce neutralization [17–23]. The preparation method of chitosan hydrogel by neutralization method is as follows. The chitosan solution is prepared by dissolving chitosan powder in acetic acid. The obtained chitosan solution is neutralized using 1% NaOH solution. The increase in pH results in the clustering of dissolute chitosan molecules to form hydrogel due to the hydrophobic interactions and formation of hydrogen bonding [24]. The kinetics of the formation of chitosan hydrogel was studied and reported [10]. This study indicates that use of chitosan in lower concentration and NaOH in higher concentration results in faster gelation rate. This is attributed to the transportation of NaOH molecules, which diffuse through the chitosan hydrogel. The hydrogels prepared from chitosan solution and neutralized using NaOH solution were stable for longer periods at the room temperature [25]. Physically cross-linked chitosan hydrogel is obtained at varying chitosan concentrations of 5% and 10% w/w using coagulation bath having 7 M NaOH [26]. Another chitosan hydrogel, which is physically cross-linked using aq. NaOH and gaseous NH_3 at different concentrations of chitosan ranging from 0.5 to 10% was reported [18]. It was also found that hydrogels obtained with chitosan concentration of 1–5% (w/w) were homogeneous when compared to the concentration of 10% (w/w). Hydrogels having a concentration of 0.5% (w/w) were found to be fragile and were also breaking into fragment during handling process and washing. Two layered structure was observed in physically cross-linked hydrogels using gaseous NH_3 owing to the fast neutralization of the surface on chitosan solution (upon the condensation of gaseous NH_3), whereas single-layered structured hydrogels was cross-linked by NaOH (Fig. 1).

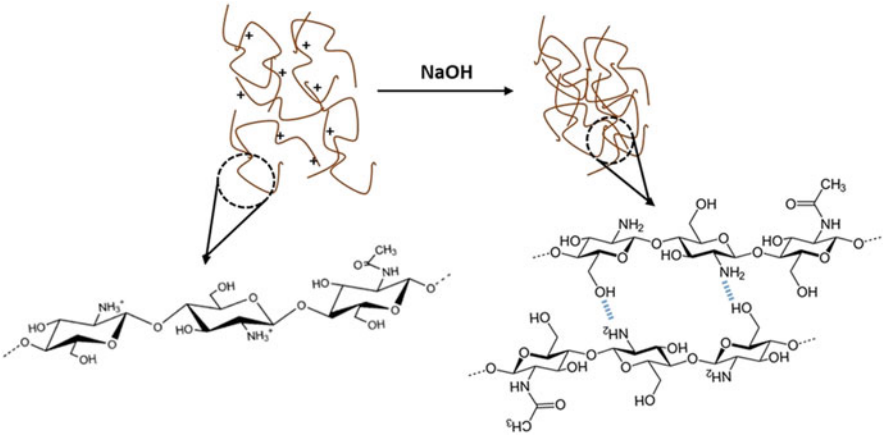

Fig. 1 The reaction of chitosan with NaOH during neutralization process

2.2 Thermo-Sensitive

When the thermo-sensitive chitosan solution is injected inside the human body, owing to phase transition and solvation it transforms into hydrogel. These thermo-sensitive hydrogels exist in an almost liquid form in a given room temperature and change to gel state when the temperature is increased [27, 28]. The addition of polyol salts can significantly improve the solubility of chitosan at neutral pH and it also promotes the formation of shield of water around the polymer [29]. A novel thermo-sensitive hydrogel, which when exposed to body temperature transforms from sol to gel, containing chitosan/GP(glycerophosphate), was reported by Chenite and group [30]. The physical hydrogel is formed at body temperature, which otherwise remains in liquid state at room temperature [31]. Here, the GP has two main roles: (a) To increase the pH value of chitosan to physiological range, i.e., 7–7.4 and to prevent the immediate gelation or precipitation of chitosan and maintains its solubility at low temperature, (b) when an increase in temperature is imposed, it initiates the controlled gelation of the chitosan [32]. There are three main interactions that take place in this chitosan/GP system: (a) electrostatic attraction between phosphate group of GP and amine group of chitosan, (b) hydrogen bonding between chitosan chain, (c) chitosan–chitosan hydrophobic interactions [33]. Upon heating of this chitosan/GP solution, the sheath of hydrated layer present over the chitosan is removed by the glycerol moiety, which induces the gelation process and letting the chitosan chains to interact among themselves through strong hydrophobic interactions [34–36]. It was studied and reported that the rheological, physicochemical and gelation properties were impacted by the chitosan concentration, degree of deacetylation and Mw as well as GP concentration [37]. The optimal deacetylation degree of chitosan that is used in the preparation of these thermo-sensitive hydrogels is 75.4% as chitosans having a different deacetylation degree showed slow gelation or remain unchanged at body temperature [38]. Owing to the transition state of gels, they are classified into

two parts: (a) thermo reversible gels: Where the gel returns back to liquid state and (b) thermo-irreversible gels: where in the gel always exists in the gel state. To an extent the chitosan/GP is a thermo-reversible hydrogel and that depends on the amount of chitosan and GP added in the solution. Under temperature stimulation hydrogen bonds are not stimulated whereas the hydrophobic interactions are reversible and it is irreversible if glycerol phosphate is present in high amounts. It was found by Ganji and group that a thermo-reversible hydrogel becomes thermo-irreversible in a 2% w/v chitosan solution having a GP concentration of 0.40 M, wherein the medium influences the gel transition temperature [39]. Another work by Suwantong reported that increasing the pH caused a reduction in the gelling speed, wherein the chitosan solution was fabricated using different concentrations and β-GP was used as the cross-linking agent [40]. Similarly it was also reported that increase in β-GP reduced the time taken for gelation and increased the chitosan's pH [41]. Stable structure networks with porous sizes 15–112 μm were observed in these chitosan hydrogels up to 28 days. Thermo-sensitive hydrogel was developed using 2% chitosan and 5% β-GP cross-linker, where the gelation time was observed to be around 9 min at 37°C [42]. This gelation time was determined by using the inverted tube test. The hydrogel showed storage modulus values approximating to 100 Pa at 100 rad/s.

Furthermore studies were carried out by using sodium bicarbonate $NaHCO_3$, a weak base, which also induces sol-gel transition as the pH is increased and in exposure to physiological temperature [43, 44]. H^+ from the acidic solution containing chitosan is reacted with HCO_3^- from $NaHCO_3$ and produces CO_2that helps in increasing the pH of the solution >6.5, and thereby inducing gelation with deprotonated chitosan at 37°C [21, 45]. It was reported by Rogina et al. that with increasing concentrations of $NaHCO_3$ there was a decrease in the gelation rate of chitosan [22]. A study by Liu et al. showed that $NaHCO_3$ concentrations in the range of 0.07 mol/L did not yield in the formation of chitosan hydrogel whereas gel was formed at a concentration of 0.08 and 0.12 mol/L with 2% w/v of chitosan [21]. In a particular study, thermo-sensitive hydrogels were prepared by taking chitosan in the concentration of 5 w/v% and 0.48 M $NaHCO_3$ as cross-linker [45]. After the gelation process the pH was 6.50 and the total time taken for the gelation process was observed to be 8 min.

2.3 Cross-Linking

Cross-linked structure present in hydrogels is formed by temporary or permanent physical entanglements, through weak interactions, microcrystallite formation or also through strong chemical bonds [46].

2.3.1 Physically Cross-Linked Hydrogels

The hydrogels falling under this category are produced easily and are also devoid of the toxicity caused by the chemical cross-linkers [17]. In physical hydrogels, the polymer network is bound together by ordered crystalline regions and also by the secondary forces including ionic, hydrophobic forces, hydrogen bonding and molecular entanglements, which by varying pH, temperature or ionic strength can be destabilized [47–49]. There exist two types of entanglement, namely cohesional and topological entanglement whereas the former is the interaction site present in between the segments of few monomer units of neighbouring polymer chains and the latter entanglement is composed of interlocked chain loops [50]. The energy of interaction at each site is minute such that they are detangled and entangled with ease, and that makes this interaction transient [51]. It was also found that with increasing cross-linking degree, the density of cross-linking also increases, which results in hydrogels having lower mesh size and swelling degree and high mechanical strength.

Ionic Cross-Linking

Owing to its mild preparation procedure and simplicity, ionic cross-linking method has more attention in the development of chitosan hydrogels. The interaction between ionic cross-linkers and chitosan can be modified by modulating solution's pH and charge density of the cross-linkers. It is a simple procedure consisting of formation of bridges between ionic cross-linker (having two reactive functional groups) and cationic group of the chitosan. Usually anionic molecules like sodium tripolyphosphate (STPP) (Fig. 2) and β-GP are used as cross-linkers [52] to prepare chitosan hydrogel. A process 'ionotropic gelation' takes place, in which they form a stable ionic complex with chitosan. The gelation process takes place immediately when the two phases are mixed together, by the formation of inter- and intra- cross-linkages within the polymer chains, and that depends on the chitosan charge density and STPP, along with ionic and pH strength of solution [53]. Hence, it can be concluded that use of different cross-linkers and their linking mechanisms emphasizes the properties and structural network of the hydrogels.

Hydrogen Bonding and Hydrophobic Interactions

Chitosan possesses many hydroxyl and amine groups in its polymer chain and easily bonds with other macromolecules through hydrogen bonding. When chitosan is blended with water-soluble non-ionic polymers, it forms hydrogel hydrogen bonding. One of the most common examples used to portray this type of bonding is using PVA, wherein mixing of PVA with chitosan results in formation of interpolymer complexes or crystallites after going through a series of freeze–thaw cycle. In this

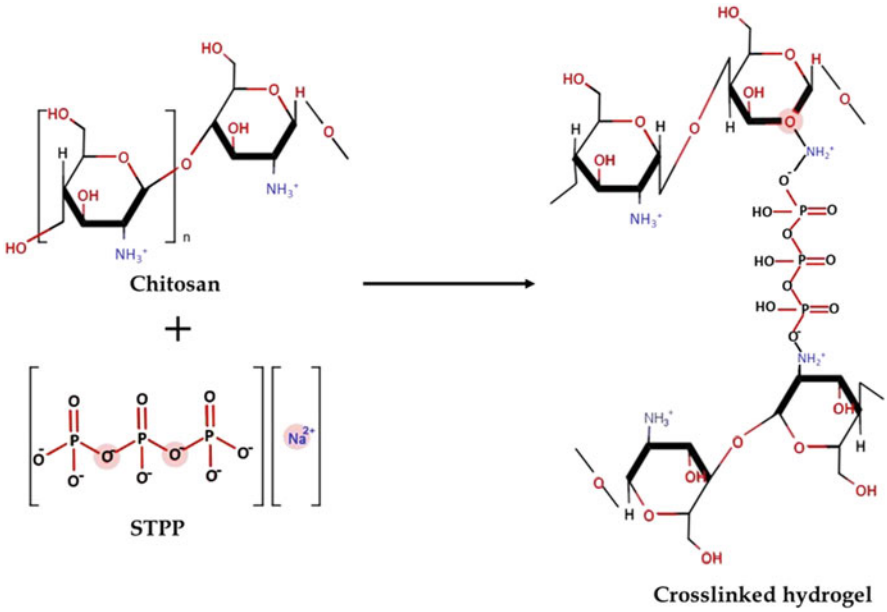

Fig. 2 The reaction of chitosan with STPP

preparation method, the chain–chain interactions of chitosan and PVA act as the cross-linking sites and form hydrogel through hydrogen bonds [54–57].

In contrast to the hydrogen bonding, polymers having hydrophobic domains are cross-linked in aqueous environment by a reverse gelation chemistry known as 'sol-gel'. They exist in liquid form at room temperature and when subjected to increased temperature condition, they transition into solid hydrogel. Owing to the temperature increase, aggregation of hydrophobic domain takes place to reduce their surface area contacting water and thereby maximizing their solvent entropy. Concentration, structure of the polymer and length of the hydrophobic section dictate the temperature at which the gelation takes place [58].

Polyelectrolyte Complexes (PECs)

Reaction between polyanions and chitosan yields in the formation of chitosan based PECs (CS-PECs), wherein the formation of these is categorized into three main stages: (a) 1° complex formation, (b) formation process within intracomplexes and (c) intercomplex aggregations. Chitosan possesses rigid stereoregular structure with bulk pyranose rings form PEC which results in a conformational change, if other polyelectrolyte has a non-rigid structure [59]. By tweaking the chemical characteristics of the polymer components such as functional group, Mw, balance between hydrophobicity and hydrophilicity, stereo compatibility and regularity, charge

density, reaction conditions (pH, mixing ratio, concentration and temperature) and flexibility can alter the CS-PECs characteristics [60]. The degree of interaction among the individual polymers determines the properties of the PECs and they mainly depend on the global charge density of the polymers which also determines the relative composition in the PEC. PEC can also form via two ionizable polymers having opposite charge, which interprets that, at the pH values in the range of pK_a interval of the two polyelectrolytes, formation reaction of PECs can take place. The various other interactions involved in the formation of these PECs structure include van der Waals interactions, hydrogen bonding, hydrophobic interactions. Above all, change of bioactive and physicochemical functions is owed to the preparation methods and diversity of structure in the chitosan based PEC. Using PEC method, chitosan based films, scaffolds and hydrogels can be prepared for variety of biomedical applications.

2.3.2 Chemically Cross-Linked Hydrogels

Hydrogels that are chemically cross-linked network by covalent bonds are formed by the reaction between cross-linking agents and functionalized monomer or polymer, which is not dissolved by any solvents until and unless there is cleavage of the covalent bond [61]. It has been observed that higher mechanical strength is present in hydrogels that are chemically cross-linked than physically cross-linked [62].

Chemical Cross-Linkers

Glutaraldehyde (GA) is widely used due to its ease of use and mild synthesis procedure, reliability, speed of reaction [63, 64]. In the case of GCCS i.e., glutaraldehyde cross-linked chitosan surface, the amount of protein adsorbed decreases as the amino groups present in chitosan which are positively charged are cross-linked by glutaraldehyde, however, by the formation of azomethine bonds by the amino groups, which binds the proteins with some of the aldehyde group present in the GCCS [65]. The use of GA as a cross-linking agent has also shown improvement in the mechanical performances of chitosan fibre and films [66]. A work reported by Gupta and group states about chitosan cross-linked with GA and spacer group glycine, where due to the higher swelling degree the cross-linked network can be hydrolysed in an acidic medium than at a basic pH [67]. It has been interpreted from the preliminary result that there exists a possibility of obtaining a drug delivery system using GCCS with desired pH sensitivity. By the process of solution casting chitosan films are formed which immerse in aq. GA solution for cross-linking. Using glycine, the remaining aldehyde groups are blocked. Though, these many things being said about GA, on the other hand it is neurotoxic. There is an existence of free unreacted dialdehyde in the system, which is completely unavoidable, though purified several times before administration.

Genipin is a natural, low-toxic water-soluble agent used for cross-linking chitosan under acidic or neutral conditions and forms a heterocyclic ring. Cross-linking chitosan with genipin had shown improvement in their stability and mechanical strength [68]. Mechanism of cross-linking involves two reactions [69, 70], (a) A faster nucleophilic attack from the amine group of chitosan on the genipin's C-3 atom, leading to the formation of an intermediate heterocyclic compound and (b) a slower reaction of nucleophilic substitution by the ester group of genipin by forming a secondary amide link with the chitosan. Chitosan hydrogels cross-linked with genipin show the ability to swell in aqueous medium, whereas they are insoluble in acidic and basic medium. When the cross-linking is established a colour change from transparent to deep blue is observed in the hydrogels, it indicated that the reaction takes place between amine group and genipin. It has also been observed that cross-linking using genipin has led to slow increase in viscosity and formation of chitosan hydrogels which are elastic.

An essential role is played by the environmental pH condition [71], which influences the reactions of cross-linking for the fabrication of genipin-cross-linked chitosan, and the networks formed swell in the aqueous media, but are in insoluble in alkaline and acidic medium. Sensitivity to temperature and environmental pH is exhibited by the film's swelling characteristics [68], where at a pH of 13 and 1.0 the swelling ratios are 240% and 315%, respectively, which is attributed to the hydrolysis by alkaline or acid of the amide linkages present in the cross-linked chitosan network and also to the regeneration of the amine groups and carboxyl acid. At neutral pH of 7 the swelling ratio is found to be 62%. The polyelectrolyte nature dictates the swelling behaviour. Below the pK_a of chitosan, amine groups become protonated and result in increased swelling. As the genipin, cross-linked chitosan networks have a high stereo hindrance, which allows for the permeation of lysozyme, than in comparison with GA cross-linked network, due to the presence of bulky heterocyclic nature of genipin, degradation rate of genipin cross-linked network is lesser than GA cross-linked network [72].

Formation of Schiff Base

Chitosan hydrogel is prepared by Schiff base reaction method. The mechanism of cross-linking involving bifunctional aldehydes involves the formation of Schiff base in them. In the reaction between the NH_2 group of chitosan and the aldehyde a Schiff base bridge is formed. In this way the chitosan molecules are bonded together and engender gelation, wherein the reaction takes place very fast and also stabilizes the hydrogel and improves the mechanical properties. A non-cytotoxic cross-linker, oxidized glucose was synthesized [73] and it is cross-linked with alkylated chitosan via Schiff base formation which resulted in hydrogel that was pH sensitive and acts as delivery vehicle for vitamins. Another work by Weng et al. showed oxidized dextran as a cross-linker of chitosan, where within 1–5 min the gelation triggered the formation of a Schiff base [74]. And this also stated that the gelation is faster given the oxidation degree of dextran is also higher. Chitosan hydrogel was prepared for

drug delivery applications which is injectable and prepared via Schiff base formation.

Michael Addition

To form cross linkages, Michael addition reactions have been used in the preparation of chitosan hydrogels [75–78], where reaction takes place between ether acrylates and thiols resulting in the formation of sulphide linkages. Recently this method has gained the interest of many researchers owing to the rapid reaction speed, benign reactivity and the biological inertness of polymeric precursors [15, 58]. Chitosan hydrogels are synthesized by Michael addition reaction of thiolated chitosan and PEG diacrylate [75]. Depending on the temperature, ratio of thiol group to aryl groups and the amount of free thiols in thiolated chitosan, the gelation time varies. Furthermore, an effective design of cross-linked hydrogels using Michael addition is facilitated by developing kinetic model approaches which will allow the prediction of rate of formation and degradation [58]. These type of models have great prospective for bottom up designing of future drug releasing vehicles.

Interpenetrating Network

When a cross-linked hydrogel is immersed into a solution containing monomers and polymerization initiators, it is subjected to polymerization results in the formation of interpenetrating networks [58]. If this process takes place in the existence of a cross-linking agent, that yields a fully interpenetrated network (Full IPN), and in the absence of a cross-linker Semi-IPNs are produced, wherein the linear polymers are entrapped within the cross-linked hydrogel as depicted in Fig.3. Based on the synthesis procedure they are classified as (a) Sequential IPN hydrogels: where the

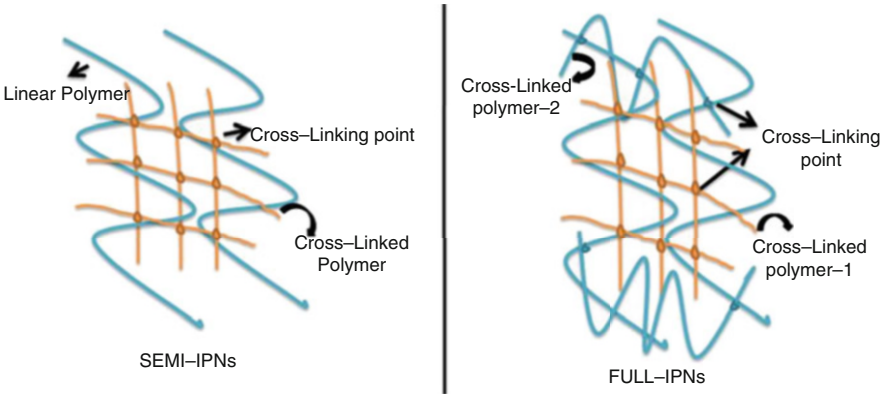

Fig. 3 Schematic diagram showing the structure of Semi-IPNs and Full-IPNs

first network is synthesized followed by the formation of the second network (b) Simultaneous IPN hydrogels: where precursors for both the networks are put together and simultaneously developed. Chitosan hydrogels synthesized using this method possess controllable physical/mechanical properties which are efficient for drug loading and delivery applications in comparison with the conventional hydrogels [79].

Agnely et al. synthesized and evaluated the mechanical and swelling performances of a chitosan/PEO semi-IPN and a control chitosan hydrogel [80]. It was concluded based on the swelling study that the semi-IPN held high potential as it possesses pH dependent swelling characteristics, which lets the pH-controlled drug release when orally administered. Using UV irradiation, full IPN hydrogels were prepared using chitosan and PVA and measured the parameters such as swelling ratio, free and bound water content of the prepared IPN hydrogel [81]. It was observed that within 1 h the gels swell swiftly and attain equilibrium. By increasing the molar ratio of chitosan's hydrophilic groups, there is an increase in free water content and swelling ratio. In response to the external stimuli i.e., temperature and pH, the PVA/chitosan IPN hydrogels exhibit swelling changes. In order to improve the mechanical strength, loading capacity and in vitro mucoadhesive force, a superporous hydrogel (SPH) based on poly(acrylic acid-co-acrylamide)/carboxymethyl chitosan(P(AA-co-AM)/*O*-CMC) IPNs(SPH-IPNs) was synthesized using GA cross-linker. Increase in *O*-CMC content, cross-linking time and GA amount decreases the swelling ratio of SPH-IPNs. Introduction of the IPN and variating the *O*-CMC content has significantly increased the loading capacity of the drug, mucoadhesive properties and mechanical strength [82].

3 Preparation Methods of Porous Chitosan Hydrogels

3.1 Freeze–Thawing Physical Cryogels

Freeze-thawing (F-T) of a concentrated polymeric aqueous solution in a single or repeated cycle, can form physical cross-links through weak interactions and results in the formation of physically crosslinked cryogels [83–85]. Chitosan combined with PVA can be established as a stable 3D hydrophilic network where PVA crystallites act as junction points. During the process of F-T, polysaccharides bind to the PVA, thereby promoting hydrogen bonds which form 3D structure [86, 87]. The porosity, gel fraction yield, wound fluid absorption and mechanical characteristics of these physically cross-linked systems are obtained using the F-T process, and these mainly depend on the number of cycles, temperature and time in an F-T cycle. Employing F-T method, PVA/chitosan/zinc oxide hydrogels were prepared [88]. The essential parameters such as the number of freeze–thaw cycle, thawing time and temperature were investigated. Increase in the cycles of F-T resulted in the increase of tensile strength, elastic modulus and decrease in pore size of PVA/ZnO/chitosan cryogels [88]. The occurrence of highly interconnected

porous structure enhanced the swelling capability and water vapour permeability of the prepared cryogels by F-T process simply indicates their applications in wound healing [89].

3.2 Chemical Cryogels

Using this technique, highly interconnected pores containing cryogel are developed in an aqueous condition by means of free radical polymerization using cross-linkers with initiators. In this case, cross-linkers with initiators are present in frozen state (partially), where the ice crystal acts as a template [90–92]. This technique helped in the generation of cryogels that are sponge like and elastic with shape memory features, owing to the highly cross-linked polymeric walls. Chitosan/CPL (clinoptilolite) [93] composite cryogels are obtained by cross-linking chitosan with GA in the presence of particles of CPL, which resulted in the heterogeneous morphology with interconnected pores also having an average porosity of 52 $\pm$ 2 μm. Employing the unidirectional freezing (UF) [94, 95] approach, the morphology of the pores can be controlled, wherein mixtures of dispersion containing inorganic particles, water-soluble polymers have been frozen in liquid nitrogen unidirectionally. This resulted in the formation of composites with lamellar or micro-honeycomb like structures. Recently chitosan/gelatin scaffolds [96], chitosan/polyacrylamide(PAAm)/zeolite cryogels [97] and chitosan/poly(vinyl amine) cryogels embedded with strong base anion exchange microspheres [98] have been prepared with aligned porous structure. The parameters that affect the size between the pore wall thickness and the channel are the Mw of chitosan, primary monomer or polymer concentration and crystallization speed. A strategy that combines the cryogelation and phase separation is induced when n-butanol is added, which helps to generate 3D networks with two generations of pores [99]. Large porous chitosan based hydrogels with less compact walls have been prepared by combining the porogen leaching along with cryogelation method. PMMA (poly (methyl methacrylate)) has been used as a polymer porogen as fractionated particles to prepare chitosan cryogel. The morphology of the prepared chitosan based cryogels was very much influenced by the mesh of fractionated PMMA particles, weight ratio of PMMA/chitosan and the crystallization rate. Inner morphology of chitosan based cryogels developed using fractionated PMMA particles revealed hierarchical porosity with larger pore diameter ranging from 75 to 90 μm which has been formed via cryogelation, whereas those produced by PMMA leaching had smaller pores range in between 10 and 15 μm [100].

3.3 Lyophilization

The technique in which aqueous polymeric solution is frozen followed by solvent sublimation under vacuum, which results in macro-porous materials [101–106]. The freeze drying pressure, degree of cross-linking and polymer concentration affect the porosity, pore size and thickness of the pore walls. In a particular work, when the chitosan concentration is increased, the prepared gelatin/carboxymethyl chitosan (CMCS)/HAP hydrogels showed wide pore walls [107]. This technique has also been used to produce pores in non-porous PAAM/CS hydrogel, thus hydrogel has been first swollen and then subjected to lyophilization. The pH and concentration of cross-linker used in the synthesis mixture has influences on the morphology of the hydrogels. A novel hydroxyethyl chitosan (HECS) and cellulose hydrogel scaffold has been developed with bubble like porous structure, by merging the lyophilization method along with porogen leaching method (SiO_2 particles as a porogen) and chemical cross-linkers. These scaffolds showed highly interconnected biporous structure, wherein the micropores having size 10 μm due to sublimation under vacuum and macropores having size 100–250 μm due to leaching by SiO_2 particles [101].

3.4 Porogen Leaching

A technique in which the cross-linking or free radical polymerization is studied in the presence of porogens, salts, solvents or inorganic particles and once the reaction is completed it is removed from the 3D matrices [108]. By altering the concentration of porogen, the pore size in the developed 3D matrices can be controlled. However, there are certain limitations in this method. The strategy of porogen leaching has been combined with the technique of cryogelation to overcome the above mentioned issues and also to obtain controlled pore architecture. The strategy of porogen leaching has been combined with cryogelation to overcome the above-mentioned issues and to obtain controlled pore architecture [99]. Using PMMA poly(methyl methacrylate) as a polymer porogen, chitosan and poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels were synthesized. Tailoring of the porous structure of these hydrogels was carried out by using porogen leaching and ice-templating process. It was observed that the swelling characteristics of the hydrogels that were incorporated with PMMA achieved equilibrium swelling much faster than those hydrogels which aren't incorporated with PMMA [109].

3.5 Gas Foaming

Another method in generating 3D matrices with macropores using foaming agents like $NaHCO_3$ or NH_4HCO_3 [110, 111]. Super-porous chitosan/HAP hydrogel scaffolds have been developed by merging gas foaming along with microwave irradiation containing $NaHCO_3$ as a blowing agent. It has also been reported that CEL/chitosan scaffold was prepared with porosity using supercritical carbon dioxide. CEL/chitosan scaffolds were prepared using two different procedures: (a) at room temperature under high pressure condition (50 bars) and (b) using gas foaming. The scaffold morphology was compared, it was observed that hydrogel prepared at room temperature had porous structures that are closed with size of 10–30 μm and the ones prepared by gas foaming exhibited pore size that were tenfold higher and had structure with interconnected pores [112, 113].

Overall, different approaches have been extensively utilized to fabricate chitosan and porous chitosan hydrogels which finds potential applications in the area of tissue engineering and regenerative medicine.

4 Applications of Chitosan Hydrogel

4.1 Drug Delivery

Chitosan hydrogel has been used for drug delivery applications [114–116]. Annapoorna et al. developed arginine and nanocurcumin complex incorporated shear thinning chitosan hydrogel for preventing hypoxia induced endothelial damage [115]. This developed composite hydrogel system is biodegradable and showed an enhanced antioxidant activity compared to chitosan control. In acidic condition, curcumin and arginine release from the chitosan hydrogel was found to be higher, which will be useful in an ischaemic site. The efficacy of the composite chitosan hydrogel system was studied in in vitro hypoxic endothelial dysfunction model to prove the angiogenic nature. The tube formation studies of the prepared hydrogel under hypoxic condition proved angiogenic nature of the developed hydrogel. The enhanced phosphorylation of eNOS at serine 1,177 was observed under hypoxic conditions in the cultured endothelial cells. This developed chitosan composite hydrogel can simultaneously deliver curcumin drug and arginine for overcoming the hypoxia induced endothelial dysfunction [115]. In another study, pro-angiogenic drug, deferoxamine nanoparticles incorporated chitosan-hyaluronic acid hydrogel was prepared for enhancing angiogenesis [116]. Controlled deferoxamine drug release was observed in the deferoxamine loaded PLGA nanoparticles incorporated chitosan-hyaluronic acid hydrogel. The prepared hydrogel is biocompatible and has shear thinning property. The in vivo angiogenic potential of the developed hydrogel was studied in the subcutaneously implanted mice for 2–4 weeks. Deferoxamine loaded PLGA nanoparticles incorporated

chitosan-hyaluronic acid hydrogel showed enhanced neovascularization compared to other groups. So the prepared deferoxamine loaded PLGA nanoparticles incorporated chitosan-hyaluronic acid hydrogel can be potentially used for therapeutic angiogenesis. Overall reported studies indicated that chitosan hydrogel is very useful in drug delivery area.

4.2 Haemostasis

Chitosan hydrogel has potential applications in haemostasis. Chitosan has shear thinning and mucoadhesive properties. The shear thinning and mucoadhesive properties of chitosan hydrogel helps the applied hydrogel to bond with the tissue and stay at the site of application [19, 117, 118]. Cationic chitosan hydrogel stops bleeding by interacting with the anionic membrane of red blood cells. The chitosan hydrogel takes 4–6 min to stop the bleeding at the injury site. The incorporation of inorganic ceramic nanoparticles can aid in enhancing the hemostatic property of chitosan [4, 104].

Whitlockite nanoparticles are an inorganic ceramic nanoparticle and have been extensively studied for bone regeneration [118–121]. Due to controlled release of calcium, magnesium and phosphate ions it has great potential in bone regeneration. Calcium, magnesium and phosphate ions are significantly contributing in haemostasis. Inorganic whitlockite nanoparticles (75 ± 5 nm) incorporated chitosan hydrogel were prepared and studied the haemostatic activity by in vitro and in vivo [117]. The prepared hydrogel is injectable, cyto- and hemo-compatible. The haemostatic effect of the prepared whitlockite nanoparticles incorporated chitosan hydrogel was studied in in vivo rat liver and femoral artery injury model and showed the reduced time for haemostasis 62 ± 3 s and 229 ± 9 s, respectively. The obtained results showed that whitlockite nanoparticles incorporated chitosan hydrogel show reduced blood clotting time than control chitosan hydrogel and commercially available haemostatic agent Floseal®. The reason for the effective haemostasis of whitlockite nanoparticles incorporated chitosan hydrogel is that the synergistic action of magnesium, calcium and phosphate ions released from whitlockite nanoparticles and amine group present in the chitosan enhanced the RBCs/platelet aggregation as well as the activation of the coagulation cascade [117]. Bioglass is an another inorganic ceramic material used for bone tissue engineering [19, 122]. It also has ionic dissolution potential similar to whitlockite nanoparticles. It is very useful in haemostasis due to calcium, silica and phosphate ions present in the bioglass nanoparticles. Nanobioglass incorporated chitosan hydrogel was developed and studied in in vivo rat liver and femoral artery injury model to know haemostatic effect [19]. The nanobioglass incorporated chitosan hydrogel exhibited the blood clotting time of 54 ± 3 s and 185 ± 9 s, respectively. Chitosan-nanobioglass hydrogel showed shorter blood clotting time when compared to chitosan hydrogel. This may be due to the presence of silica, phosphorous and calcium ions released from nanobioglass and amine group of chitosan synergistically enhanced the RBCs/

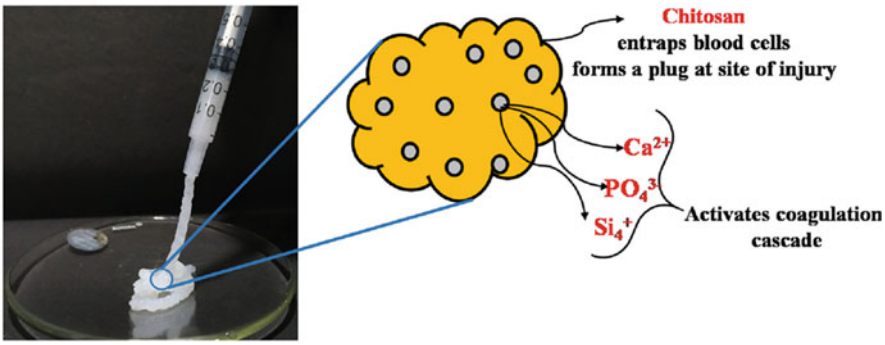

Fig. 4 Schematic representation of mechanism of haemostasis by nanobioglass incorporated chitosan hydrogel. Reproduced with permission, Copyright Elsevier

platelet aggregation as well as the activation of the coagulation cascade [19] (Fig. 4). Vasoconstrictor (0.25% potassium aluminium sulphate) and coagulation activator (0.25% calcium chloride) incorporated injectable composite chitosan hydrogel (2%) was developed for rapid haemostasis [123]. The developed composite hydrogel haemostasis study was done using in vivo rat femoral and liver injury model and the developed potassium aluminium sulphate and calcium chloride incorporated injectable chitosan hydrogel stopped the bleeding in 105 ± 31 s and 20 ± 10 s, respectively. The composite hydrogel showed rapid haemostasis when compared with chitosan control hydrogel as well as commercially available Fibrin® and Floseal®. This may be due to the synergistic effect of chitosan and potassium aluminium sulphate, which also accelerates RBC/platelet aggregation, activates the coagulation cascade thereby inducing rapid haemostasis [123]. All the reported studies clearly indicated that the addition of inorganic agents improved the chitosan haemostatic property.

4.3 Tissue Engineering

The applications of chitosan hydrogel are extensively studied for tendon, ligament, adipose, cartilage and bone tissue engineering. In one of the studies, a bi-layer chitosan-hyaluronic acid hydrogel coated multiscale fibrous scaffold of PCL aligned microfibres/random nanofibres and PCL random microfibres/nfibres were developed by electrospinning for ligament regeneration and characterized. Chitosan-hyaluronic acid hydrogel coated multiscale fibrous scaffold has aided in cell viability, proliferation and infiltration with enhanced protein adsorption. Chitosan-hyaluronic acid hydrogel coating acts as reservoir for cells and nutrients and this bi-layered hydrogel coated multiscale fibrous scaffold has applications in ligament regeneration [124]. In another study, electrospun aligned poly (l-lactic acid) (PLLA)nanofibres (for mimicking the aligned collagen fibre bundles in tendon) and chitosan-collagen hydrogel

coated on layered PLLA fibres with (for mimicking the glycosaminoglycans of sheath ECM) for tendon tissue regeneration [125]. Alginate gel was coated (as an outer layer) on chitosan-collagen hydrogel coated electrospun fibrous membrane to inhibit the peritendinous adhesion. The prepared scaffolds are biocompatible to tenocytes as well as showed good cell proliferation, attachment and spreading on the developed fibrous scaffolds. This biocompatible chitosan-collagen/PLLA/alginate scaffold has potential application in flexor tendon regeneration [125]. Similarly, Twisted Chitosan hydrogel scaffold with reinforced PLLA aligned microfibrous bundle was developed and characterized for tendon tissue regeneration [126]. Nanofibrin incorporated alginate-*O*-Carboxymethyl chitosan hydrogels were developed for adipose tissue engineering [127]. The developed hydrogel has showed adipose derived stem cells adhesion, proliferation. The adipocytes differentiation was proven by Oil Red O staining. The nanofibrin incorporated alginate-*O*-Carboxymethyl chitosan hydrogels have applicability in adipose as well as other soft tissue engineering. Strontium ranelate nanoparticles (160 ± 30 nm) incorporated chitosan/alginate/fibrin injectable gel were developed and characterized for cartilage tissue regeneration [128]. The prepared hydrogel was found to be biocompatible with human mesenchymal stem cells and it has also exhibited chondrogenic differentiation potential with enhanced proteoglycan and collagen synthesis. This strontium ranelate nanoparticles incorporated chitosan/alginate/fibrin injectable gel has potential application in cartilage tissue engineering. Overall these studies reported that chitosan hydrogel is very useful in ligament, tendon, cartilage and adipose tissue engineering.

4.4 Wound Healing

Chitosan based scaffolds, sponges and hydrogels have the applicability in infectious wound healing [129–133]. Chitosan hydrogel is potentially used in the treatment of infectious wounds [132, 133]. Lysostaphin incorporated chitosan hydrogel was developed in the treatment of *Staphylococcus aureus* (*S. aureus*) infected wounds [132]. The interaction between lysostaphin and chitosan was studied using molecular docking analysis. The developed gel were found to be smooth in texture and also biocompatible in nature. The in vitro and ex vivo antibacterial studies confirmed that the developed hydrogel has significant reduction of *S. aureus* survival by $\sim$3 Log10CFU/mL. The prepared hydrogel has the ability to disrupt the biofilm formed in catheter tubes and results in a reduced biofilm formation with higher percentage of lysostaphin (Fig. 5). These results demonstrated that lysostaphin incorporated chitosan hydrogel has the applicability in the treatment of infectious wounds.

In another work, tigecycline nanoparticles (93 ± 13 nm) and platelet-rich plasma incorporated hydrogel were developed for treating *S. aureus* infected wounds and characterized [133]. The addition of tigecycline nanoparticles inhibits the *S. aureus*. The platelet-rich plasma incorporation in the chitosan helps in wound healing. The prepared hydrogel is cytocompatible, injectable and showed a sustained tigecycline

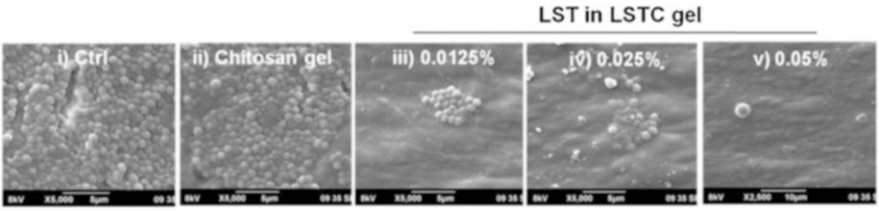

Fig. 5 SEM images of catheter tubes showing the inhibition of bacterial growth by lysostaphin. Reproduced with permission, Copyright Elsevier

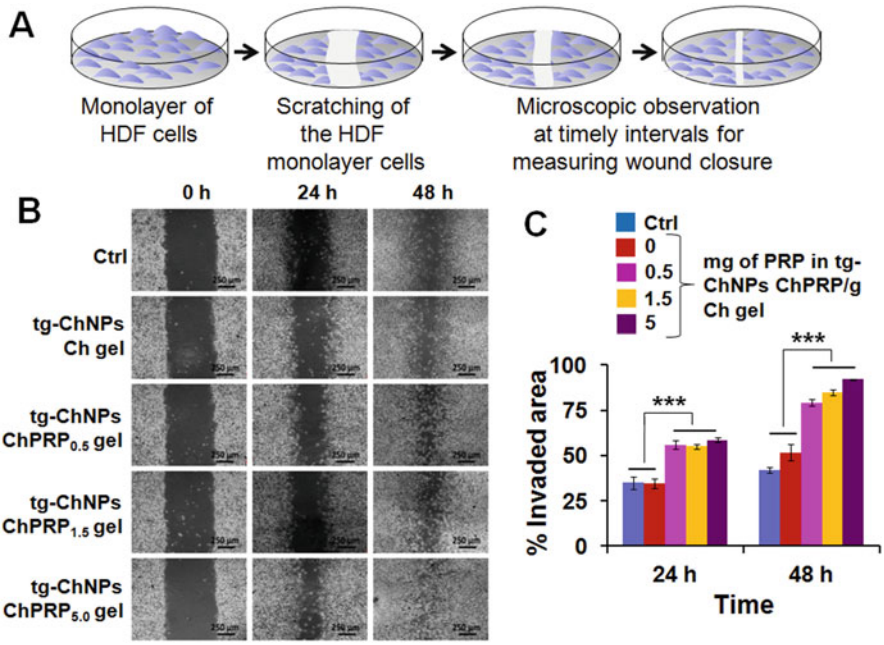

Fig. 6 Scratch assay performed to confirm the wound healing effects of the hydrogel. Reproduced with permission, American Chemical Society

drug release from chitosan hydrogel. The increasing concentration of platelet-rich plasma enhanced the cell proliferation and migration (Fig. 6). Finally, in vivo *S. aureus* inhibition studies of the developed hydrogels were analysed in *S. aureus* infected *Drosophila melanogaster* model. The tigecycline nanoparticles (93 ± 13 nm) and tigecycline drug incorporated chitosan hydrogel showed an enhanced *S. aureus* inhibition. This developed hydrogel has the ability to inhibit the bacteria as well as promote the healing of wounds. These reported studies clearly indicated the applicability of chitosan hydrogel in the area of infectious wound healing.

5 Summary

In this review, we overviewed the different procedures that have been reported in the synthesis of chitosan and porous chitosan hydrogels. The mechanism behind the development of these hydrogels using different methods has also been discussed. These preparation methods significantly influenced the parameters such as swelling, degradation, porosity, solubility, stability and mechanical strength of the hydrogels. This change in parameters results in chitosan hydrogel possessing unique characteristics, which has vivid use in different biomedical applications. In addition, we have overviewed the applications of chitosan hydrogel in haemostasis, drug delivery, tissue engineering and wound healing. This review is very useful for the researchers who are working in the area of chitosan and chitosan based hydrogel for biomedical applications.

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Antimicrobial Properties of Chitosan and Its Derivatives

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Abstract The investigations of chitosan as an antimicrobial substance came into focus in the 1990s. The number of publications on this topic has been rapidly increasing since then, with more than 2,500 papers published in 2020. Initially, the interest was mainly related to potential applications in agriculture and food products, but currently, the emphasis on the medical use of chitosan and chitosan derivatives is continuously increasing. Common derivatives of chitosan include carboxymethyl chitosan (CMC), *N*,*N*,*N*-trimethyl chitosan (TMC), *N*-(2-hydroxyl), propyl-3-trimethyl ammonium chitosan chloride (HTCC), hydroxypropyl chitosan (HPC), and glycol chitosan. All of these derivatives have been investigated as antimicrobial polymers in various applications, but most such studies involve using the cationic derivatives TMC and HTCC. Countless other antimicrobial chitosan derivatives have been reported in at least one publication. Many researchers have studied the antimicrobial mechanism of action against bacteria and fungi and found that the polymers affect the cell membrane, but the details of the interaction are still debated. Other studies have indicated intracellular nucleic acids, surface proteins, and lipopolysaccharides as possible targets. Various applications, including plant protection, food preservation, wound treatment, and water purification, have been considered to utilize the antimicrobial properties of chitosan-based materials. Structure-activity relationship studies are helpful to elucidate the function and importance of different structural characteristics that may influence activity, including the degree of acetylation (DA), charge, the structure of the substituted, degree of substitution (DS), and molecular weight (Mw).

Keywords Antibacterial · Antifungal · Antimicrobial · Chemical modification · Degree of acetylation (DA) · Degree of substitution (DS) · Mechanism of action · Molecular weight (Mw) · Structure-activity relationship

1 Introduction

The antimicrobial properties of chitosan were discovered early. The first reporting of the potential activity of chitosan and chitosan derivatives against bacteria was probably in a 1950 paper in the *Japanese Medical Journal*, titled "*Macramin, a new high molecular antibacterial substance derived from chitin*" [1]. Remarkably this early publication did not report the antibacterial activity of chitosan but of the methylated derivative, which only became an active research topic in the last decades of the twentieth century. Later, in 1964, it was reported that chitosan could inhibit fermentation in baker's yeast [2]. However, there were few publications on research into this topic in the following decades. Search in the ISI web of science reveals only 11 publications relevant to this topic in the years up to 1990. Perhaps the most notable work was described in papers by Lee A. Hadwiger and co-workers, focusing on the activity of chitosan against fungi [3–6]. This research may have been motivated by interest in the fungal cell wall composition, which

contains chitin in many species and chitosan in some species [7]. Thus it was reported by Allan and Hadwiger [3] that chitosan was active against fungal species with chitin-containing cell walls but lacked activity against fungi with chitosan-containing cell walls. In the 1990s, antimicrobial properties and applications of chitosan finally came more into focus as a subject for scientific research. Two papers published at the beginning of the decade had a significant impact. The first of these studies was by Muzzarrelli and co-workers published in a paper entitled: "*Antimicrobial properties of N-Carboxybutyl chitosan*," which appeared in *Antimicrobial Agents and Chemotherapy* in 1990 [8]. In this work, they described the investigation of the antimicrobial activity of *N*-carboxybutyl chitosan against 298 strains of Gram-positive and Gram-negative pathogens and *Candida ssp*. Furthermore, the study included some investigations of the mechanism of action based on electron microscopy imaging. This revealed that the derivative caused fraying, weakening, and local thickening of staphylococci's cell wall. They also observed that the periplasmic space of Gram-negative species expanded, and the intracellular material appeared to be less densely packed after exposure to *N*-carboxybutyl chitosan. The latter paper was by Sudarshan, Hoover, and Knorr entitled "*antibacterial action of chitosan*" published in *Food Biotechnology* in 1992 [9]. They showed that chitosan hydroglutamate and chitosan lactate could inactivate Gram-negative and Gram-positive bacteria in 1 h. Leakage of intracellular beta-galactosidase from *E. coli* exposed to chitosan was also detected, indicating cell membrane permeabilization. Chitosan also caused cell agglutination at pH 5.8. In 2021 the Muzzarelli paper had been cited more than 340 times and the Sudarshan, Hoover, and Knorr paper more than 550 times. Much of the work in the following decade was focused on the potential use of chitosan as a preservative for food products [10] and plant protection in agriculture [11]. Research into the potential use for chitosan in health care and pharmaceuticals was also intensifying, but chitosan's antimicrobial properties were in general, not the main focus of this work. However, antimicrobial action can be an added benefit of using chitosan and chitosan derivatives for wound treatment [12], for bone repair [13], to improve osseointegration of implants [14], and as a mucoadhesive component in pharmaceutical formulations [15]. The research into antimicrobial properties and chitosan and chitosan derivatives applications has continuously increased in the last few decades. Whereas there was only one paper published focusing on this topic in 1990, there were between 40 and 50 ISI listed papers published in the year 2000. The number had increased in 2010 to more than 450, and in 2020, papers describing investigations into the antimicrobial properties of chitosan and chitosan derivatives were more than 2,500. Many publications are looking at the antimicrobial action of unmodified chitosan with varying degrees of acetylation and molecular weight, which is sometimes cross-linked and often mixed with other materials to prepare liquid formulations, hydrogels, coatings and finishing, microparticles, and nanoparticles [16]. These have been prepared for application in agriculture, water treatment, food products, and health care [16]. Furthermore, the chemical derivation of chitosan is a very active research field. Chitosan polymers are insoluble in alkaline aqueous solutions with pH above 7 and this limits the application. Chemical derivatives with additional polar or charged group can be

highly soluble independent of pH, which can be a significant benefit. The purpose of the chemical modification is also often to enhance the antimicrobial activity and therapeutic index, especially the activity against microorganism relative to cytotoxicity toward mammalian cells. Commonly, derivation to enhance antimicrobial activity introduces groups with cationic charge [17], but other derivatives such as lipophilic *N* and *O* acyl derivatives [18], a hydroxypropyl chitosan [19], and carboxymethyl chitosan [20] have also been investigated as antimicrobial agents. Furthermore chitosan conjugates can be prepared by linking bioactive moiety with the polymer and thus modifying the biological profile. Antioxidants are commonly introduced in this way and such conjugates have also been investigated for antimicrobial activity [21]. Other antimicrobial agents such as antimicrobial peptides have also been conjugated with chitosan, chitosan derivatives, and chitosan nanoparticles. Chitosan has also been used in drug delivery systems to aid in the delivery of antimicrobial drugs.

2 The Antimicrobial Mechanism of Action

2.1 Overview

Chitosan and many chitosan derivatives have broad-spectrum activity against various microorganisms, including Gram-positive and Gram-negative bacteria and fungi. It has also been shown that these polymers can inactivate viruses such as tobacco mosaic virus [22], norovirus, calicivirus [23], and coronaviruses [24]. The mechanism for chitosan and chitosan derivatives antimicrobial action is still debated. Multiple different mechanisms have been proposed. The mechanisms implicated in the antimicrobial action include disruption of the cell membrane, blockage of the nutrient flow, binding to intracellular RNA and DNA, chelation binding of metal ions, and electrostatic binding lipoteichoic acid [17, 25]. Not all of these mechanisms are mutually exclusive, and in general, the number of possible mechanisms discussed in review papers seems to have increased over time. However, not all of these mechanisms are equally well supported by experimental data.

2.2 First Studies

The first significant study into the cytotoxic mechanism action focused on soya bean (*Glycine max*) and common bean (*Phaseolus vulgaris*) plant cells, not microorganisms [26, 27]. Interest in the effect of chitosan on plants stems from the importance of fungi as pests causing plant disease. Fungi contain chitin and chitosan in their cell wall, and thus the researchers were interested in finding out if polycationic chitosan released by fungi could adversely affect plant cells. These studies showed that chitosan increased membrane permeability of the membrane and caused leakage of

the electrolytes, proteins, and UV-absorbing species (such as co-enzymes and nucleic acid). Other polycations such as poly-L-lysine, DEAE-dextran, and spermine caused similar leakage. It was found that bivalent cations inhibited the effect of chitosan in the order $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$. The results indicated that chitosan induced the formation of quite large "pores" in the cell membrane. The latter paper also showed radiolabeled Ca^{2+}, bound to the cell surface, was released before chitosan-induced leakage of the cytosol content could be detected [26].

2.3 *Later Work*

Some significant studies on the antimicrobial mechanism of chitosan and chitosan derivatives were published in 2001. Helander et al. [28] studied the effect of chitosan on the Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium*. Exposure to chitosan allowed the entry of 1-*N*-phenyl-naphtylamine (NPN), a hydrophobic probe normally excluded from Gram-negative bacteria, into the periplasmic space between the inner and outer membrane. This showed that chitosan, similar to other polycations, weakened the outer membrane barrier function. The effect was reduced or eliminated by $MgCl_2$. Chitosan did not increase the release of lipid material or lipopolysaccharides (LPS), which indicated a reversible pouring of the outer membrane. Electron micrographs showed that the outer membrane of chitosan-treated cells was covered by vesicular structures and by additional material so that the cell envelope appeared considerably thickened (Fig. 1a). Se-Kwon Kim and co-workers [29] showed that low molecular weight

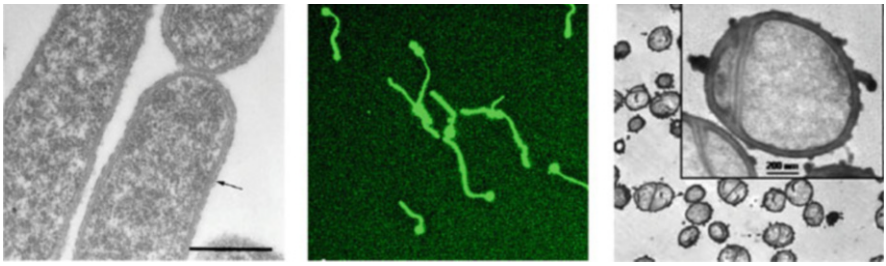


Fig. 1 Microscopy observation of the effect of chitosan on bacteria and fungi: (**a**) from Helander et al. [28] showing undulating outer membrane of *E. coli* and vesicles caused by treatment with chitosan. (**b**) from Xu et al. [35] showing the internalization of fluorescein-labeled 2-AMAC-oligochitosan in cysts and germ tubes of *P. capsici*. (**c**) from Raafat et al. [37] showing *S. stimulans* cells treated for 60 min with chitosan. Irregular structures are protruding from the cell surface, which might be chitosan deposits still attached to the negatively charged surface polymers. The cell membrane became locally detached from the cell wall, giving rise to "vacuole-like" structures underneath the cell wall, possibly resulting from ion and water efflux and decreased internal pressure. (Published with permission from Elsevier and American Society for Microbiology)

chitosan (degree of polymerization (DP) 5–7) was less effective in inhibiting Gram-positive bacteria, including lactic acid bacteria and Gram-negative bacteria than high molecular weight chitosan (Mw 24–7 KD). Muzarrelli et al. [30] showed that methyl pyrrolidinone chitosan, *N*-carboxymethyl chitosan, and *N*-phosphonomethyl chitosan exerted effective fungistatic action against *Saprolegnia parasitica*. Electron microscopy showed that the chitosan derivatives altered the morphology of the fungi, and at 4 mg/ml concentration, they seemed to precipitate together with the fungus. The cell was thickened and frayed, often coated with chitosan. Partial disappearance of the plasma membrane and damage to the internal organelles was also observed in some cases. Liu et al. [31] studied carboxymethylated chitosan and chitosan activity against *E. coli*. They observed fluorescently labeled oligomers inside the cells. Based on this observation the authors suggested that antimicrobial effect was mainly due to inhibition of DNA transcription. Later studies on the effect of chitosan on *E. coli* not only confirmed the observation made by Helander et al. that the permeability of the outer membrane was increased but also showed that the inner cell membrane was also damaged, causing leakage of the cytosol content [32, 33]. These studies also showed that chitosan formed complexes with phosphatidylcholine in liposomes, and this suggested interaction with anionic lipids was causing membrane damage. One of the studies also indicated that chitosan disrupted the cell membrane of Gram-positive *S. aureus* and mainly affecting dividing cells. In 2006 [34], similar results were also obtained for the mechanism of action for antimicrobial dimethylaminoethyl-chitosan (DMAEC) against *E. coli* and *S. aureus*. In 2007 Xu et al. [35] reported that fluorescently labeled oligochitosans, with DP 3–9, localized mainly in the cytoplasm of *P. capsici* (Fig. 1b) and showed no binding to the cell wall or the cell membrane. It was also demonstrated the oligochitosan could affect the electrophoretic mobility of the genomic DNA and total RNA. The authors, therefore, suggested that small antifungal chitosan oligomers could cross the fungal cell wall and cell membrane and bind to intracellular targets such as DNA or RNA. However, these results were contrasted by Park et al. [36] which showed that chitosan oligomers (1KD) were, in general, less effective against 9 fungal strains than higher Mw chitosan (3, 5, or 10 KD). Fluorescence studies and transmission electron microscopy studies indicated that chitosan penetrated the cell wall and accumulated in the plasma membrane. The membrane disrupting ability could also be correlated with molecular weight, and that chitosan with higher molecular weight showed increased activity and membrane disrupting ability. A major study into chitosan's mode of action against Gram-positive bacteria was published in 2008 [37]. The study included *S. stimulans* and two strains and different mutants of *S. aureus*. The average Mw of the chitosan used in the study was 243 KD, but a prior investigation had shown that molecules larger than 10 KD were required for activity. Chitosan had a depolarizing effect on the cell membrane of *S. stimulans* and caused the K^+ efflux to increase and a gradual leakage of UV-absorbing substances. However, no protein leakage was detected. The depolarizing effect of chitosan is much slower and incomplete compared to nisin, an antimicrobial peptide known to form pores in the cell membrane. Transmission electron microscopy investigations revealed that the membrane was intact but

became locally detached from the cell wall (Fig. 1c). No cell wall lysis was detected. Investigations with different mutants revealed that *tagO* mutants, which lack genes for teichoic acid biosynthesis were least susceptible to chitosan with five-fold reduction relative to parent strain. The exposure to chitosan caused up-regulation or down-regulation of various bacterial genes. The authors therefore speculated that chitosan binds to teichoic acid on the cell wall surface and this causes extraction of membrane lipids. The proposed mechanism rests on the assumption that high molecular weight chitosan cannot cross the cell wall and that chitosan is not active against Gram-negative bacteria, which lack this negatively charged copolymer on the cell surface. The latter assertion was made in a later publication by the main authors [38], but this is clearly contradicted by data from other investigators that show that chitosan is also highly active against *E. coli* and many other Gram-negative bacteria. The susceptibility of bacteria to chitosan can vary considerably according to species or strain. No statistical analysis has been carried out to show that either Gram-negative or Gram-positive bacteria are more susceptible to chitosan. It would also be hard to make a representative sample of the thousands of bacterial strains known to man. Studies have also been carried out for the antibacterial mechanism of chitosan and chitosan derivative microspheres against *E. coli* [39] and other bacteria [40]. These studies confirmed that this form of the polymer also disrupts the bacterial cell membrane. Studies with 21 genes deleted and complemented *E. coli* strain and showed that activity was reduced against the *ΔompA* strain lacking the Outer Membrane Protein A (OMPA) [40]. The authors speculated that chitosan binds to OMPA, but interestingly, this protein is nearly neutral and lacks a negative charge. They therefore proposed hydrogen binding as the main interaction.

2.4 Other Considerations and Summary

In addition to the direct effect of inhibiting microbial growth and killing microbes, chitosan can also act indirectly by modulating infection response. Thus there is considerable research into the use of chitosan as an adjuvant in vaccinations [41] and the effect of chitosan on the human and animal immune systems [42]. The presence of chitosan and chitin oligomers and polymers may also indicate a fungal infection and stimulate host defense mechanisms in plants [43]. This topic will not be covered in the chapter, but insightful, relevant review articles are included in the references list [42, 43].

This review of past investigations into the mechanisms of action should show that it is most likely that chitosan and chitosan derivatives act to disrupt the cell membrane of bacteria and fungi. Other mechanisms, such as intracellular effect on DNA or RNA function or binding to teichoic acid, are much more speculative and often contradicted by other published work. It has been shown that chitosan can cross the cell membrane to enter into the cytosol, but it is not certain that this causes harm to the microorganism.

However, the precise mechanism by which chitosan disrupts the membrane is much less well known. The replacement of membrane-bound Ca^{2+} to destabilize the membrane may play a role, and chitosan may also cause pore formation in the membrane similar to the effect of many antimicrobial peptides [44]. Further detailed studies will be required to clarify this issue.

3 Synthesis of Antimicrobial Chitosan Derivatives

3.1 Reaction Conditions and Selectivity

Chitosan can be chemically modified to improve biological properties, including antimicrobial activity. The introduction of polar substituents can enhance aqueous solubility, contributing to higher antimicrobial activity, especially at pH above 7. Besides, the purpose of the modification is usually also that substituents should, in some way, contribute directly to increased activity.

The glucosamine monomer of chitosan has three nucleophilic groups, the C2-amino group and the C6 and C3 hydroxyl groups (Fig. 2). The amino group is commonly the target for modification. It is the most nucleophilic group under neutral or mildly basic conditions and will preferably react with electrophiles like alkyl halides, sulfonates, and epoxides [16]. However, the reaction is not always very selective, and the hydroxyl groups, especially the C6-hydroxyl group, may also react, especially when the degree of substitution is high. The amino group can react

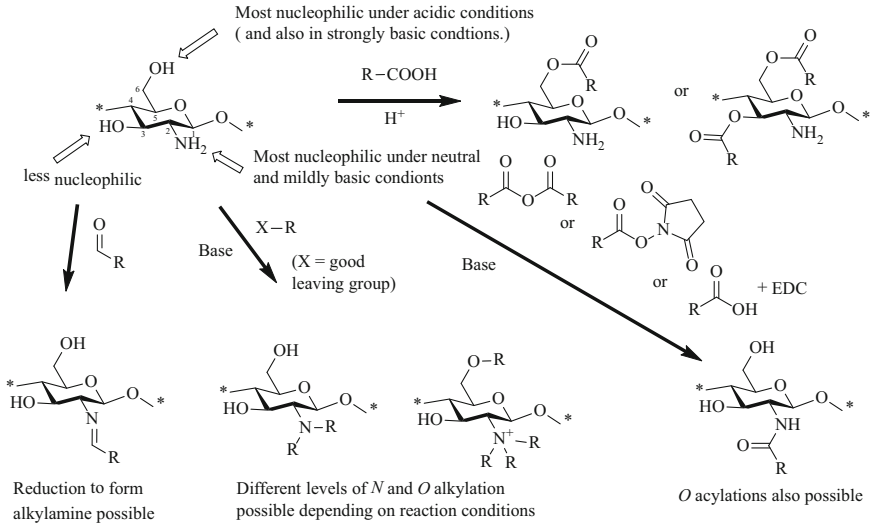


Fig. 2 Nucleophilic groups in the glucosamine monomer and some common reactions used to modify the chitosan structure

selectively with aldehydes and ketones to form imine derivatives [45], and this can be coupled with a reduction to obtain N-alkyl derivatives [46, 47]. However, this reaction can only be utilized to form monoalkyl or dialkyl derivatives. Monoalkyl and dialkyl derivatives can react with alkyl halides to obtain quaternized *N,N,N*-trialkyl derivatives [46, 48]. The amino group can also be *N*-acylated by reaction with acyl halides, [49] anhydrides [50, 51], N-hydroxysuccinimide esters (NHS-esters) [52], or with carboxylic acids in the presence of suitable coupling reagent such as 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [53]. As in the case of electrophilic reagents, these reactions are not necessarily selective for the amino group, and esters can be formed with the C6 and C3 hydroxyl groups. However, amides are more stable than esters. Unwanted ester linkages can therefore be hydrolyzed by treatment with aqueous acid or base without affecting the amide bonds [49]. The reaction conditions can also be adjusted so that the modification of the hydroxyl groups, especially the more reactive primary C6 hydroxyl group, is preferred. In anhydrous acidic conditions, the hydroxyl groups will be condensed with carboxylic acid to form esters, whereas the protonated amino group will not react. Chitosan has thus dissolved in methane sulfonic acid and reacted with carboxylic acids to form C6-O-esters [54, 55]. In more alkaline conditions (high pH), the C6 OH group will become ionized and more nucleophilic than the amino group. Then O-alkylation will be preferred in reaction with alkyl halides and other electrophiles [56, 57].

Chitosan is soluble in acidic aqueous solution and poorly soluble or insoluble in organic solvents, especially non-polar solvents. The required conditions for *N*-alkylation with electrophiles or *N*-acylation with acyl halides or anhydrides are usually basic. Conventional organic chemistry reactions are mainly carried out in aprotic organic solvents to avoid interference, especially from water, and also because many reagents are only soluble in such solvents. However, these conditions are not compatible with the solubility of chitosan. Therefore, many reported that reactions to obtain chitosan derivatives are carried out in organic solvent or organic solvent water mixtures with chitosan only partially in solution. These inhomogeneous conditions can limit the efficacy of the reaction and the selectivity.

3.2 Degree of Substitution and Structural Characterization

Numerous chitosan derivatives have been reported. There are probably dozens or hundreds of new types of derivatives reported every year, depending on the definition. The derivatives can vary in the substituent's chemical structure and the degree of substitution (DS), defined as the number of substituents on each monomer unit and reported in the range 0–1 or 0–100%. Furthermore, the chitosan starting material can also vary in the degree of acetylation and molecular weight, and this will influence the final structure, molecular weight, and the general properties of the resulting chitosan derivative. Also, the distribution of the substituents along the polymer can be a factor. It is generally assumed that the distribution is more or less

random, but this is not certain. Derivatives with multiple substituents are also more common now, and the number of possible structures is therefore limitless.

In many cases, derivatives are only characterized by FTIR or elemental analysis, which gives somewhat limited information about the structure. The molecular weight or degree of acetylation for the final product is rarely reported. Information about the chitosan starting material can also be limited. Thus it is often difficult to assess the relevance of the reported antimicrobial activity of chitosan derivatives. Limited data is sometimes provided, and the actual structure of the derivative can be different from what is reported. Thus, a detailed NMR analysis should be carried out and preferably size exclusion chromatography, to determine molecular weight and size distribution. However, even when such data exist, correct interpretation can be a challenge, and errors are not uncommon.

4 Antimicrobial Chitosan Derivatives

Although a large number of structures derived from chitosan have been reported, some structures are much more common than others and studied in several publications. Some of these derivatives are also commercially available, which makes them accessible for antimicrobial research.

4.1 Carboxymethyl Chitosan (CMC)

Carboxymethyl chitosan (CMC) is one of the more common chitosan derivatives. Muzzarelli first reported it in 1982 [58, 59], and in 2020 there were more than 300 ISI publications where CMC was used in some way. It is commercially available from many companies and can be synthesized by reaction with 2-chloroacetic acid in water/isopropanol solvent with NaOH as a base to give *N, O* alkylated CMC [60] (Fig. 3). Alternatively, an *N*-selective modification can be done by reductive alkylation with glyoxylic acid [61]. It has diverse uses, including CMC-based nanoparticles for drug delivery applications [62], absorbent to remove dye from waste-water [63], and for coating and stabilizing metallic catalytic nanoparticles [64]. The part of the structure is similar to ethylenediaminetetraacetic acid (EDTA), and CMC can therefore chelate metal ions. The binding to Ca^{2+} has been utilized to stabilize CMC nanoparticles [20] and bind the polymer to dental pulp [65]. CMC–zinc complexes have been prepared and applied as antimicrobials against *S. aureus* and *E. coli* [66]. This derivative has also been used in combination with silver nanoparticles to prepare durable antibacterial cotton fabric [67], to prepare tetracycline loaded nanoparticles to treat intracellular *S. aureus* infections [68], and to prevent biofilm formation by Gram-positive and Gram-negative bacteria [69]. Carboxymethyl chitosan has also further modified for enhanced antimicrobial activity, and this has included grafting with poly(*N*-vinylimidazole) [70] and

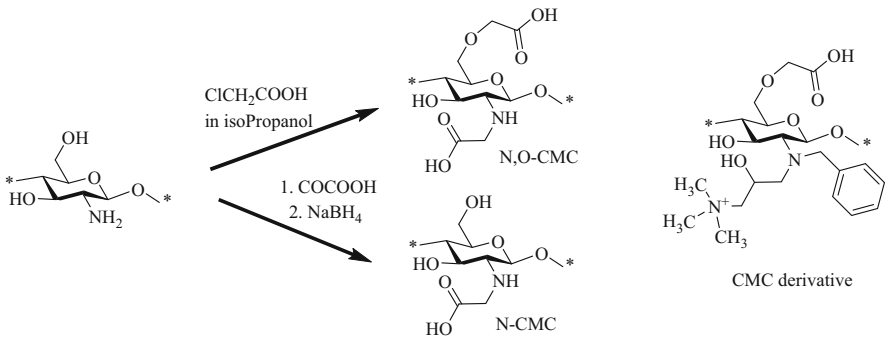


Fig. 3 Carboxymethyl chitosan (CMC) synthesis and an example of a reported antimicrobial CMC derivative [71]

N-alkylation and further reaction with *N*-(3-chloro-2-hydroxy-propyl) trimethylammonium chloride to introduce both lipophilic and quaternary ammonium groups [71].

4.2 N,N,N-Trimethyl Chitosan (TMC)

N,*N*,*N*-trimethyl chitosan is probably the second most studied chitosan derivative. It can be synthesized by reacting chitosan with suitable methylation reagents, such as methyl iodide or dimethyl sulfate. The solvent *N*-methyl pyrrolidone (NMP) is most commonly used for this reaction in combination with NaOH as the base [72]. This reaction is not fully selective, and a high level of *N,N,N*-trimethylation is usually accompanied by a high level of *O*-methylation, which can reduce the solubility of the product [73]. The *O*-methylation can be avoided by using alternative procedures, including reductive dimethylation followed by *N*-methylation in a second step [48] or by using DMF/H_2O as the solvent for the reaction [74]. (Fig. 4) There has been considerable interest in TMC as an absorption enhancer affecting tight junctions [75]. It has been used as an absorption enhancer on the intestinal [76], nasal [77, 78], and pulmonary [75, 79] for drug delivery. It has also been used for non-viral gene delivery [80]. TMC nanoparticles have been used for drug delivery, including insulin [81, 82] and siRNA delivery [83]. *N*,*N*,*N*-trimethyl chitosan has a permanent cationic charge and has good aqueous solubility independent of pH. It is more active against Gram-positive and Gram-negative bacteria than chitosan, especially at pH above 7 [49, 84, 85]. Antimicrobial TMC fibers have been used in wound treatment [86], antibacterial coating for polypropylene and polylactic acid nonwovens [87], and for antimicrobial filtration membranes for drinking water treatment [88]. This derivative has also been used in combination with antimicrobial drugs. Xanthar gum/TMC hydrogel has been prepared for encapsulation and delivery of ciprofloxacin and investigated for antimicrobial activity against *S. aureus* and *E. coli* and

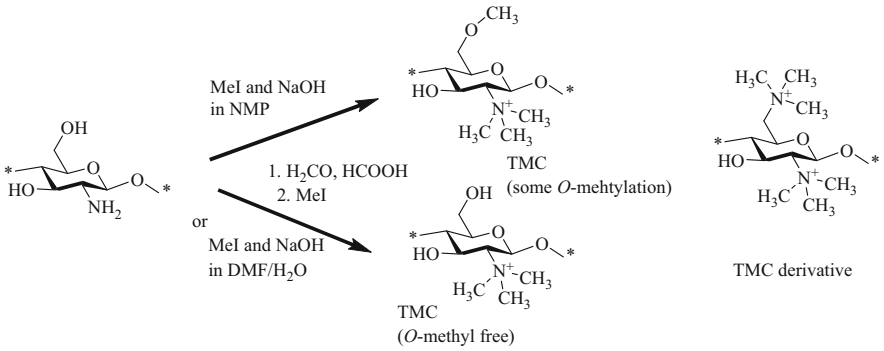


Fig. 4 *N*,*N*,*N*-trimethyl chitosan (TMC) synthesis and an example of a reported antimicrobial TMC derivative [91]

cytotoxicity toward human lung cell lines [89]. Vancomycin-loaded nanoparticles based on TMC have been reported and investigated for drug release kinetics and antimicrobial efficacy in vitro [90]. Investigations have also focused on analogous *N*-alkyl derivatives with longer alkyl chains such as ethyl, propyl, and hexyl. Thus *N*, *N*,*N* triethyl, *N*, *N*-dialkyl-*N*-methyl, and *N*-alkyl-*N*-methyl derivatives of chitosan have also been reported and investigated for antimicrobial activity [91–93]. Further derivation of TMC has also been reported, including C6-NH_3 and C6-$N(CH_3)_3$ deoxy derivatives [91] and 6-O-2-hydroxypropyl-3-(trimethylammonium) derivatives [94]. According to these publications, the increased cationic charge contributed to enhanced antibacterial activity relative to TMC and chitosan. This derivative has also been used for vaccinations. This includes the use of TMC microparticles as a delivery system for mucosal adjuvant LTK63 in intranasal immunization of mice with meningococcal conjugated vaccine [95]. Recently a self-assembly of a self-adjuvanting nanovaccine delivery system from TMC and polyanionic oligopeptide has been reported [96].

4.3 N-(2-Hydroxyl) Propyl-3-Trimethyl Ammonium Chitosan (HTC)

N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan (HTC) or HTC chloride (HTCC) is another common quaternary ammonium derivative of chitosan with good aqueous solubility in acidic, neutral, and alkaline solutions. It is synthesized by reacting chitosan with glycidyl trimethyl ammonium chloride (Fig. 5). Pharmaceutical applications include its use in hydrogels for nasal delivery of macromolecular drugs [97] and gene delivery [98]. It has been reported to possess significant activity against Gram-positive and Gram-negative bacteria and pathogenic fungi, with a minimal toxic effect on human erythrocytes and embryo kidney cells [99]. It can therefore be considered as a suitable antimicrobial agent for topical applications.

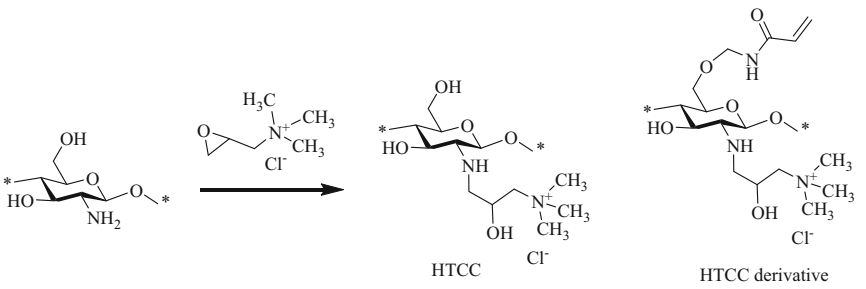


Fig. 5 *N-*(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) synthesis and an HTCC derivative with a fiber-reactive group [100]

It has been modified with fiber-active group and used as antimicrobial textile finishing [100, 101] and included in nanofibrous antimicrobial mats produced by electrospinning from blends with rectorite and polyvinyl alcohol [102]. Carboxymethyl cellulose/HTTCC films have been developed and shown to be effective against *S. aureus* and *E. coli* in vitro [103]. The blend was used to coating for bananas to extend the shelf life. This chitosan derivative has also been mixed with silver nanoparticles and graphene oxide and used to produce antibacterial and antifungal contact lens [104]. These were also loaded with the antifungal drug voriconazole, to further increase the activity. The materials showed antimicrobial efficacy in vitro, and in vivo tests of the lenses demonstrated efficacy for treating fungal keratitis in a mouse model. The disinfectant chlorhexidine has been included in injectable HTCC hydrogel for periodontal application to prevent infections caused by bacteria in dental plaque [105]. HTCC capped mesoporous silica nanoparticles have been investigated as delivery system for the fungicide pyraclostrobin to be used as an agricultural pesticide to control *Phomopsis asparagi* (Sacc.) infestation [106]. Nanoclay modified with HTCC has been investigated for tissue engineering application and shown to be highly biocompatible and increase cell growth in tissue culture as well as having a bactericidal effect on *S. aureus* and *E. coli* [107]. HTCC has also been reported as a broad range inhibitor of coronavirus entry [108].

4.4 Hydroxypropyl Chitosan (HPC)

Hydroxypropyl chitosan (HPC) is a derivative of chitosan with improved water solubility and film-forming properties. It is synthesized by the reaction of propylene epoxide with chitosan under alkaline conditions (Fig. 6). It has been reported to be active against *S. aureus* and *E. coli* as well as the fungal species *A. mali*, *C. diplodiella, F. oxysporum*, and *P. piricola* [19]. This derivative has been used to formulate medicated nail lacquers containing antifungal agents such as ciclopirox and amorolfine for treating onychomycosis, a fungal infection of the nails [109–

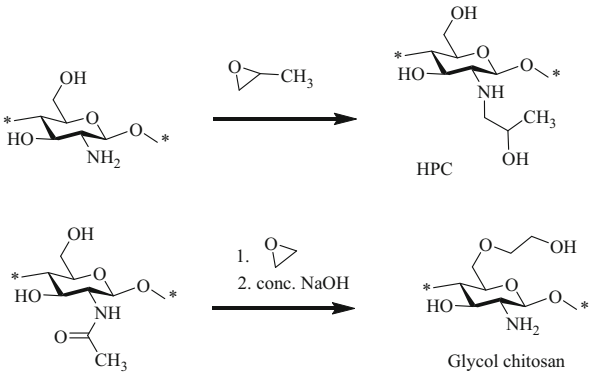


Fig. 6 Synthesis of hydroxypropyl chitosan (HPC) and glycol chitosan

111]. HPC has been photo-crosslinked for application in wound dressing and conjugated [112] with cyclodextrin for drug delivery applications [113]. Antimicrobial derivatives prepared by graft co-polymerization with maleic acid [114] and HPC grafted with the antimicrobial peptide nisin have also been reported [115].

4.5 Glycol Chitosan

Water-soluble glycol chitosan is prepared by treating chitin with oxirane (ethylene oxide) followed by deacetylation with a strong base (Fig. 6) [116]. This derivative is commonly used in drug delivery applications and has been modified with deoxycholic acid to self-assemble into nanoparticles [117] that have been used for tumor-specific delivery of anticancer drugs [118, 119]. It has also been *N*-acetylated with palmitic acid NHS ester and quaternized to make polysoap intended for drug delivery applications [120] and to coat liposomes to make pH-responsive drug delivery systems [121] and injectable hydrogels [122]. In contrast to other commercially available derivatives, there are relatively few investigations into the antimicrobial properties of glycol chitosan. However, there are a few examples of such application of glycol chitosan. It has been cross-linked with PEG-diglycidyl ether to form pro-angiogenic hydrogels that could inhibit *S. aureus* [123], and it has been used to coat iron oxide nanoparticles to form antibacterial superparamagnetic nanoparticles [124].

4.6 Other Derivatives

Other notable chitosan derivatives include *N*-carboxybutyl chitosan. This derivative was originally reported by Muzzarelli et al. [125] and subsequently studied for wound healing [126] and as an antimicrobial substance [8] (Fig. 7). It is obtained

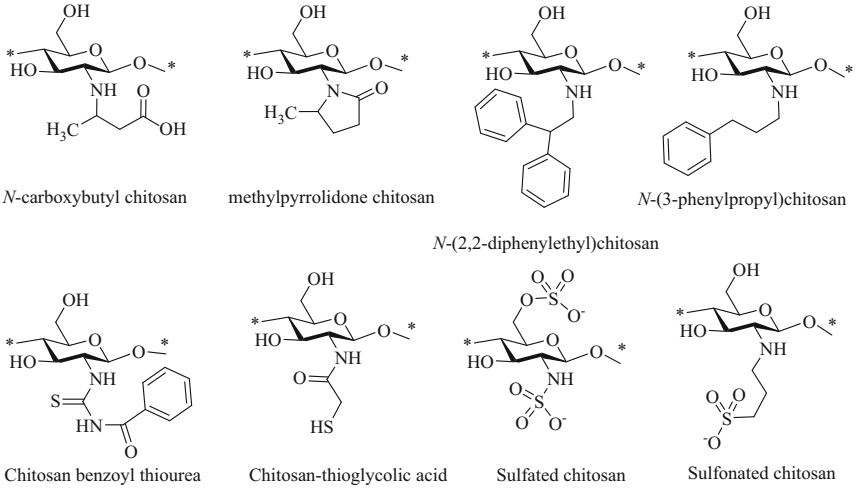


Fig. 7 Structure of some chitosan derivatives with reported antimicrobial activity (many of the reported derivatives had DS that is considerably <1)

by reaction with levulinic acid (4-oxo-pentanoic acid) in the presence of reducing agent. This also results in a ring formation to give 5-methyl pyrrolidone chitosan, especially with a slow addition of the reducing agent [127]. Raeba et al. [128] investigated a series of *N*-alkyl derivatives obtained by reductive alkylation and found that s *N*-(2,2-diphenylethyl) chitosan was most active against the gray mold *Botrytis cinerea* fungi and the rice leaf blast pathogenic fungi *Pyricularia grisea*. Another such derivative, *N*-(3-phenylpropyl) chitosan, strongly inhibited larval weight gain of the cotton leafworm *Spodoptera littoralis*. Benzoyl thiourea derivatives of chitosan have been synthesized and found to be active against fungi and bacteria [129, 130]. Geisberger et al. [131] reported that chitosan-thioglycolic acid (amide derivative) was considerably more effective against Gram-positive *Streptococcus sobrinus*, Gram-negative *Neisseria subflava,* and the fungi *Candida albicans* than either TMC or CMC. In contrast other researchers have found that similar thioglycolic acid derivatives of chitosan were not more active against bacteria than chitosan [132, 133]. The anionic derivatives chitosan sulfate [134] and sulfonated chitosan [135] have also been found to be active against *E. coli* and *S. aureus*.

4.7 Chitosan Conjugates

Some bioactive moieties can also be covalently linked to chitosan polymer to form conjugates. Chitosan conjugates are perhaps most commonly prepared by covalently linking natural antioxidants to the polymer. Many publications report grafting of antioxidants such as gallic acid, ferulic acid, caffeic acid, cinnamic acid, p-coumaric

acid, salicylic acid, and catechin to chitosan [136]. The grafting of antioxidants results in increased antioxidant and radical scavenging activity of polymer. Different methods have been used to graft these groups on chitosan, including the free-radical grafting method, enzyme-mediated synthesis, and activated ester mediated synthesis. These investigations are often inspired by the potential use of these chitosan conjugates in food products to serve as preservatives, reduce oxidation, or as neutraceuticals. Some investigators have reported increased antimicrobial activity resulting from the conjugation of antioxidants. Different flavonoids were enzymatically grafted onto chitosan fibers using tyrosinase and investigated for antioxidant and antimicrobial activity [137]. Some of the flavonoids increased antimicrobial activity of chitosan against *Bacillus subtilis* and *Pseudomonas aeruginosa*. Vanillin and 4-hydroxybenzaldhyde were linked to chitosan by reductive alkylation [138]. Films prepared from *N*-vanillyl chitosan and *N*-4-hydroxybenzyl chitosan were similarly effective in reducing the biomass of *Aspergillus flavus* fungi as films prepared from unmodified chitosan, but the films prepared from the conjugates had a marked effect of reducing B_1 and B_2 aflatoxins produced by the fungus by 98.9% and to non-detectable levels, respectively. Laccase from *Trametes versicolor* was used to graft gallic acid and caffeic acid onto chitosan [139]. This modification increased antioxidant activity and activity against *Escherichia coli* and *Listeria monocytogenes*.

Antimicrobial peptides are a part of the innate immune defense of vertebrates, plants, and invertebrates. Some peptide chitosan conjugates have been reported and investigated for antimicrobial activity. Antimicrobial ε-poly-L-lysine peptides were grafted onto vinyl modified chitosan using thiol-ene click chemistry [140]. The resulting conjugates showed enhanced, selective, broad-spectrum antibacterial activity toward Gram-negative *E. coli* and *Pseudomonas aeruginosa,* and Gram-positive bacteria *Enterococcus faecalis* and methicillin-resistant *S. aureus* (MRSA), and antifungal activity against *Candida albicans* and *Fusarium solani*. The antimicrobial CysHHC10 peptide was grafted to the C-2 (amino) or C-6 (hydroxyl) position of chitosan backbone via thiol-maleimide "click" conjugation, utilizing the maleimidohexanoic linkers [55]. Both peptidopolysaccharides exhibited reduced hemolytic activity and cytotoxicity relative to unmodified CysHHC10 peptide. The peptidopolysaccharides were used to prepare coatings formed via layer-by-layer assembly with tannic acid. These coatings killed the adhered bacteria upon contact while maintaining more than 60% viability for the adhered fibroblasts. We have reported grafting of a short antimicrobial peptide, anoplin, to chitosan *N*-(2-azido) acetyl polymers by copper-catalyzed alkyne–azide "click chemistry" coupling (CuAAC) to give conjugates with different degree of substitution (6–23%) [141] (Fig. 8). This resulted in increased activity against *S. aureus, E. coli*, *E. faecalis*, *P. aeruginosa* relative to chitosan and anoplin and a remarkable reduction in the hemolytic activity (HC_{50}) for human erythrocytes. Thus, more than a 100-fold increase in selectivity ($HC_{50}/MIC_{E.\ coli}$) relative to anoplin for four conjugates and more than a 10-fold reduction for the other two conjugates.

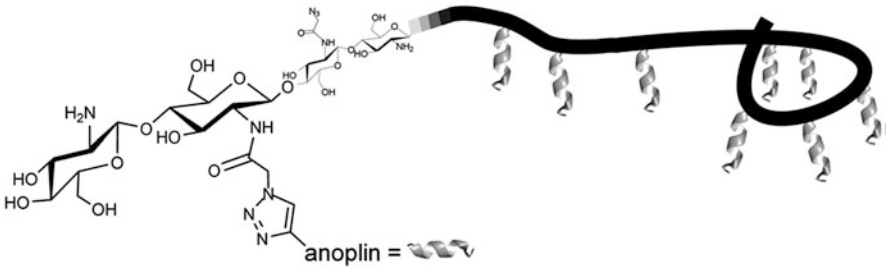


Fig. 8 Schematic representation of anoplin–chitosan conjugates. From reference [141]. (Published with permission from Royal Society for Chemistry)

4.8 Nanoparticles, Hydrogels, and Coatings

Nanoparticles based on chitosan are a very active research field. Chitosan-based nanoparticles have been developed for various gene and drug delivery applications [142–144], and there are also numerous reported applications for nanoparticles based on chitosan derivatives such as CMC [20], HTCC [145], and TMC [146]. These nanoparticles are often functionalized with moieties targeting cellular receptors to provide more selective delivery to specific cell types or tissue. Cancer treatment is probably the most common aim for therapeutic nanoparticle development, but chitosan-based nanoparticles have also been developed as a potential treatment for infectious disease and as disinfectants. In most cases, such nanoparticles are reported as delivery systems for other antimicrobial compounds or antibiotic drugs. Examples of this include chitosan-based nanoparticles imine-grafted with the antiseptic essential oil components eugenol and carvacrol [147]. The eugenol and carvacrol grafted chitosan nanoparticles had equivalent or better antibacterial activity than that of the unmodified chitosan nanoparticles. The grafted nanoparticles were less cytotoxic toward 3T3 mouse fibroblast than pure essential oils. Then frog-skin derived antimicrobial peptide temporin B has been encapsulated in chitosan nanoparticles [148]. The encapsulation increased antibacterial activity and reduced the peptide's cytotoxicity against mammalian cells. The nanocarrier provided sustained antibacterial action against *Staphylococcus epidermidis* for at least 4 days, with up to 4-log reduction in the number of viable bacteria compared to chitosan nanoparticles that did not contain the peptide. Although most studies have similarly used chitosan nanoparticles to encapsulate or as substrate to graft other antimicrobial substances, there are some reports where chitosan is the only antimicrobial material used in the preparation of nanoparticles. Chitosan-alginate nanoparticles were prepared and found to be active against *Propionibacterium acnes* [149]. The nanoparticles also inhibited *P. acnes*-induced inflammatory cytokine production in human monocytes and keratinocytes. Chitosan nanoparticles were prepared by mixing the cationic polymer with anionic proteins isolated from *Penicillium oxalicum* [150]. These particles inhibited the growth of the fungi *Pyricularia grisea*, *Alternaria solani,* and *Fusarium oxysporum.*

Chitosan and chitosan derivatives have also been formulated into hydrogels intended for antimicrobial treatments. Such hydrogels are often intended for wound treatment to provide dual action for stimulating wound healing and providing antimicrobial action to prevent or treat wound infections. As with nanoparticles, it is common to include some other antimicrobial substance in such formulations, but there are also reports where chitosan or chitosan derivatives are the only antimicrobial substance. An example of this is chitosan hydrogels with deposited hydroxyapatite [151]. The biocompatibility of chitosan-hydroxyapatite composite membranes was evaluated in MTT assay and it was found that they had no effect on the growth of MG-63 osteosarcoma cells. Superabsorbent hydrogels have been prepared form HTCC-g-poly(acrylic acid-co-acrylamide) co-polymers, including N,N-′-methylenebisacrylamide (MBA) as a cross-linker [152]. The antibacterial activity against *S. aureus* and *E. coli* increased with the HTCC weight content up to 50–60% but decreased with higher content.

Chitosan and chitosan derivatives have also been used to coat other materials with the aim of reducing antimicrobial fouling of the surface. Thus HTCC has been used to coat titanium implants [153]. The coating prevented adherence of *S. epidermidis* to the surface. Medullary cavities of rat femora were contaminated with *S. epidermidis*, and a coated and untreated titanium rods were simultaneously implanted into the medullary canals. Implant-associated infection occurred in the control group with uncoated titanium implants, while the infection was prevented with HTCC-coated implants.

4.9 Synthesis of Antimicrobial Derivatives Utilizing Protection Groups

It can be challenging to control the synthesis of chitosan derivatives to obtain the desired DS for the substituent and avoid modification of groups (-NH_2 or –OH) that are not a target for the reaction. Protection groups have been introduced to allow fully regioselective and chemoselective modification of the polymer. The protection groups can also improve solubility in organic solvents. This is also beneficial because some reagents will react with water and some reactions are not compatible with aqueous solutions. This approach was mainly pioneered by Kurita and co-workers [154–156]. They reported the synthesis of various chitosan derivatives where the phthaloyl protection of the amino groups was utilized to achieve C6 regioselective modification (Fig. 9). This protection group can be introduced by reaction with phthalic anhydride in DMF. The phthaloyl protection group can be removed in the final step, following the C6 modification, by reaction with aqueous hydrazine (N_2H_4), to obtain the desired derivative. Thus phthaloyl chitosan has been used as precursors for the synthesis of antimicrobial derivatives such as maltoside and mannoside branched chitosan [157]. The maltoside branched chitosan was less active than chitosan in suppressing *Bacillus subtilis, S. aureus, E. coli, Streptococcus*

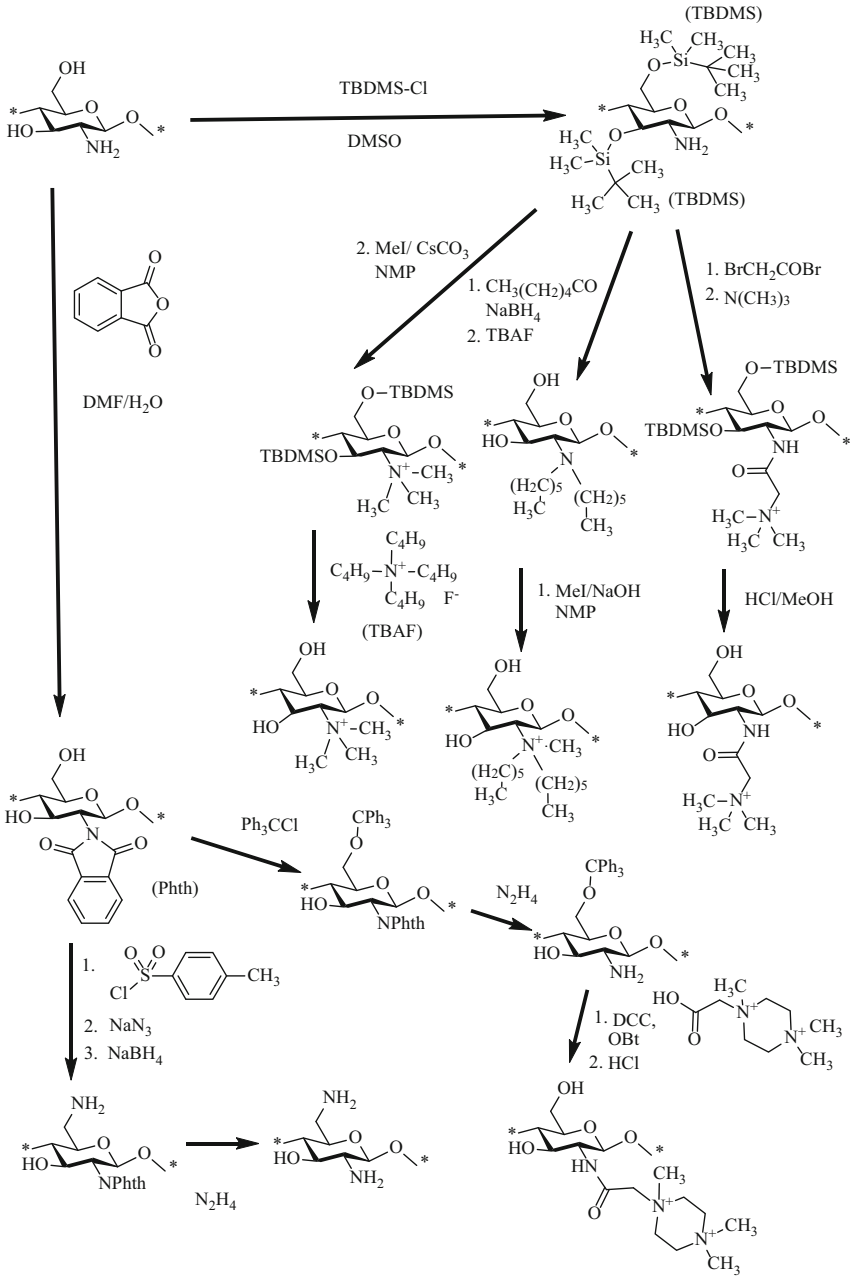


Fig. 9 Some examples of synthesis of antimicrobial chitosan derivatives utilizing protection groups

mutans, and *C. albicans* growth, but mannoside chitosan was more active than chitosan against *B. subtilis* and *P. aeruginosa*. The phthaloyl protection was also utilized for the synthesis of 6-amino-6-deoxychitosan, which was soluble and active against *S. aureus*, *E. coli*, *P. aeruginosa,* and *A. niger* at pH 6.5 and 7.5 [158]. *N*-phthaloyl chitosan has been mixed with polyethersulfone to prepare an ultrafiltration membrane with antifouling property [159]. Fully acetylated chitin can also be used as *N*-protected chitosan, like in the synthesis of glycol chitosan, but C6 modification reactions are usually more efficient with *N*-phthaloyl chitosan [160].

Furthermore, *N*-phthaloyl chitosan can be used as a precursor for the synthesis of 6-O-triphenylmethyl (trityl) protected chitosan [156]. The trityl-protected chitosan can be used for *N*-selective modifications in NMP and the trityl group then removed by treatment with acid. This approach was for the synthesis of a series of chitosan betainates [161], *N,N,N*-trimethylated chitosan [84], and piperazine derivatives [162] at different degrees of substitution. These derivatives were further studied to show that the antibacterial activity is dependent on the DS [84, 163, 164]. Our group has introduced the *O*-selective protection of chitosan by reaction of chitosan mesylate with tert-butyldimethylsilyl chloride (TBDM-Cl) in DMSO [165, 166]. (Fig. 9) The advantage of this protection strategy is that it allows for fully chemoselective modification of the amino groups and that di-TBDMS chitosan is fully soluble in CH_2Cl_2. Therefore, reactions can be carried out in a solvent that is commonly used in organic chemistry and would otherwise not be compatible with chitosan as a reagent. The TBDMS protection group can be fully removed in the final step by treatment with acid or suitable fluoride ion reagent such as tetrabutylammonium fluoride (TBAF). TBDMS chitosan has been used for fully selective synthesis of *N*-alkyl, *N*-dialkyl, and *N,N,N*-trialkyl derivatives, quanidilated and quaternary *N*-acyl derivatives with up to 100% degree of substitution, which were used in the investigation of the structure-antibacterial activity relationship [85, 93, 167] and efficacy for eradicating *S. aureus* biofilms [168].

5 Structure-Antimicrobial Activity Relationship

5.1 Degree of Acetylation (DA)

It is generally agreed that an increase in the degree of acetylation (DA) will reduce the antimicrobial activity of chitosan. Fully *N*-acetylated chitosan (chitin) is not active against bacteria and also insoluble aqueous solutions. However, the effect of a moderated DA is less clear. Younes et al. [169] studied the influence of DA and molecular weight of chitosan on antimicrobial activity. They prepared 15 samples of chitosan with DA ranging from 2 to 61% and Mw ranging from 43 to 135 KD. They measured the MIC values for four Gram-positive bacteria (*S. aureus*, *B. cereus*, *E. faecalis,* and *M. luteus*) and four Gram-negative bacteria (*E. coli, P. aeruginosa,*

K. pneumoniae, and *S. typhi*) as well as three types of fungi (*A. niger*, *F. oxysporum*, and *A. solani*). In addition, they did an agar diffusion assay and investigated the reduction in colony count. The conclusion of the study was that DA reduced activity against bacteria, whereas it had a variable effect on fungi. However, a closer look at the data shows that there was no increase or only one dilution (two-fold) increase in the MIC values with DA = 24% or less in most cases. In the case of fungi, the DA had hardly any effect with a one-dilution difference or no difference in the MIC values. Blagodatskikh et al. [170] investigated the activity of *N*-reacetyated oligochitosan against *S. aureus* and *E. coli* and found maximal activity when DA was 16–28%. We have investigated the antibacterial activity of *N*,*N*,*N*-trimethylated, and *N*-acyl quaternary chitosan derivatives where each derivative was synthesized from five different chitosan starting materials with DA ranging from 6 to 34% [85]. There was some difference (up to two dilutions or more) between derivatives prepared from different starting materials, but overall there was no clear trend in the influence of DA on activity. Thus it can be concluded that although a significant increase in DA may have a negative effect on the antimicrobial activity, the influence of moderated DA (up to 20–30%) is limited and can even be beneficial.

5.2 Charge

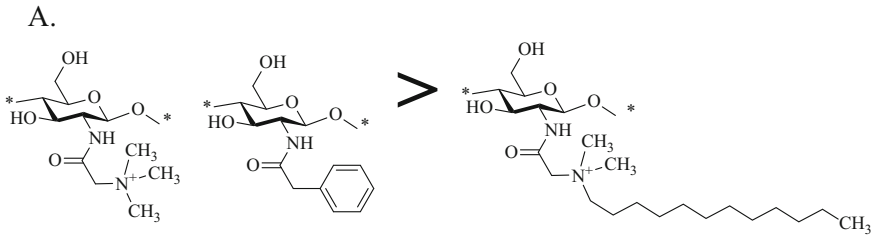
Chitosan is polycationic in acidic solution, which is a unique property for a natural polysaccharide. The polycationic charge is generally thought to be the most significant factor causing an antimicrobial effect due to electrostatic interaction with the cell surface. This is also supported by the fact that other well-known antimicrobial polymers such as polyethyleneimine, poly-lysine, and DEAE dextran are also polycationic. The pKa of chitosan is around 6.5. It is often stated that chitosan is only active at pH below this value because significant electrostatic interaction between the anionic cell surface and the polymer is required for activity. However, our results and some reported work in the literature do not fully support these statements. Investigations of antimicrobial activity are complicated because the chitosan has limited solubility above pH 6. The activity may seem low simply because the sample is not fully in solution. Recently we used chitosan (Mw 103 KD, DA = 17%) as reference material for investigations of antibacterial chitosan derivatives and observed that chitosan was equally active against *S. aureus* at pH 5.5 and 7.2 (MIC = 256 μg/ml) [133]. Chitosan was also effective against *E. coli* at 7.2. However, the MIC value for *E. coli* was four times higher at pH 7.2 (2,048 μg/ml) than at pH 5.5 (512 μg/ml). Some publications support that chitosan lacks significant activity at pH 7 [171, 172], whereas others have reported activity at and above pH 7 [170, 173, 174]. It has also been observed that chitosan microparticles are active

against *E. coli* at pH 7 [40]. Most well-known antimicrobial chitosan derivatives such as TMC and HTCC have a quaternized ammonium group with permanent cationic charge, and this is the most common feature of many reported antimicrobial chitosan derivatives. However, some studies have reported the antimicrobial activity of derivatives like CMC and sulfonates that will be zwitterionic or have net – anionic charge under some conditions. The importance of the polycationic charge is therefore not fully understood.

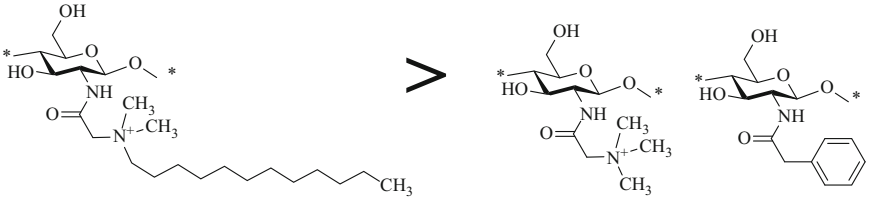
5.3 Substituent Structure

When new derivatives are introduced and discussed in publications, it is often implied that the increase in positive charge and/or lipophilic moieties is causing the observed increase in activity. However, there are few systematic studies to investigate the structural requirements for an active chitosan derivative. Ideally, such derivatives should be studied at around 100% DS so that different structures can be compared more accurately. We have done some studies to compare the effect of the structure of different substituents in chitosan with 100% or very high DS, on antibacterial activity. Thus we have studied the activity of *N*-(2-quaternary ammonium) acetyl derivatives of chitosan for activity against bacteria [49]. The N-acetyl (2-*N*,*N*,*N*-trimethyl ammonium) and the *N*-acetyl (2-pyridinium) polymer derivatives, which had a small cationic substituent, were 4 to 128 times more active, based on MIC at pH 7.2, than the amphiphilic *N*-acetyl (dimethyl-dodecyl ammonium) derivative (Fig. 10a). Interestingly the reverse relationship was observed with analog derivatives of glucosamine monomer and short chitosan oligomer. In this case, the *N*-acetyl (2-dimethyl dodecyl ammonium) derivative was active against bacteria, and the N-acetyl (2-*N*,*N*,*N*-trimethyl ammonium, and 2-pyridinium) derivatives lacked activity.

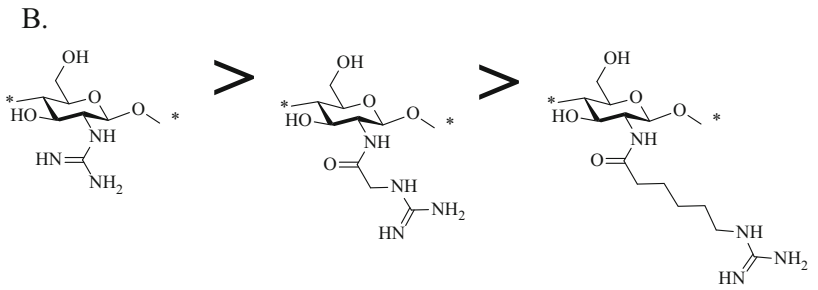
Similarly, we investigated the antimicrobial activity of a series of fully substituted *N*,*N*-dimethyl, *N*-alkyl and *N*-methyl, *N*,*N*-dialkyl, with methyl, ethyl, butyl, and hexyl alkyl chains against Gram-positive *S. aureus* and *E. faecalis* and Gram-negative *E. coli* and *P. aeruginosa* at pH 7.2 [93]. It was found that derivatives with shorter alkyl chains were most active against *S. aureus.* The *N*,*N*-dimethyl *N*-ethyl derivative was the most active derivative against this bacteria, whereas the activity against the other bacterial increased with alkyl chain length and the chain and in this case the *N*,*N*-dimethyl-*N*-hexyl derivative was most active. Investigation of hemolytic activity indicated that the *N*,*N*,*N*-trimethyl and *N*,*N*-dimethyl-*N*-dimethyl chitosan derivatives were more than 25 times less hemolytic and thus less toxic to erythrocytes than longer chain derivatives. A similar relationship was also observed for cytotoxicity against the Caco-2 cell line. It was concluded that *N*,*N*,*N*-alkyl quaternary chitosan derivatives with monomethyl and monoethyl chains exhibited significantly higher selectivity in antibacterial activity relative to cytotoxicity than the longer alkyl chain derivatives.



Chitosan polymer derivatives. Activity against *S. aureus* at pH 7.2



Glucosamine and chitosan oligomer derivatives. Activity against *S. aureus* at pH 7.2



Chitosan polymer derivatives. Activity against *S. aureus* and *E. coli* at pH 7.2

Fig. 10 Schematic illustration of the substituent structure-activity relationship for derivatives reported in Rúnarsson et al. [49] and Sahariah et al. [167]

We have also studied the influence of the position of the cationic charge relative to the polymer backbone. Thus *N*,*N*,*N*-trimethyl chitosan (TMC), where the cationic charge was on the polymer backbone, was compared to *N*-acetyl(2-trimethyl ammonium and 2-pyridinium) and *N*-hexanoyl (trimethyl ammonium and 2-pyridinium) derivatives where the charges were separated from the backbone with a two- and six-carbon spacer, respectively [85]. In the case of *S. aureus* the TMC derivative was most active, followed by the derivatives with two-carbon spacers. The six-carbon spacer derivatives were least active. A similar but not as clear relationship was observed for *E. coli*. A later study also confirmed these results and showed that *N*-guanidyl chitosan was highly active against *S. aureus* and *E. coli*, *N*-acetyl guanidyl chitosan was less active, and *N*-hexanoyl guanidyl chitosan lacked activity [167]

(Fig. 10b). Thus it can be concluded that activity is highly dependent on the substituent structure in fully quaternized chitosan derivatives. In polymer derivatives, small cationic substituents close to the polymer backbone seem, in general, the most active and less cytotoxic to mammalian cells than more amphiphilic derivatives. The most active derivatives have the cationic charge located on the polymer backbone, like TMC. Derivatives, where the charge is moved away from the polymer backbone by linking it through a flexible spacer, are expected to be less active.

5.4 Degree of Substitution (DS)

Most new antimicrobial chitosan derivatives are reported without studying the effect of the degree of substitution on activity. However, such studies are critical to confirm the potential benefit of the reported substituent. If a substituent is genuinely contributing to the activity, a positive relationship between activity and DS should be observed. We have studied the relationship between antibacterial activity and *N*,*N*,*N*-trimethylation of chitosan. A positive relationship between DS and activity against *S. aureus*, *S. aureus* MRSA, and *P. aeruginosa* at pH 7.2 was observed [84, 175] (Fig. 11a). The maximum activity (defined as 1/MIC) was reached with DS = 20–30% for the quaternary group, and further increase did not increase the activity. At pH 5.5, the influence of trimethylation was much less marked, and highly trimethylated derivatives were only slightly or not more active than chitosan against *S. aureus* and *E. coli* [84, 133]. A similar relationship was observed for *N*-quanidylated chitosan synthesized at 15%, 35%, 55%, and 100% degree of substitution [167] (Fig. 11b). There was about a 100-fold increase in activity when DS was increased from 15 to 55%. A further increase in the DS to 100% only doubled the activity (1 dilution difference).

Interestingly our recent study has shown a negative relationship between DS, for the hydroxypropyl group (in HPC), and antibacterial activity [133] (Fig. 11c). This was also the case for HTCC, where activity against *S. aureus* at pH 5.5 and *E. coli* at pH 5.5 and 7.2 was negatively correlated with DS. In contrast, DS in the range 37–110% did not affect activity against *S. aureus* at pH 7.2. These results contradict other studies which have reported HPC and HTCC as antimicrobial derivatives. However, the derivatization has a solubilizing effect, and this may cause these derivatives appear to be more active than chitosan in some conditions. Studies of the effect of DS on activity are important, and they have conclusively shown that the *N*,*N*,*N*-trimethylation, *N*-guanidylation, and N-acetyl 2-*N*,*N*,*N*-trimethylation contribute to antibacterial activity at pH 7.2.

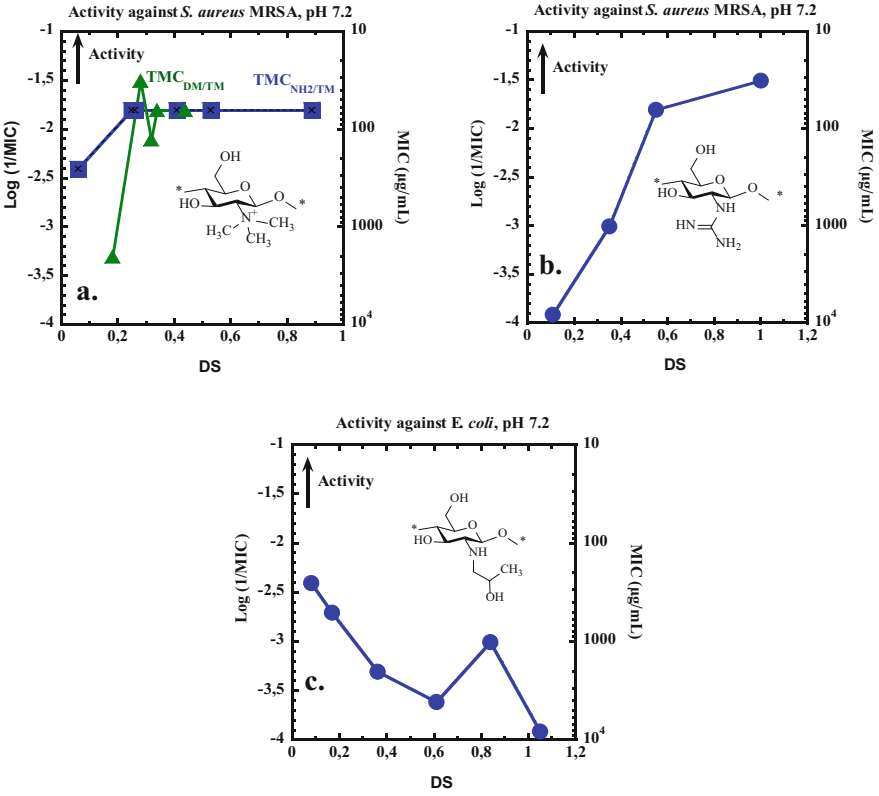


Fig. 11 Relationship between DS and antibacterial activity. Based on: (**a**) data for TMC for Rathinam et al. [175] ($TMC_{TM/DM}$ is partially *N,N,N*-trimethylated and the remaining amino groups are *N,N*-dimethylated. $TMC_{TM/NH2}$ is partially trimethylated, whereas there is no methylation on the remaining amino groups), (**b**) data for guanidyl chitosan from Sahariah et al. (2015), and (c) data for hydroxypropyl chitosan (HPC) from Rathinam et al. [133]

5.5 *Molecular Weight*

Almost all reviews on chitosan state that molecular weight is an important factor influencing chitosan's antimicrobial activity. However, further details on the relationship between molecular weight and activity are often not given in these reviews. Sometimes it is stated that the effect can vary considerably depending on the microorganisms. However, there is no lack of studies focusing on this topic. As early as 1984, Kendra and Hadwiger studied the antifungal effect of chitosan oligomers (DP 1–7) and chitosan polymers against fungi [6]. Since then, there have been many more studies into the relationship between antimicrobial effect

and Mw of chitosan and chitosan derivatives. Most of these have been published in the last two decades. Rhoades and Roller studied the effect of chemically and enzymatically degraded chitosan against microbes causing food spoilage such as *Candida sp*., *Rhodotorula sp*., *S. ludwigii*, *Z. bailii,* and *S. cerevisiae* [176]. No et al. studied the effect of chitosan with Mw ranging from 28 to 1,671 KD against 11 species of Gram-positive and Gram-negative bacteria [177]. Zheng and Zhu studied the inhibition of *S. aureus* and *E. coli* growth with chitosan with Mw from <5 KD to 305 KD [178]. Omura et al. studied the antibacterial activity of chitosan oligomers and polymers with Mw ranging from 0.179 to 300–400 KD [179]. Qin et al. studied the effect of Mw on the growth of *S. aureus*, *E. coli,* and *C. albicans* with nine chitosan samples with Mw ranging from 1.4 to 400 KD [180]. Mellegard et al. studied the influence of DA and Mw ranging from 2.3 to 224 KD on activity against *B. cereus, E. coli,* and S. *typhimurium* [181]. Tikhonov and co-workers studied the effect of pH and Mw of chitosan against *S. aureus* (MRSA). Later they also studied the effect of Mw ranging from 0.73 to 600 KD against five species of fungi [182]. In 2014 Younes et al. reported a study of the degree of acetylation and Mw in the range 40KD to 110 KD on the activity against eight bacteria species and three species of fungi [169]. Tikhonov and co-workers also studied the effect of Mw on the antibacterial activity of reacetylated chitosan [170] and betainated oligochitosan [183]. This is not an exhaustive list of all important studies but indicates the number of high-quality research works on the relationship between molecular weight and chitosan. Notwithstanding this large number of studies, there is no clear consensus on the relationship between Mw and activity. Researchers have come to different conclusions from studies that involve the same organisms. However, a closer look at the published data may explain the differences. The conclusions can differ because the Mw may be limited and also differ between studies, and sometimes there is too much emphasis on small variations in the activity that may be only 1–2 dilutions (2–4 difference in MIC), which is not necessarily a significant difference in our experience. In a study published in 2019, we reported the relationship between the MW of TMC (93% DS) and activity against *S. aureus*. The study was done with 49 samples with Mw ranging from 2 to 144 KD. The advantage of using such a high number of samples is that results for just one sample should not significantly affect the observed relationship. We found a strong positive correlation between activity at Mw and activity at Mw below molecular weight which we defined as the critical molecular weight for activity (CMW). When the molecular weight was more than CMW the activity did not change, and the activity was not influenced by a further increase in Mw (Fig. 12a). The CMW for TMC was found to be around 20 KD. When data from other studies are examined a similar relationship can be observed. This is illustrated by the examples shown in Fig. 12b, c.

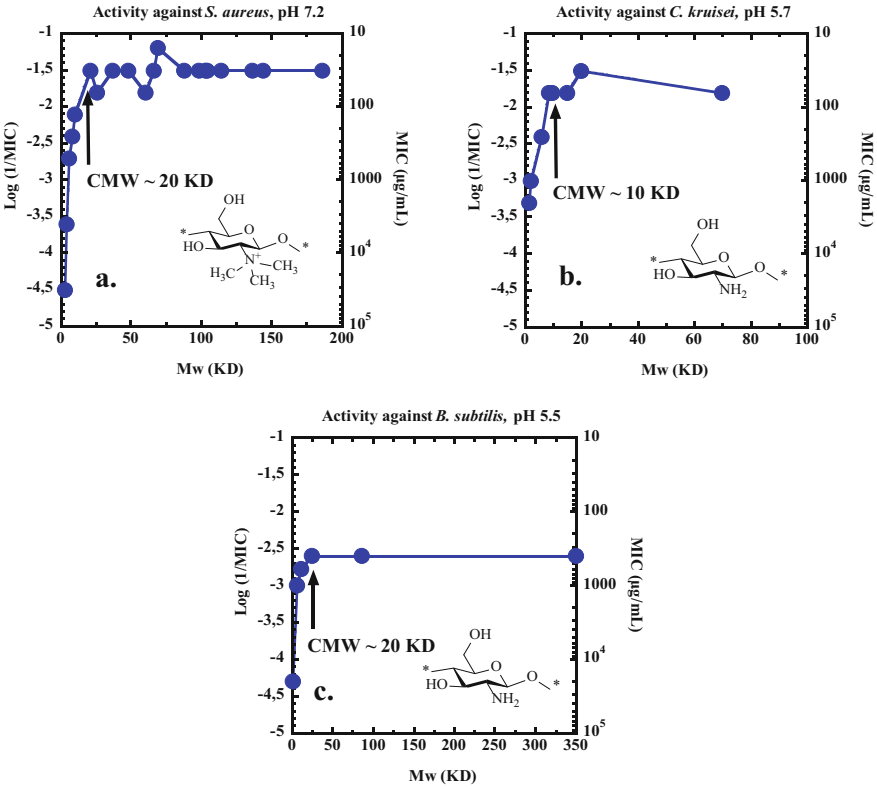


Fig. 12 Molecular weight activity relationship. (**a**) Based on data from Sahariah et al. [184], (**b**) based on data from Kulikov et al. [182], and (**c**) on data from Omura et al. [179]

5.6 Multiple Substituents

Some studies report chitosan derivatives with two or more substituents introduced on the chitosan backbone. In theory, the number of possible combinations with different DS for each substituent can be huge. This number becomes virtually limitless if there are many possible substituents and if the starting material's DA and Mw are also considered as additional variants. This large number of possibilities presents a challenge when it comes to the study of the structure-activity relationship and optimization of the structure. We have proposed to use the Design of Experiment (DOE) approach to obtain this information with a library containing a minimal number of representative chitosan derivatives. In a 2015 study, we used TBDMS protected chitosan for one-pot synthesis of derivatives with three types of substituents, *N*,*N*,N-trimethyl, *N*-stearoyl, and *N*-acetyl, introduced at different degree of substitution. A DEO software was used to propose a matrix of 15 derivatives, which were then synthesized and analyzed with the software to isolate factors with a

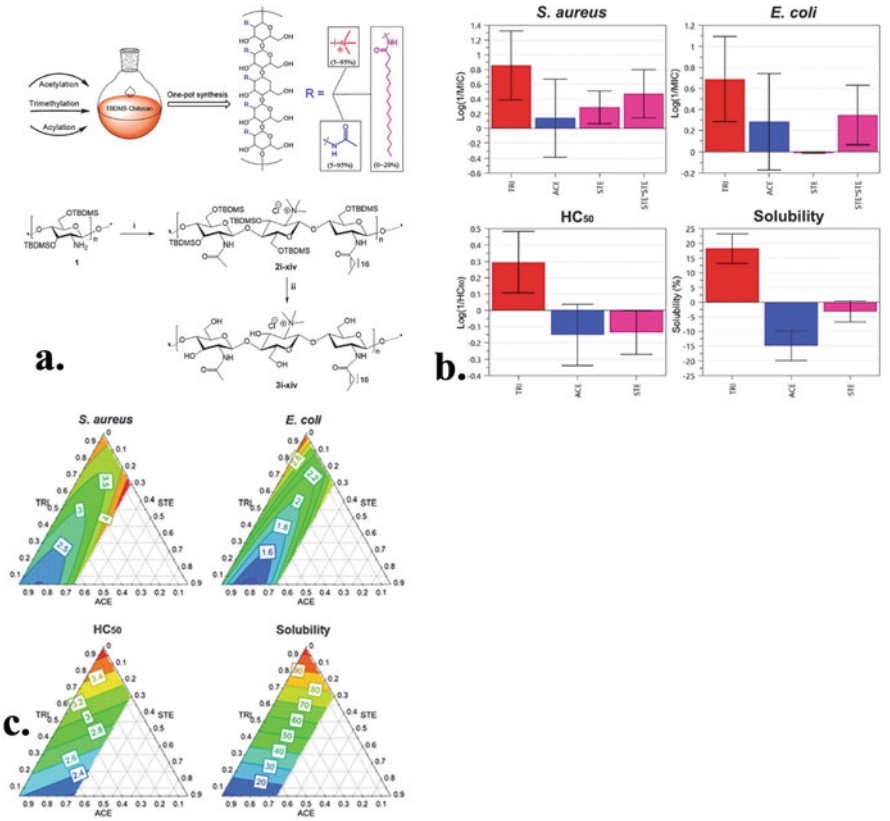


Fig. 13 (**a**) One-pot synthesis of chitosan of a series of chitosan derivatives with three different substituents, with different DS for each substituent, (**b**) coefficient plot for significant factors and interactions influencing the antibacterial activity, solubility, and hemolytic activity, (**c**) Response contour plots for antibacterial activity, hemolytic activity, and solubility. From Sahariah et al. [185]. (Published with permission from Royal Society for Chemistry)

statistically significant effect on solubility, antibacterial activity, and hemolytic effect. It was found that the optimized chitosan derivatives had around 65% degree of trimethylation, 5% degree of acetylation, and 30% degree of steroylation (Fig. 13).

6 Summary

The number of studies into the antimicrobial properties of chitosan and chitosan derivatives and potential applications in different fields such as the food industry, agriculture, health care, water treatment is continuously increasing. The

antimicrobial mechanisms of action have also been studied for more than four decades. It is often suggested that there are many different mechanisms of action and that these can vary depending on the microorganism. An impartial examination of published studies favors the cell membrane as the primary target, although the precise action mechanism is not fully understood. A large number of chitosan derivatives have been synthesized and investigated for antimicrobial properties. The more common chitosan derivatives, including CMC, TMC, HTCC, HPC, and glycol chitosan, have all been examined for antimicrobial application, but most such studies have involved using the cationic chitosan derivatives TMC and HTCC. Other reported derivatives have very diverse structures and can be either cationic, lipophilic, anionic, or zwitterionic. The drawback of many such studies is that they only report a single of few derivatives, which are often synthesized with just one DS. Investigations with a series of derivatives with different DS and prepared from starting materials with different DA and Mw are rare. In future research, there should be more emphasis on detailed studies of the structure-activity relationship. It is vital to show either a positive or a negative relationship between the DS and activity to understand the value of a new derivative fully. The Mw activity relationship has been studied for many decades, but there is still no clear consensus on the Mw activity relationship. Our recent study and reanalysis of published data suggest that most studies are showing a relationship where there is a positive relationship between Mw and activity until a specific molecular weight (CMW) is reached, and after that, there is no further increase in activity with increased Mw.

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Flavor-Related Applications of Chitin and Chitosan in Foods: Effect of Structure and Properties on the Efficacy

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Contents

Abstract Chitin and chitosan are natural and abundant polymers with extensive structural possibilities for physical and chemical modification. The well-known inherent astringency of chitin and chitosan limits their use in the food industry. However, structural and chemical modifications may be explored for their potential in improving the flavor of foods. This chapter reviews the flavor-related applications of chitin and chitosan including enhancing saltiness perception, improving flavor

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stability, and reducing the bitter taste of food products. Also, it focuses on the effects of the physical and chemical properties on the potency of utilization.

Keywords Chitin · Chitosan · Debitterization · Flavor modulator · Flavor stability · Saltiness perception · Warmed-over flavor

1 Introduction

Chitosan is a biopolymer derived from chitin which can be found in many places in nature, including the exoskeletons of crustaceans, insects, mollusks, and fungi [1–3]. The exoskeleton of crustaceans is the major source of biomass for chitin and chitosan production in industry [4] with an estimated 1.5 billion tons available worldwide. Derived from N-deacetylation of chitin, chitosan is the most abundant cationic natural polysaccharide. It consists of β 1–4-linked D-glucosamine and N-acetyl-D-glucosamine [5] (Fig. 1). The degree of deacetylation (DD) ranges from 40 to 98%, and the molecular weight ranges from 5×10^4 to 2×10^6 Da [3, 6, 7]. Chitosan has a broad array of applications such as in agricultural, water purification, food, cosmetics, textile, and biomedical industries, owing to its biodegradable, biocompatible, biofunctional, low toxicity, gelatinous, and antimicrobial properties [1, 3, 4, 8, 9]. The DD and the molecular weight of the polymer are two important parameters dictating the use of chitosan for various applications. For example, a high DD and low molecular weight lead to a relatively high ionization

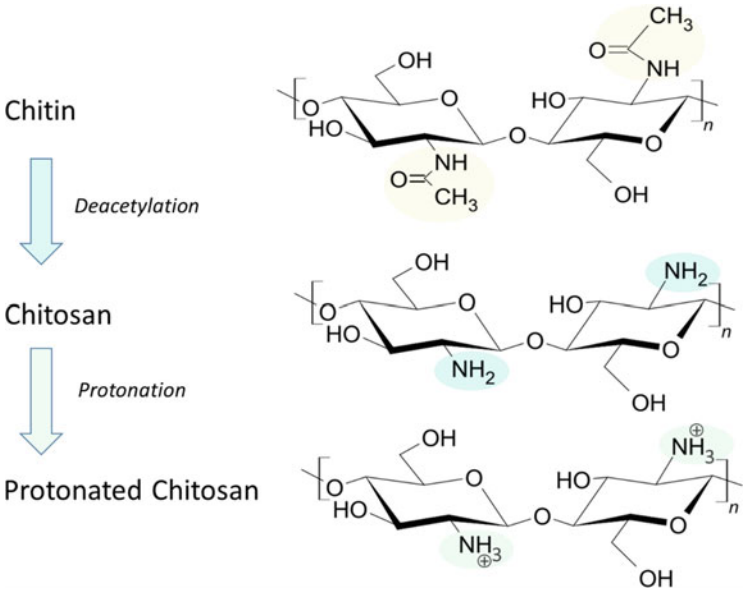

Fig. 1 Structure of chitin and chitosan

capability of chitosan, which could make it soluble in biological fluids and suitable for use in pharmaceutical fields.

Recently, the development of chitosan for use in food applications has progressed significantly and rapidly. In the food industry field, chitosan has an advantage over synthetic polymers, as it is considered to be GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA). In food-related products, it functions as a clarifying agent, preservative, pasting agent, dietary fiber, and flavor modulator. Among these applications, chitosan has received increasing attention and has been extensively explored in flavor modulation [7, 10]. The flavor of food is largely a combination of taste and odor, those are sensations that result when specialized nerve receptors in the mouth and nose detect molecules. It can be the primary quality attribute that contributes to consumer acceptance and preference. There is increasing evidence to suggest that flavor is becoming more important, perhaps because of more demanding consumers who ask for food with desirable organoleptic properties [10]. Unfortunately, some healthy food choices and ingredients with specialized biological functions are often viewed to be in conflict with enjoyable flavor. Moreover, food processing and storage are associated with losing preferable flavors and generating undesirable flavors. Therefore, it is a big challenge for academic and industrial researchers to design food products without sacrificing food flavor [11]. This review focuses on the most recent advances in the flavor-improving applications of chitosan in the food industry. This includes its use in taste masking, as a saltiness enhancer, and in preventing foods from developing off-flavors.

2 Applications of Chitin and Chitosan for Improving Saltiness Perception

2.1 Strategies for Reducing Sodium Intake

Sodium chloride (NaCl) is commonly used to season food. When dissolved in biological fluids, NaCl provides free Na^+ ions to the system which may bind to the taste bud receptor to cause a saltiness perception. As a food additive, NaCl not only provides a saltiness perception, but it also reduces the water activity of foods, which may inhibit microbial growth and therefore prolong the shelf-life of foods [12]. Moreover, it is implicated in several enzymatic reactions in food systems to modulate the texture, color, taste, and odor of food substances [13].

For the human body, sodium intake is necessary to maintain fluid balance in the body, and it is vital to muscle and nerve function. Research has indicated that 75–80% of sodium intake is from processed food products. Bakery products accounted for 13% of the sodium intake, while cereal products for 5%, processed meats for 18%, and sauces and spreads for about 11%. Spices, herbs, and salt purchased separately were responsible for 23% of the sodium intake [14]. However, excess sodium intake is associated with increased risk of non-communicable

diseases (NCDs) including stroke, hypertension, and cardiovascular disease, which are the leading causes of death globally. The World Health Organization (WHO) (2007) has recommended a daily NaCl intake (RDI) of less than 5 g. Nevertheless, the average daily NaCl intake in the USA, United Kingdom, and Asia is approximately 8.2–9.4 g, 9.4 g, and 12.0 g [15], respectively, which are much higher than the RDI suggested by WHO. Hence, techniques to reduce the salt levels in foods should be considered in order to help bring dietary sodium intake closer to those recommended for promoting public health.

Common methods for reducing the sodium level and improving the saltiness perception of foods include using substitutes such as KCl, $CaCl_2$, and $MgSO_4$ [16] as well as using flavor enhancers such as citric acid and monosodium glutamate [17]. However, the use of those metal salts and acids provides astringency and metallic flavors in food systems, thus their actual applications in food preparations are limited. Moreover, the release of sodium from the food matrix and its dissolution rate in the oral cavity have proven to be crucial for saltiness perception. Consequently, approaches such as modulating the size and shapes of salt crystals and altering the food texture have been explored recently for their potential as salt reduction substitutes [18, 19]. Saltiness is primarily perceived from free sodium ions rather than those bound with the food matrix. When consuming foods, a high proportion of sodium may be retained in the food matrix in a bound form even after being swallowed. This suggests that a significant amount of sodium might be taken into the body without being perceived [20]. Based on this principle, a higher proportion of free sodium ions released from the food matrix means that less salt addition is required in foods systems, and less sodium would be consumed [21]. Accordingly, the presence of negatively charged molecules in the food matrix (e.g., milk protein, soy protein, xanthan gum, and κ-carrageenan) would reduce saltiness perception, owing to the increased electrostatic interactions between sodium ions and the food matrix [22]. In contrast, positively charged groups interact with negatively charged groups through ionic interactions, which may therefore help release more free sodium ions and enhance saltiness perception [10].

2.2 Chitin and Chitosan as Saltiness Enhancers

The scientific publications reporting chitin and chitosan for saltiness enhancement applications are addressed in Table 1.

2.2.1 Particle-Shaped Chitin and Chitosan as Saltiness Enhancers

Free Na^+ generates the salty taste in food. Negatively charged food ingredients adsorb Na^+ through static electricity, creating bound Na^+ and reducing the level of saltiness [22]. However, positively charged food ingredients interact with negatively charged food ingredients to decrease the amount of bound Na^+, resulting in more

Table 1 Scientific publications reporting the saltiness enhancement applications of chitin and chitosan

Materials	Physicochemical properties	Forms	Applied foods	Outcome	Reference
Chitin	21–22% DD, 5.1–9.3 nm diameters	Nanofiber	Model solution	0.15 and 0.3 g/L of CNF showed remarkable saltiness enhancement potential (scored at 4.4 and 4.0 compared to 3.3 and 2.8 for groups without CNF, respectively)	[10]
	22.89–53.36 DD, 15.01–17.24 nm diameters	Nanocrystal, nanofiber	Model solution	The saltiness perception of CNC and DACNF suspensions performed higher than that of CNF suspension, inferring that lower aspect ratio and higher DD result in higher saltiness	[6]
	16.2% DD, 111 nm diameter	Nanofiber	Tilapia fillets	Curing solutions with 0.12–0.18 g/L of CNF are capable of providing stronger saltiness perception in the fillet than that without addition of CNF	[23]
	32.67–50.67 particle sizes	Nanoparticles	Model solution	Compared with the control group, the saltiness intensity of 0.03% chitin nanoparticle solution treated with ultrasonication was increased by about 30%	[5]
Chitosan	78.3% DD, 186.3 kDa molecular weight	Microparticles	Popcorn	The use of chitosan/NaCl microparticles provided a saltiness taste stronger than the general NaCl group. The level of saltiness can be maintained while the NaCl used can be decreased by up to 54.4%	[24]
	76.5% DD, 213.2 kDa molecular weight	Microparticles	Popcorn	Up to 45% of salt reduction can be achieved without a reduction of the saltiness perception of seasoned popcorn; the yield could be improved with addition of maltodextrin	[25]

DD degree of deacetylation, *CNF* chitin nanofibers, *CNC* chitin nanocrystals, *DACNF* deacetylated chitin nanofibers

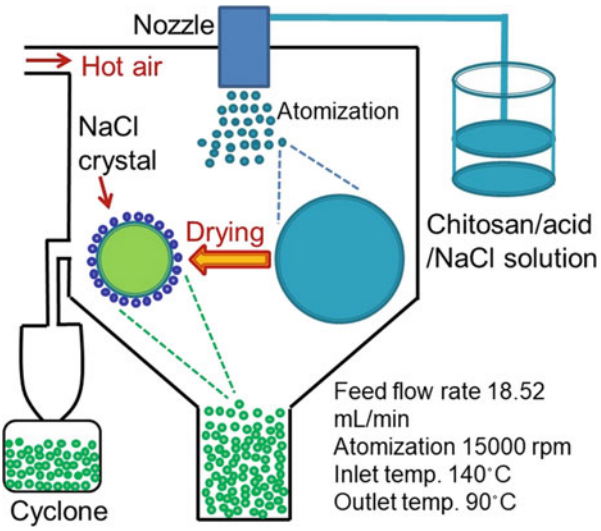

Fig. 2 The process diagram and conditions for preparing chitosan/organic acid/NaCl microparticles

free Na^+ and enhancing the level of saltiness [10]. Chitin and chitosan are nitrogen-containing linear polysaccharides composed of β-1,4 linked units of N-acetyl glucosamine, which have a positive charge under a weakly acidic environment. The positive charges from protonated amino groups may interact with the negatively charged ions in food systems, such as chloride ions from NaCl and carboxylate ions from proteins. The quantity of free sodium ions in solution may therefore be elevated, and this would be followed by an increased saltiness perception.

Recently, research has been conducted to explore the opportunities and challenges in the use of chitin and chitosan for enhancing the saltiness perception in food systems. Yi et al. [24] prepared chitosan (78.3 ± 2.5% degree of deacetylation (DD), 186.3 ± 12.4 kDa weight average molecular weight)/acid/NaCl microparticles by the spray drying method (Fig. 2) for popcorn seasoning. It showed a promising capability for maintaining saltiness sensory perception with reduced salt addition. They dissolved chitosan and NaCl into various organic acid solutions including acetic acid, citric acid, and lactic acid. This was followed by a spray drying process to obtain the microparticles. Loss of the organic acids via evaporation occurred along with the spray drying process. The NaCl content was slightly raised from 25.0–66.7% to 30.2–80.8% in the microparticle systems. The rate of moisture absorption of the prepared microparticles was between 0.83 and 3.19 g/100 g/h, representing that these particles may rapidly dissolve in the oral cavity to provide the saltiness perception. Moreover, the prepared chitosan/acid/NaCl composites are hollow particles with relatively large sizes, and NaCl concentrations in the system ranged from 15.4 to 32.0 μm. A comparison of the saltiness in sensory evaluations of ground NaCl, chitosan/acid/NaCl microparticles, and general (ungrounded) NaCl has been carried out. Postgraduate students of the Department of Food Science at National Taiwan Ocean University (Keelung, Taiwan) were volunteers who

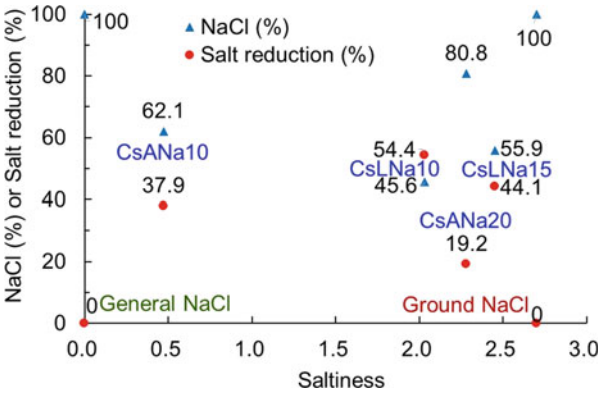

Fig. 3 The relationship between the saltiness score of sensory evaluations and NaCl content (%) or salt reduction (%) for chitosan/acid/NaCl microparticles. *Cs* Chitosan, *A* Acetic acid, *L* Lactic acid, *Na10* Use 10 g of NaCl in the solution formulation

participated in a hedonic taste test. The panel consisted of 20 untrained subjects (10 females and 10 males, 23–26 years old). Twenty grams of corn kernels was popped, and 1 g of test substances was added. The mixture was stirred for 3 min to allow the seasoning agents to spread over the popcorn. The salinity of the unground NaCl group was given a score of 0 and set as the standard for comparison with the other groups. Groups with less saltiness than that of the NaCl group were given a negative score as low as −5, whereas those with saltier taste were given a positive score as high as 5. The results shown in Fig. 3 indicated that the use of chitosan/acid/NaCl microparticles provided a saltiness taste comparable to the ground NaCl group and stronger than the general NaCl group. Because the tested microparticles had only 45.6–80.8% NaCl content, this result indicated that the level of saltiness can be maintained while the amount of NaCl used can be decreased by 19.2–54.4%. The decrease in NaCl used may be associated with the numerous small NaCl crystals on the surfaces of the chitosan/acid/NaCl. Also, the cationic effect of chitosan may play a role in increased release of free Na^+ ions. Thus, these microparticles show promise for reducing sodium addition to surface-salted foods.

Lu et al. [25] used a similar strategy and further incorporated maltodextrin into microparticles to optimize the shape and physical properties of products. Chitosan was prepared from shrimp shells and had an average DD of 76.5% and a weight average molecular weight of 213.2 ± 3.2 kDa. It was subsequently mixed with maltodextrin at weight ratios of 2:0, 1:1, and 0:2 for further use. The mixtures were then dissolved in 1% organic acid solution (acetic acid and lactic acid) at a concentration of 1%. Three percent NaCl was then dissolved in the solution as well, and the microparticles were obtained by a spray drying method. In addition, microparticles without chitosan and organic acid were prepared as the blank group. The size, density, organic acid content, NaCl content, rate of moisture absorption, and surface morphology of the particles were investigated. Along with the different acids used and the ratio of chitosan/maltodextrin, different morphological changes were observed by microscopy. Sodium chloride crystals were attached to the surface of both acetic acid and lactic acid groups, and the surface of both groups presented depressions and wrinkles, while groups without chitosan showed obvious cohesion

and could not form solid microparticles. The size of each group was between 2.3 and 9.7 μm. The average hygroscopic rates ranged from 3.4 to 5.7 g/100 g/h, and the apparent densities were between 1.7 and 1.9 g/cm^3; these results were similar to Yi et al. [24]. However, with addition of maltodextrin, the yield of microparticles was greatly improved. Sensory evaluation was carried out by panelists at the laboratory level using a 5-point hedonic rating scale. The salinity of popcorn mixed with ground NaCl was given a score of 0 and set as the standard for comparison with those mixed with microparticles. As a result, chitosan/maltodextrin/NaCl particles could effectively provide salinity, and it is worth mentioning that up to 45% salt reduction could be conducted without a reduction of the saltiness perception of seasoned popcorn.

Results from Yi et al. [24] and Lu et al. [25] indicated that chitosan/NaCl and chitosan/maltodextrin/NaCl microparticles can provide a better saltiness perception than ordinary table salt. It could be concluded that the average particle size (<32 μm) is less than ordinary salt (about 500 μm), and there are a large number of micrometer-sized NaCl crystals (<4 μm) distributed on the surface of the particles. Therefore, NaCl crystals can rapidly dissolve in the oral cavity and elevate the concentration of Na^+, which results in a strong saltiness perception. However, both studies revealed that this strategy is not suitable for use in a liquid food system such as soups and stews where the dissolved particles do not retain this dominant structure and therefore do not enhance the perception of saltiness but instead have an astringent taste owing to the chitosan in the system.

Interestingly, Somsak et al. [5] revealed that chitin nanoparticles are capable of acting as a saltiness enhancer in a solution system. They prepared α- and β-chitin from shrimp shells and squid pens, respectively. Chitins were then introduced into an ultrasonication treatment of 30, 45, and 60 min, respectively, followed by homogenization at 12,000 rpm for 20 min to produce chitin nanoparticle suspensions. Afterwards, the chitin nanoparticle suspensions were freeze-dried and ground into powder to obtain the final products. The particle sizes of the α-chitin nanoparticles were between 32.67 and 41.33 nm, while those of the β-chitin nanoparticles were between 42.60 and 50.67 nm. The particle sizes decreased with an increase in the ultrasonication treatment time. Then, the test solutions were prepared by mixing 0.03% dried nanoparticles and 0.3% NaCl in deionized water; a 0.3% NaCl solution without chitin nanoparticles was used as the control group. A panel of eight trained panelists was selected for sensory evaluation to test the ability of chitin nanocrystals to enhance the saltiness perception. The salty intensity ratings of α-chitin nanoparticles/NaCl solutions were 35.79, 43.70, and 47.33 mm on a 150-mm scale, while those of β-chitin nanoparticles/NaCl solutions were 35.89, 41.91, and 45.66 mm, respectively, for samples after 30, 45, and 60-min of ultrasonication treatment. The ratings were comparable between control groups and the 30 min-treated group, whereas there were significant increases in saltiness intensities in 45–60 min-treated groups. Compared with the control group, the saltiness intensity of 60 min-treated groups increased by about 30%. This may be related to the extremely small particle size of chitin nanoparticles by which chitin could rapidly provide amine (-NH^{3+}) groups in the solution system to bind to Cl^- and form a Stern

layer by electrostatic interactions that caused an elevated ratio of free Na^+ in the diffuse layer and, in turn, improved saltiness. In addition, increasing ultrasonication time may improve the high specific surface area and high porosity of chitin nanoparticles, which could improve their ability to adsorb negative ions in the solution system.

2.2.2 Chitin Nanofibers/Nanocrystals for Improving Saltiness Perception

Due to the existence of numerous intramolecular and intermolecular hydrogen bonds, the chitin molecules are difficult to dissolve in water and general solvents. Chitin can only be dissolved in a few special solvents such as LiCl/dimethylacetamide [26], NaOH/urea solvent systems [27], and ionic liquids [28]. This greatly limits its application and development in biomedical fields [2]. To deal with this issue, chitin could be processed into nanomaterials, such as chitin nanofibers (CNF) and chitin nanocrystals (CNC), which can increase their dispersion in water and form a colloidal solution. Methods used for preparing chitin nanomaterials include mechanical disassembly, chemical modification, and electrospinning. The mechanical disassembly methods feature the grinding method [29, 30], ultrasonication [10, 25], the starburst system [31], dynamic high pressure homogenization [32], and micro-fluidization technology [33], whereas acid hydrolysis [34], 2,2,6,6-tetramethylpiperidine-1-oxy radial (TEMPO)-mediated oxidation [35], and partial deacetylation [36] could be involved in chemical modification. These prepared chitin nanomaterials are generally stable in aqueous systems and may therefore be introduced as saltiness enhancers in liquid foods.

Nanofibers are generally defined as those with a diameter less than 100 nm and an aspect ratio greater than 100. Chitin nanofiber is composed of approximately 18–25 chitin molecular chains arranged in parallel and bonded by strong hydrogen bonds (crystallization region) to form a fiber with a diameter of about 2–5 nm and a length of about 300 nm [10, 37, 38]. Due to their size and structure, chitin nanofibers not only maintain chitin properties but also have advantages such as high surface area to volume ratio, excellent mechanical properties, high flexibility, easy chemical modification, and antiviral and anti-tumor activity [34, 39]. They could potentially be used in filtration, recovery of metal ions, controlled release of drugs, tissue engineering, enzyme carriers, wound healing, cosmetics, biosensors, and medical implants [25, 37, 40, 41].

Jiang et al. [10] prepared a 0.3 g/L β-chitin aqueous suspension, and then treated it by ultrasonication at 20 kHz and 200 W for 30, 45, and 60 min. After centrifugation, the supernatant was collected as chitin nanofibers, represented as CNF30, CNF45, and CNF60, respectively. The DD were determined by FTIR at 21–22%, and the diameters of CNF30, CNF45, and CNF60 were analyzed by TEM and found to be 9.3, 5.6, and 5.1 nm, respectively. Sensory evaluation was conducted; 0.075–0.3 g/L chitin nanofiber suspensions were added with 3 g/L sodium chloride to obtain the test sample. The panel consisted of 20 untrained and volunteer subjects (10 females

and 10 males, 23–26 years of age). Every panelist was asked to drink 5 mL of 3.0 g/L NaCl as well as the CNF and CNF/NaCl solutions of varying concentrations before assessing the saltiness and astringency of the solutions which were evaluated using a 7-point scale (1 = very weak, 4 = moderate, 7 = very strong). The results showed that there was no significant effect for promoting saltiness perception observed in the 0.075 g/L CNF group, whereas the 0.15 and 0.3 g/L CNF groups showed remarkable saltiness enhancement potential (scored at 4.4 and 4.0 compared to 3.3 and 2.8 of groups without CNF), indicating that CNF had the ability to improve the salty taste. In addition, CNF60, the group with longest ultrasonication treatment time, had the best saltiness promoting effect. This was possibly because CNF can provide -$NH3^+$ to adsorb anions such as Cl^- and OH^- in the Stern layer, so that the concentration of free Na^+ in the solution is higher than that of the control group. Moreover, chitin and chitosan are often hindered in the food industry by their astringency. However, with the participation of NaCl, the astringency of CNF30, CNF45, and CNF60 groups was significantly reduced compared to the group without NaCl. The astringency of chitin and chitosan could increase from the increasing in positive charges on the molecular chain [42]. When sodium chloride is added, the positive charge on chitin nanofibers will be neutralized by chlorine ions due to the interaction of charge, so its astringency will be decreased [10].

To explore the relationship between physicochemical properties and the saltiness-improving capacity of chitin nanomaterials, Tsai et al. [6] used ultrasonication treatment to prepare deacetylated chitin nanofibers (DACNF) and acid hydrolysis to prepare chitin nanocrystals (CNC) (rod-shaped in morphology with lower aspect ratios than the original nanofibers). The DDs of the original nanofibers (CNF, CNC, and DACNF) were 22.89%, 23.43%, and 53.36%, respectively. CNF, CNC, and DACNF had diameters of 17.24, 16.05, and 15.01 nm and lengths of 1.73, 0.12, and 1.81 μm, respectively. When suspended in deionized water, the overall zeta potential of chitin nanomaterials is 19.73–30.08 mV, and the concentration (0.04–0.074 mg/mL) has no obvious influence on the tested value. Sensory evaluation was also conducted using a 7-point scale to evaluate the saltiness of each group. Accordingly, the DD and the aspect ratio played roles in saltiness-improving capacity. The saltiness perception of CNC and DACNF suspensions was higher than that of the CNF suspension, inferring that lower aspect ratio and a higher DD result in higher saltiness. This may be due to the ionization of the surface amine group from deacetylated molecules under the pH < 7 environment. These amine groups are able to adsorb Cl^-, OH^-, and other negative ions, increasing the proportion of free Na^+ in the solution and thus improving the saltiness perception (Fig. 4). Moreover, the number of molecules and total surface area of CNC and DACNF were both larger than CNF, so more negative ions could be absorbed, resulting in more free Na^+ in the solution and a strong salty perception.

Another approach to using CNF for replacing NaCl from a curing solution of tilapia fillets was conducted by Hsueh et al. [23]. Curing solutions with CNF (with a DD of 16.2% and mean diameter of 111.6 ± 45.4 nm) at concentrations between 0.12 and 0.18 g/L were able to provide a stronger saltiness perception than those without the addition of CNF. More free Na^+ ions are assumed to be present in the

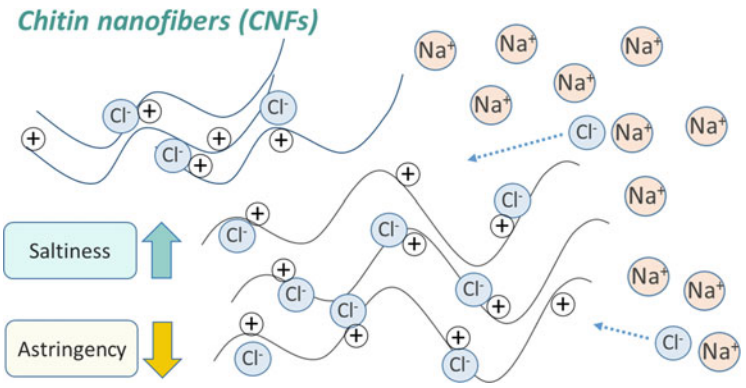

Fig. 4 The mechanism of chitin nanofibers for enhancing saltiness perception

CNF/NaCl curing solution, resulting in a higher proportion of a combination of these ions on the surface of tilapia fillets. Moreover, adding 3 g/L citric acid or 4 g/L malic acid to CNF/NaCl curing solution could effectively improve the saltiness of fish fillets without generating obvious sourness. The addition of these organic acids provided environments with a lower pH value. This may facilitate the ($-NH_3^+$) degree of protonation of amine groups in the molecular chain. As evidenced, the addition of lactic acid in CNF/NaCl curing solution elevated the zeta potential from 0.7 to 3.8 mV, which may increase the adsorption capacity of anions. It could also be concluded that the saltiness of sodium chloride at a specific concentration range may be enhanced by the addition of a small amount of acid [43, 44].

3 Chitin and Chitosan Prevent Food from Loss in the Quality of Flavor

3.1 Flavor Loss and Off-Flavor Development of Foods

The most important factor that maximizes food quality is its flavor, and there is increasing attention on the stability of flavor. The quality attribute of food flavor stability can be affected by the chemical reactivity of food flavors and the environment of food such as the availability of light and atmospheric oxygen [45]. Flavor compounds can be susceptible to chemical changes occurring from various kinds of interactions. Among the many reactions taking place in the food system, oxidation is generally considered to affect flavor stability during storage [46]. For example, meat or meat products are highly susceptible to lipid oxidation which leads to rapid generation of rancidity or a warmed-over flavor [47]. When meat is stored after cooking, lipid oxidation occurs rapidly, and this is followed by a deterioration in flavor; this phenomenon is termed warmed-over flavor (WOF). The flavor is described as “rancid,” “stale,” and like “cardboard” and even compared to “damp

dog hair" [48]. WOF was scientifically considered to be an organoleptic challenge to meat products, as cooking and subsequent refrigeration is commonly introduced when preserving foods with meat. Once cooked meat is exposed to oxygen, it could develop off-flavors in a short time. Irreversible conversion of iron from the ferrous to the ferric form of myoglobin pigments occurs during heating, and this is an important cause of rapid oxidation of lipids in meats during cooking. Concern about flavor deterioration of oxidized lipids in meats has prompted the study of antioxidants for preventing or controlling lipid oxidation [49].

Another crucial factor that causes negative effects on flavor stability is microbial spoilage. Under particular storage conditions, specific spoilage organisms (SSOs) produce metabolites responsible for off-flavors and cause organoleptic rejection of the product [50, 51]. Off-flavor production is one of the major indicators used by consumers when they evaluate the freshness of raw food materials [52]. Compounds with a specific smell such as nitrogen-related compounds, sulfuric compounds, ketones, aldehydes, and esters are mainly produced by various microorganisms during food spoilage [50, 51, 53]. One of the highly perishable foods is fish which readily undergoes autolysis followed by consumption by the microorganism. The chemical changes of flavor compound precursors, including amino acids, nucleotides, trimethylamine oxide (TMAO), and volatile organic compounds (VOC) may generate off-flavors and cause organoleptic rejection [54]. The post-mortem chemical changes related to biochemical reactions and microbial metabolism can cause deterioration of texture and flavor and finally loss of edibility.

3.2 Chitin and Chitosan for Improving the Flavor Stability of Foods

Chitosan has a remarkable antioxidant and antibacterial capacity [55, 56], and it may attenuate lipid oxidation and inhibit the growth of spoilage bacteria in food systems during storage. Since chitosan is reported to possess better antioxidation and antimicrobial capacity than chitin [57], it was preferred over chitin for use in preventing the generation of off-flavors in foods. The action of chitosan for improving the flavor stability of foods would involve antioxidant and antimicrobial pathways. Chitosan is able to adhere to the membranes of microorganisms, subsequently changing its permeability. Moreover, it can act as a trace metal chelating agent, which may inhibit the growth of microbes, or it may effectively bind to the microbial DNA and interfere with the synthesis of mRNA and proteins. Chitosan tends to adhere to or penetrate the cells depending on whether its molecular weight is high or low, respectively (Fig. 5) [58]. The addition of 0.05 and 0.1% chitosan inhibited the growth of common spoilage microorganisms, including *B. cereus*, *S. aureus*, *E. coli,* and *P. fluorescens* in vitro, while *S. typhimurium* was partially inhibited [59]. In addition, chitosan is able to chelate the free iron released from meat heme proteins, thereby inhibiting the catalytic potential of iron ions in the initial stage of lipid

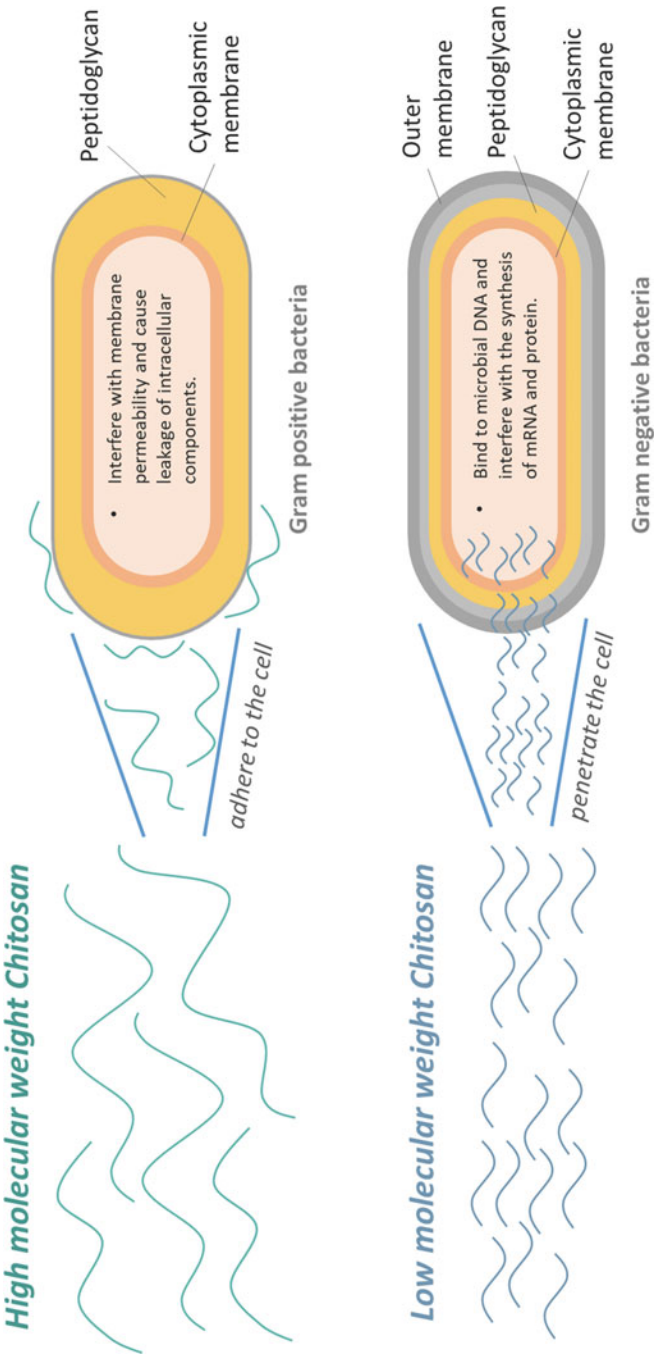

Fig. 5 The antimicrobial mechanisms of high and low molecular weight chitosan

autoxidation [60]. Xue et al. [61] reported that the protective action of chitosan toward the deterioration of foods is also effective when it is used as a protective film, where it acts as a barrier against oxygen [62]. The scientific publications reporting chitin and chitosan for improving flavor stability are addressed in Table 2.

3.2.1 Chitin and Chitosan Promote Flavor Stability in Muscle Food Products

Muscle food products are highly susceptible to off-flavor and rancidity development by the autoxidation of their unsaturated lipids. St. Angelo and Vercellotti [63] introduced chitosan at a concentration of 1% in meat and this resulted in a 70% decrease of 2-thiobarbituric acid (TBA) values, while a 93% reduction of TBA and a 99% inhibition in hexanal content were observed with N-carboxymethyl chitosan treatment after 3 days of storage at 4°C. N,O-carboxymethyl chitosan (NOCC) showed a similar effect in controlling the flavor deterioration of cooked meat within a 9-day storage period [64]. Researchers from Korea conducted a series of studies to reveal the application of chitosan as preservative agent in processed sausage, where it could potentially reduce or replace the use of nitrite [78–81]. Interestingly, they found that the preservative capacity of chitosan is increased along with increasing its molecular weight [80]. This phenomenon could be completely interpreted as chitosan with high molecular weight having bioadhesive properties such that it can attach to cells and form a covering around them. Subsequently it can alter their structure, permeability, and functioning and inhibit the survival of microorganisms. This action takes place mainly against Gram-positive bacteria because their cell wall is composed of a thick layer of peptidoglycan rather than the outer membrane [58]. The most frequent spoilage bacteria that can be identified in sausages are *Lactobacillus sakei* and *Brochothrix thermosphacta* [82], which are classified as Gram-positive bacteria. Therefore, these bacteria could be affected by high molecular weight chitosan. In addition, as a food additive, chitosan is known to form an astringent taste in a food system. However, research has indicated that addition of 0.1% chitosan with relatively high molecular weights of 150–1,250 kDa (DD = 85–87%) into reduced-fat Chinese-style sausage had no adverse effect on the sensory characteristics [66]. Also, addition of chitosan with low molecular weight (5 kDa, 0.2%) did not negatively affect the sensory characteristics of sausage, and it was certainly able to prevent the sausage from lipid oxidation within 3-week storage [66]. These results suggest a promising application of chitosan for improving flavor stability in sausage.

Serrano and Bañón [67] reported that the use of 0.02% or 0.05% chitosan (molecular weight of 340 kDa, 80% DD) can reduce the SO_2 required to preserve pork burgers and effectively enhance the flavor stability at 2°C storage for up to 21 days. The addition of either 0.02% or 0.05% chitosan was not detected by sensory analysis, but it significantly reduced the generation of undesirable odors including rancid, acidic, and putrid odors within the storage time. In the quantitative descriptive analysis, a linear intensity five-point scale was used to quantify the sensory

Table 2 Scientific publications reporting the flavor stability improving applications of chitin and chitosan

Applied methods	Materials	Physicochemical properties	Applied foods	Outcome	Reference
Immersing	NCC	Not available	Meat	93% decrease of TBA and a 99% reduction in hexanal content by treating with 3% N-carboxymethyl chitosan after 3 days of storage at 4°C	[63]
	NOCC	Not available	Cooked meat	The mean deterioration inhibitory effect of NOCC at 500–3,000 ppm was 43.4–69.9% at 4°C, for 9 days as reflected in their TBA values	[64]
	Water-soluble chitosan	80–82% DD	Oysters	Retained better freshness flavor during storage	[65]
Intact addition	Chitosan	Chitosans of different viscosity (14 cP, 57 cP, and 360 Cp)	Comminuted flesh of herring	The formation of hydroperoxides and TBARS in herring samples containing 200 ppm chitosan was reduced after 8 days of storage by 61% and 52%, respectively	[60]
	Chitosan	85–87% DD, 5 kDa molecular weight	Reduced-fat Chinese-style sausage	Addition of chitosan (0.2%) did not negatively affect the sensory characteristics of sausage, and it was certainly able to prevent the sausage from lipid oxidation within 3-week storage	[66]
	Chitosan	80% DD, 340 kDa molecular weight	Pork burger	The addition of either 0.02% or 0.05% chitosan was not detected by sensory analysis, but it significantly reduced the generation of undesirable odors including rancid, acidic, and putrid odors at 2°C storage for up to 21 days	[67]
	Chitosan nanoparticles	562 nm of average diameter	Milk	All of the sensory parameters were not significantly affected by the addition of	[68]

(continued)

Table 2 (continued)

Applied methods	Materials	Physicochemical properties	Applied foods	Outcome	Reference
				the nano-chitosan solution when stored for 15 days at 4°C	
Edible coating	Chitin nanofibers	50–70 nm of diameters	Raw beef	The odor and overall acceptability were significantly improved by coating the gelatin-CMC films incorporated with chitin nanofiber for 12 days of storage at 4°C	[69]
	Chitosan film	Not available	Horse mackerel	The flavor could be retained well (scored at 3.5 compared to 2.2 of sample without chitosan packing, in a five-point scaled quantitative descriptive analysis) after 8 days of storage	[70]
	Chitosan film	85% DD, 400 kDa molecular weight	Grass carp fillets	Chitosan coatings resulted in significant attenuation of off-flavor compounds, such as trimethylamine, hypoxanthine, and histidine. They promoted the overall acceptance from 1.00 to up to 3.24 in a five-point scaled sensory evaluation at the 15th day of storage at 4°C	[54]
	Chitosan coating incorporated with the lactoperoxidase system	85% DD, medium molecular weight	Rainbow trout	The sensory score of odor can be improved from 2.58 and 2.53 to 6.87 and 6.70 and overall acceptability in a 9-point scaled description by this coating system	[71]
	Chitosan	85% DD, 160 kDa molecular weight	Silver carp	After 30 days of refrigerated storage, the chitosan-coated group received a better score (about 4.8) than the control group (about 3.8) in a nine-point hedonic scale	[72]

	Chitosan	89% DD	Strawberries	1% of chitosan treatment showed no significant improvement in flavor stability when stored for 1 week at 2°C	[73]
	Chitosan	High molecular weight, 82.7% DD	Strawberries	2% of chitosan treatment could maintain edible quality of tested sample from 3 days to 21 days of storage	[74]
	Chitosan	95% DD	Papaya	The fruits showed acceptable quality in flavor, scored at 3.13 and 4.24 in a 5-point scale, for samples treated with 1.0 and 1.5% of chitosan solution, respectively	[75]
	Moringa oil/chitosan nanoparticles embedded gelatin nanofibers	85% DD	Cheshire cheddar cheese	Sensory scores for taste and overall acceptability were significantly improved with this treatment	[76]
	Water-soluble azido chitin derivative film	70 kDa molecular weight, degree of substitution of the azido derivative was 0.8	Ricotta Cheese	After 28 days of the storage experiment, the flavor and odor scored at 4-excellent for the 2% chitin treated sample whereas it was 2-fair for the untreated one	[77]

NCC N-carboxymethyl chitosan, *NOCC* N,O-carboxymethyl chitosan, *CMC* carboxymethyl cellulose, *DD* degree of deacetylation, *TBA* thiobarbituric acid, *TBARS* thiobarbituric acid reactive substances

descriptors. In the fresh burgers preserved with low SO_2 (150 mg/kg), the sensory scores of rancid, acidic, and putrid odors developed were from 1.08, 1.08, and 1.05 to 2.14, 2.15, and 2.12, respectively, at the 21st day of storage, whereas with 0.02% and 0.05% chitosan addition, the scores of those odors were 1.76, 1.76, 1.56 and 1.59, 1.59, 1.85, respectively. Similar results were found in the storage of cooked pork burgers. The addition of chitosan could reduce the development of rancid odors, rancid flavors, putrid odors, and putrid flavors and retard the loss of meat odor and meat flavor during storage.

On the other hand, although chitin is considered to possess lower antioxidant and antimicrobial capabilities than chitosan, its nanofiber (CNF) formed materials could still be effective for improving the flavor stability of foods. Azarifa et al. [69] prepared a gelatin-CMC film incorporated with chitin nanofiber and reported that it could have a promising effect on the flavor stability of refrigerated raw beef. The odor and overall acceptability were significantly improved during 12 days of storage by coating the beef with the nanocomposite. The interaction between positively charged groups of CNF and the negative charges of the microbial membrane affected the permeability of the cell membrane, and this is considered to be the major mechanism for the antimicrobial effect of CNF.

Chitosan applications also promoted the flavor stability of aquatic products efficiently. Ahn and Lee [70] used chitosan film for packing lightly-salted horse mackerel, and they found that the flavor could be retained well (scored 3.5 compared to 2.2 for a sample without chitosan packing, in a five-point scaled quantitative descriptive analysis) after 8 days of storage. Yu et al. [54] investigated the effects of chitosan (400 kDa molecular weight, 85% DD) based coatings on flavor retention of refrigerated grass carp fillets. The results indicated that chitosan coatings resulted in a significant attenuation of off-flavor compounds such as trimethylamine, hypoxanthine, and histidine. Meanwhile, favorable flavor compounds such as inosine monophosphate and umami-associated free amino acid could be accumulated with this treatment. In sensory evaluation, chitosan coatings significantly retarded the sensory deterioration during storage, which could improve the overall acceptance from 1.00 to up to 3.24 in a five-point scaled quantitative descriptive analysis at the 15th day of storage at 4°C. Jasour et al. [71] used a chitosan (medium molecular weight, 85% DD) coating incorporated with the lactoperoxidase system to prevent rainbow trout from flavor deterioration during refrigerated storage for a period of 16 days. They evaluated the color, odor, texture, and general acceptability using a 9-point scaled description. The sensory score for odor could be improved from 2.58 and 2.53 to 6.87 and 6.70 of odor and overall acceptability by this coating system. Even coating with chitosan alone revealed a positive effect for improving the flavor stability; both the odor and acceptability were scored at 5.54. Fan et al. [72] compared the capability of chitosan (molecular weight of 160 kDa, 85% DD) and glacial acetic acid for improving the flavor stability of silver carp. After 30 days of refrigerated storage, the chitosan-coated group received a better score (about 4.8) than did the glacial acetic acid group (about 3.8) in a nine-point hedonic scale, owing to its ability to reduce the generation of TBA and total volatile basic nitrogen (TVB-N). Cao et al. [65] revealed that oysters pretreated by immersing in water-

soluble chitosan (80–82% DD) solution retained better freshness flavor during storage. This was due to the growth inhibition effect of chitosan toward microorganisms.

3.2.2 Other Foods

Due to their notable antioxidation and antimicrobial activities, chitin and chitosan can also be used to preserve the flavor of fruits, dairy, and cereal products. Strawberries could be one of the most perishable fruits, and they are characterized by a short shelf-life. Han et al. [73] developed 1% chitosan (89% DD) based solutions (containing 6% of organic acids) for coating strawberries. A trained panel developed appearance, texture, and flavor descriptors by evaluating chitosan-coated strawberries stored for 1 week at 2°C. However, the results indicated that no significant amelioration in the flavor of treated strawberries after the storage period. Another approach was conducted by Jesmin et al. [74], 2% of gamma radiation treated high molecular weight chitosan solution (82.7% DD, viscosity less than 200 mPa s and 2% acetic acid) was used to treat the test strawberries. They suggested that the treatment could remarkably prevent strawberries from weight loss (17% to about 2%) and the growth of microbes (2.00×10^5 to 1.04×10^4 CFU/g). Results from sensory evaluation showed that the treatment could maintain the edible quality of the tested samples from 3 days to 21 days of storage. From these results, it can be concluded that the concentration of chitosan used may play a crucial role in improving the flavor stability of strawberries. Han et al. [83] suggested that a 2% chitosan solution treatment could control the mold growth of strawberries, whereas the mold decay incidence of 1% chitosan treated strawberries was estimated to be over 70% after 2 weeks [73]. Cell wall degrading enzymes are suggested to be secreted onto the surface of strawberries along with the growth of microbes, and this results in the disruption of receptacles and the loss of flavor compounds. Furthermore, chitosan treatment can also help retard the flavor loss of papaya. Ali et al. [75] revealed that treatments with 1.0 and 1.5% of chitosan (95% DD) solution promoted the flavor stability of papaya. The fruits showed acceptable flavor quality; samples treated with 1.0 and 1.5% of chitosan solution scored 3.13 and 4.24 (in a 5-point scale), respectively. Interestingly, samples treated with 2.0% chitosan solution were not ripened properly during the experimental period of cold storage, and they were removed from the test due to unacceptable quality.

Dairy products are rich in nutrients and are good medium for growth of various microbes. The presence of undesirable bacteria in milk may cause a deterioration in flavor such as souring of milk, which may further lead to economic loss [84]. Several studies were conducted to use chitin and chitosan in controlling the development of undesirable flavors in dairy products. Seo et al. [68] prepared chitosan nanoparticles with an average diameter of 562 nm by a grinding method, dissolved them into a 0.3% ascorbic acid solution, and then added them into commercial milk at various concentrations. In the sensory evaluation, 8 trained sensory panelists were recruited and the rancidity, bitterness, astringency, and overall acceptability were investigated

on a 7-point scale. However, all of the sensory parameters were not significantly affected by the addition of the nano-chitosan solution when the milk was stored for 15 days at 4°C. Lin et al. [76] introduced chitosan (85% DD) moringa oil/chitosan nanoparticle embedded gelatin nanofibers as a preserving agent for reducing the flavor loss of Cheshire cheddar cheese. The cheese samples were wrapped with the nanofibers and stored at 25°C for 4 days. The sensory score for taste and overall acceptability were significantly improved with this treatment, owing to the inhibition activity toward the growth of *L. monocytogenes* and *S. aureus*. Most recently, Kritchenkov et al. [77] developed water-soluble azido chitin derivatives, which possessed high antibacterial activity, to preserve Ricotta cheese. This novel material was prepared by an ultrasound-assisted treatment of chitin (70 kDa) with 1-azido-3-chloropropan-2-ol at 80°C. The prepared chitin had a degree of substitution of the azido derivative of 0.8, and it could be dissolved in water. Two percent of the azido chitin solution was used to treat the cheese sample. After 28 days of the storage experiment, the flavor and odor scored at 4-excellent for the treated sample, whereas it was 2-fair for the untreated one.

4 Chitin and Chitosan for Food Debittering

4.1 The Bitter Taste in Food and debitterization Technologies

Bitterness is widely distributed in foods, and basically each chemical class contains bitter molecules [85]. This sensation originates from taste receptors (TR) localized on taste buds in the oral cavity. Humans have the ability to identify a broad range of materials as bitter, which indicates that bitter molecules occur in many variations. The most important representative bitter molecules are identified as certain alkaloids (e.g., nicotine, quinine, caffeine), terpenoids (e.g., isoalpha acid, amarogentine, limonoids), and flavonoids (e.g., naringin, neohesperidin, epigallocatechin gallate) [85]. Bitter taste can be considered a major problem in the food and pharmaceutical industries, owing to its negative hedonic impact on ingestion [86, 87]. In the past, bitterness reduction technology was focused on pharmaceuticals, however, recently, most research is conducted on the reduction of bitter taste or astringency in functional food or beverage applications. These foods and beverages possess inherent off-tastes because of their fortification with healthy but poor-tasting additives. Only in limited cases will consumers accept a strong bitter taste in food and beverages (e.g., in black or green tea, black coffee, beer, grapefruit products, or bitter lemons). For most other cases, a bitter taste has to be eliminated from food or masked since it is not desirable [85].

Foods, unlike drugs, are mainly selected by the consumer based on their sensory properties. Hindering the taste of drugs should be acceptable, whereas foods should taste delicious [88]. Recently, due to the demand for healthier foods or beverages, the problem of bitter-tasting food products is surfacing again. The ingestion of these foods/beverages is not only perceived as bitter but also as astringent and/or sour.

Reduced sugar, fat, and sodium for healthy benefits can also indicate sourness, astringency, and bitterness in the base matrices. Each modality is translated by different molecular sensing mechanisms in the oral cavity, and the sensation consciously recognized is a mixture that is difficult to separate into individual sensory tastes. Therefore, the complex mixture of sensations of foods makes masking off-tastes a big challenge [85].

Methods for dealing with this issue can be varied. In general, plant breeding has selected for less-bitter varieties and processing often involves chopping and peeling to remove the bitterest parts of the plant [88]. Another example is in the juice industry. Raw orange juices are debittered by using naringinase to cleavage the bitter naringin into the less bitter naringenin or naringin-7-O-glucoside. On the other hand, most of cloudy raw apple juices are processed to remove most of the bitter-tasting or astringent polyphenols to yield clear beverages [85]. Moreover, most commonly, bitterness is masked by additives. Among the most used additives are sodium ions which antagonize the bitter taste of a broad array of compounds. However, their activity varies a great deal, depending on the chemical nature of the bitter-tasting molecule, and in some cases, they have been proven to be quite inactive. Although bitter-taste blockers have been much explored, only a few have shown a wide spectrum of activity. In fact, none of them is known to be effective against all bitter compounds. Among other bitter-taste blockers, we can mention cyclodextrins, adenosine monophosphate, and certain protein combinations such as lactoglobulin bound to phosphatidic acid [89].

4.2 Chitin and Chitosan for Debitterization Use in Foods and Herbal Extracts

Due to its natural polycationic property, chitosan is known to act as clarification agent in the juice and beverage industries [90]. It could be effective in coagulating suspended particles through ionic interaction with negatively charged molecules and thus help separate them from juice and beverages. A wide array of successful applications have been reported in the literature such as in apple, grape, lemon, passion fruit, and pomegranate juices [90–92]. Along with the clarification process, some of the major bitter-tasting molecules, such as tannins and flavonoids in juice, could be removed and give products with a lower bitterness perception.

Another debitterization technology feature in chitosan application is encapsulation, which was frequently used in masking bitter-tasting bioactive molecules in herbal extracts and functional foods. Chitosan acts as a bitter taste blocker in this technology. It enables the bitter bioactive molecules to be encapsulated into the form of free-flowing micro/nano-capsules and then reduces the solubility of such bitter bioactives by providing a physical barrier to the taste buds [87, 93–98]. The resulting microcapsules can be blended with other ingredients for food formulation and processing. The advantage of encapsulation for taste-masking of bitter bioactives

in functional foods is in the wide variety of dosage forms and product applications. In general, the coating polymer, chitosan, can be insoluble in a salivary environment at pH 6.8 and either readily dissolve in gastric fluid at pH 1.2 or it may be insoluble in gastric fluid but decomposed in the intestine fluids to release the bioactives [93]. This strategy may prevent the release of bitter-tasting bioactive molecules in the oral cavity after ingestion but allow their release where they should be absorbed. The technology can be achieved by dissolving/dispersing chitosan in a solvent or by incorporating the bitter-tasting bioactive molecule in a chitosan solution. Nowadays, most encapsulation in industry is achieved using fluidized bed processors where the coating solution is sprayed through a nozzle and dried with warm air.

Moreover, because of their high protein affinity, chitin and chitosan are promising supports for the immobilization of specific debitterization enzymes such as naringinase and exopeptidases in the food industry [99, 100]. The basic applications of chitosan for debitterization can thus be included in the aforementioned strategies (Fig. 6). The scientific publications of chitin and chitosan for Debitterization applications are addressed in Table 3.

4.2.1 Chitin and Chitosan as Carriers for Debittering Enzymes

In the food industry, immobilized naringinase is used for enzymatic debittering of several citrus juices. It can promote the hydrolysis of bitter-tasting naringin into rhamnose and prunin and further cleave prunin to glucose and naringenin [109]. In this aspect, chitin and chitosan were both used as carriers for immobilization of naringinase. Tsen and Tsai [107] used a one-step method employing glutaraldehyde and sodium borohydride to immobilize naringinase from *Penicillium* sp. on chitin. The naringin content in grapefruit juice could be reduced to about 40% within 60 min of treatment with a capacity of about 10 times the column volume. Bodakowska-Boczniewicz and Garncarek [108] obtained chitosan (medium molecular weight) microspheres by reversed-phase suspension methodology, whereby glutaraldehyde was introduced as a cross-linking reagent for the immobilization of naringinase. The immobilization yields could be up to 31.97%, and the immobilized enzyme microspheres were used for debittering grapefruit juice. Compared to the soluble form of enzyme, the Km value of the immobilized naringinase was higher (2.56 vs. 6.59 mM), and the immobilized naringinase had good recycling stability. It retained 88.1% of its initial activity after 10 runs of the hydrolysis process from fresh grapefruit juice.

4.2.2 The Removal of Bitter-Tasting Molecules in Foods by Chitosan

Chang and Juang [104] proposed chitosan (1,850 kDa molecular weight, 97.2% DD)/activated clay composite beads for removing tannic acid, the most important bitter-tasting molecules in various juices, from a model liquid system. The beads were found to effectively adsorb tannic acid with a quantitated capacity of 1,490 g/

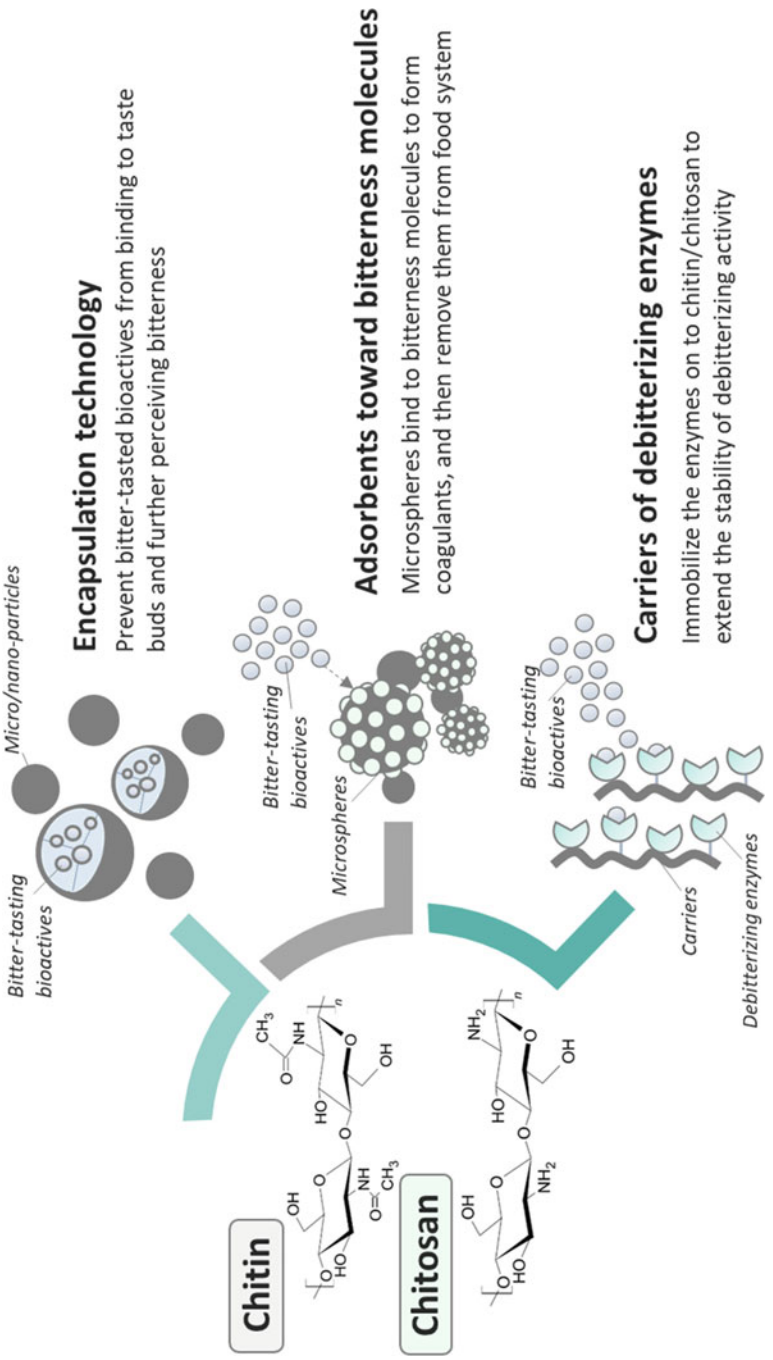

Fig. 6 The applications of chitin and chitosan for debitterization

Table 3 Scientific publications reporting the Debitterization applications of chitin and chitosan

Mechanism	Materials	Physicochemical properties	Applied foods	Outcome	Reference
Encapsulation	Chitosan-CDs adduct	70 kDa molecular weight	Caffeine and artichoke leaves, aloe and gentian extracts	Chitosan-β-CD adduct conferred the better debittering capacity than other tested groups (chitosan alone, CDs alone, and chitosan-γ-CD). It remarkably reduced the bitterness scores from 4.0 to 0.2 against caffeine, 5.0 to 0.9 against artichoke extract, 3.0 to 0.0 against gentian extract, and 3.0 to 0.0 against aloe extract in a 5-point scaled sensory evaluation	[89]
	Chitosan-coated alginate microcapsules	Medium-molecular weight, 75–85% DD	Flavoniods-rich herbal extract	Chitosan-coated alginate microcapsules had an efficiency for flavonoids up to 77.97% at optimum conditions: sodium alginate concentration 1.49%, extract concentration 1.58%, and $CaCl_2$ concentration 0.84%. The loaded flavonoids could be released gently in simulated gastric fluid and rapidly in simulated intestinal fluid	[101]
	Chitosan nanoparticles	75–98% DD, 10–235 kDa molecular weight	Xanthohumol and lupulone from hop extract	Nanoparticles composed of chitosan with the lowest DD and highest molecular weight can retain a small amount of bitter compounds, indicating that the encapsulation may prevent those bitter-tasting molecules from attaching to the taste buds to give a bitter perception. The release of bitter-tasting bioactives increased by increasing the molecular weight of chitosan in a pH = 7 buffer system	[102]
	Chitosan nanoparticles	Not available	Bacoside-rich *Bacopa monnieri* extract	Nanoparticles composed of 0.1% chitosan, 0.15% TPP, and 15 mg/mL of extract have an average particle size about 220 nm, and the efficiency of encapsulation was 52.0%	[103]

Adsorption	Chitosan/activated clay composite	97.2% DD, 1,850 kDa molecular weight	Model liquid system	Chitosan/activated clay composite beads were found to effectively adsorb tannic acid with a quantitated capacity of 1,490 g/kg	[104]
	Chitosan	81.23–90.54% DD	Lime juice	Chitosan with the lowest DD of 81.23% could have the highest capacity for reducing the contents of major bitterness molecules, limonin (58.56%) and naringin (22.26%)	[105]
	Carboxymethyl chitosan	85% DD, 80% degree of substitution	Yeast paste derived from beer process	2% of carboxymethyl chitosan at pH 7.5 for 58 min may greatly reduce the content of α-acid, β-acid, and iso-α-acid	[106]
Enzyme carriers	Chitin	Not available	Grapefruit juice	Naringin content could be reduced to about 40% within 60 min of treatment with a capacity of about 10 times of column volume	[107]
	Chitosan microspheres	Medium molecular weight	Grapefruit juice	Immobilized naringinase had a km value of 6.59 mM towards naringin; it retained 88.1% of its initial activity after 10 runs of hydrolysis process	[108]
	Chitin	Not available	Casein hydrolysates	The immobilized porcine pancreatic exopeptidases was effective in releasing the free amino acids from peptides, leading to the removal or a decrease in bitterness	[99]

DD degree of deacetylation, *CD* cyclodextrin, *TPP* tripolyphosphate

kg, which is much higher than those previously reported on organoclay and activated carbon, 110 and 25 g/kg, respectively [110]. Deatcheewa and Buri [105] investigated the effect of DD and concentrated chitosan on the reduction of lime juice bitterness. They found that chitosan with the lowest DD of 81.23% could have the highest capacity for reducing the content of the major bitterness molecules, limonin (58.56%) and naringin (22.26%), among all tested groups. Consequently, the decrease in the DD of chitosan resulted in a decrease of bitterness scoring in sensory evaluation. However, the molecular weights of these tested chitosans were addressed in this study. In general, the deacetylation process is frequently followed by a significant decrement in the molecular weight of chitosan, hinting that the chitosan tested with relatively high DD may have lower molecular weight than others. Therefore, the molecular weight of chitosan may also play a role in this study. Jiang et al. [106] attempted to recover the yeast paste derived from beer processing and use it as a novel healthy food. Carboxymethyl chitosan (85% DD, 80% of degree of substitution) was used for debittering the paste, focusing on the removal of α-acid, β-acid, and iso-α-acid. The optimal conditions determined by response surface methodology (RSM) experiments were treatment by adding 2% of carboxymethyl chitosan at pH 7.5 for 58 min. Ye et al. [111] further described the debittering capacity of carboxymethyl chitosan in this food system. They found that a 15.19–19.49 mg/L reduction of iso-α-acid concentration could be achieved by 1 g of carboxymethyl chitosan. Li et al. [112] constructed a chitosan (550 kDa molecular weight, 85.3% DD)-Ce^{4+} microsphere resin system to remove the bitter-tasting molecules in processed orange juice. The adsorption system could effectively remove 43.2% of limonin and 54.86% of naringin from juice without significant nutrition loss.

Taking the aforementioned studies into consideration, chitosan was not only capable of removing bitter-tasting phenolics in juice products, but it also shows a high capacity for interacting with non-phenolic compounds such as limonin and iso-α-acid which provide significant bitterness in foods.

4.2.3 Encapsulation Technology

Chitosan itself presents strong astringency when dissolved in acidic medium, and this limits its use in oral ingestion [113]. However, when integrated with other polymers into composites or by reducing the particle size to nano-scale, it is capable of acting as an effective bitter-taste-blocker for bitterness bioactives instead of providing an astringent perception. Binello et al. [89] prepared chitosan (70 kDa molecular weight) cyclodextrin (CD) adducts using a malonyl or a succinyl bridge between these two polymers by a series of chemical syntheses, and they tested the bitter-masking potential of these materials against caffeine and artichoke leaves and aloe and gentian extracts. As a result, the chitosan-β-CD adduct conferred better debittering capacity than other tested groups (chitosan alone, CDs alone, and chitosan-γ-CD). It remarkably reduced the bitterness scores from 4.0 to 0.2 against caffeine, 5.0 to 0.9 against artichoke extract, 3.0 to 0.0 against gentian extract, and

3.0 to 0.0 against aloe extract in a 5-point scaled sensory evaluation. It is worth mentioning that chitosan alone provided a notable debittering effect as well, but it was accompanied by a strong unpleasant astringent sensation which may limit its use. Another composite proposed by Khorshidian et al. [101] also demonstrated its capability for encapsulating bitter-tasting molecules. Medium-molecular weight chitosan (75–85% DD) was introduced to prepare chitosan-coated alginate microcapsules by using the $CaCl_2$ method followed by filtration and centrifugation. The microcapsules were then loaded with herbal galactagogue extract (containing *Foeniculum vulgare*, *Cuminum cyminum*, *Trigonella foenum,* and *Anethum graveolens* extracts, rich in flavonoids). The encapsulation efficiency of flavonoids can be up to 77.97% at the optimum conditions: sodium alginate concentration of 1.49%, extract concentration of 1.58%, and $CaCl_2$ concentration of 0.84%. The loaded flavonoids could be released gently in simulated gastric fluid (SGF) and rapidly in simulated intestinal fluid (SIF); this demonstrated a desirable controlled release profile.

Leonida et al. [102] investigated the encapsulation properties of chitosan toward two specific compounds in hop extracts, xanthohumol and lupulone. These are known for their antimicrobial activity, but this is accompanied by an extremely bitter taste. Researchers prepared chitosan (varied in molecular weights and DDs) nanoparticles by ultrasonication-assisted ionic interaction with tripolyphosphate (TPP). The particle sizes ranged from 28.1 to 49.4 nm when loaded with the bioactive compounds. Notably, two different types of chitosan were used in this study, including chitosan prepared from shrimp shells (190–235 kDa molecular weight, 75% DD) and from enzymatic treatment (4, 10, and 50 kDa molecular weight, 98% DD). In the in vitro release study, they found that the release of bitter-tasting bioactives was increased by increasing the molecular weight of chitosan in a pH = 7 buffer system. Nanoparticles composed of shrimp shell chitin (with a relatively high molecular weight and low DD) can retain a small amount of bitter compounds. This indicated that encapsulation may prevent those bitter-tasting molecules from attaching to the taste bud to perform the bitter perception because the pH value of saliva is around 6.2–7.6, which is similar to the test condition. On the other hand, it may also hint that the release would be low in a neutral intestinal environment as well, which could limit the absorption and further bioavailability. However, the results still recommend the nanocomposites as interesting for further study in food applications as preservatives and for oral administration. Anand et al. [103] also demonstrated the potential of chitosan-TPP nanoparticles as encapsulation matrices that reduce the bitterness of bacoside-rich *Bacopa monnieri* extract (BME). They used RSM experiments to find the optimal conditions which were 0.1% chitosan, 0.15% TPP, and 15 mg/mL of BME. The nanocomposites prepared in these conditions had an average particle size of about 220 nm, and the efficiency of encapsulation was 52.0%. These technologies may facilitate the supplementation of bioactive compounds in various functional food formulations through their biochemical and physiological activity.

5 Summary

In food industry, products are mainly preferred by consumers based on their sensory qualities, and flavor is the most important among all indices. Chitin and chitosan are versatile materials with proven flavor-improving capabilities for specific food products. Physical and chemical modifications including three flavor-improving strategies using chitin and chitosan have been reviewed: (1) the particle or fibril form of materials as saltiness enhancers; (2) intact addition or edible film/coatings for improving flavor stability; (3) removing or encapsulating bitter-tasting molecules.

For use in saltiness enhancement, chitosan/NaCl and chitosan/maltodextrin/NaCl microparticles had a stronger salty perception than common market table salt. This is mainly because the average particle size (<32 μm) is less than the general salt products (about 500 μm), and there are a large number of NaCl crystals (<4 μm) distributed on the surface of the particle matrix. Therefore, in the oral cavity, NaCl crystal can quickly dissolve and rapidly improve the concentration of Na^+ and have a strong salty perception. This strategy belongs to the particle size and shape alteration/enhanced dissolution rate in the aspect of sodium reduction applications. It can be used as highly salty sprinkle salt with low sodium content to season the surface of dried foods such as popcorn and chips. However, it is not suitable for use in liquid foods where the dissolved particles do not retain this dominant structure and therefore do not enhance the perception of saltiness. Instead the particles produce an astringent taste due to the presence of chitosan in the system. Chitin is particularly hard to dissolve in water. By mechanical disassembly or chemical modification methods, chitin nanomaterials, including chitin nanofibers and chitin nanocrystals, can be obtained, and these are able to form a stable suspension in water. When NaCl is added to a chitin nanomaterial suspension, the amine groups on chitin are able to absorb chloride ions due to the electrostatic interaction, resulting in a decrease in the zeta potential of the suspension system, which could reduce the astringency of the suspension as well as increase the concentration of free Na+ in the solution, resulting in an improved saltiness perception. This could be categorized into the alteration of food composition sodium reduction strategy, which can be used in soups, sauces (such as soy sauce, oyster sauce, bean paste), pickled foods, and seasoning bags of ready-to-eat food to achieve the purpose of reducing sodium.

Furthermore, chitosan possesses significant antioxidation and antimicrobial activities. It is able to improve the flavor stability of various foods by directly incorporating it into the composition or by coating it onto the surface. Chitosan with high molecular weight can inhibit the growth of Gram-positive bacteria, which would likely make it suitable for use in meat foods such as sausage. When used as a coating solution, the concentration of chitosan may significantly affect the preserving capacity. The use of chitin in this field is limited due to its lower DD than that of chitosan, resulting in relatively low antioxidation and antimicrobial activities. However, it could be applicable when it undergoes specific structural or chemical modification.

Moreover, chitosan revealed high adsorption capacity toward specific bitter-tasting molecules. Therefore, it could act as a coagulant and remove them from the

food system. Also, due to the remarkable binding capacity toward negatively charged compounds, it could effectively encapsulate the bitterness bioactives to prevent them from being perceived by bitter sensors in the oral cavity. Chitin and chitosan could also be used as carriers for Debittering enzyme immobilization in order to obtain products with desirable flavor.

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Modified Chitosan Films/Coatings for Active Food Packaging

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Contents

Abstract Chitosan (glucosamine) the first derivative of chitin is considered more promising in food packaging due to its versatility in all aspects. Chitosan can easily be modified by chemical reactions, forming composite by incorporating many active

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components to obtain film/coating. The bio-molecular architecture of chitosan provides excellent properties which are very much essential to active food packaging. This chapter aims to review the application of modified chitosan films/coatings with an emphasis on the structure–property relationship, a crucial component in search for the best composite material as a green substitute for plastics. The future course of research in the area of active food packaging and the significant role of chitosan as an important biomaterial in advanced techniques such as plasma and digitized 3D printing processes have also been highlighted in brief.

Keywords 3D printing · Chitosan · Food packaging · Nanocomposite film/coating · Plasma techniques

1 History of Packaging: An Introduction

The word packaging, in general, refers to the materials such as boxes, bags, papers, etc., in which goods are to be covered or protected before they are transported and sold. This definition clearly indicates that the goods need to be protected from any other external aggression so that they reach up to the consumer in as original shape and quality as possible. With regard to food stuffs, the packaging not only protects food but also very often enhances its shelf-life and helps in keeping its nutritional value intact. In earlier times wood/metal/glass/ceramic containers or foils, papers, clothes, etc., were used for packaging of foods. In recent couple of decades, however, all of those packaging materials got replaced by plastics. All kinds of man-made polymeric materials like polyethylene, polypropylene, PVC, polyesters, etc., have been employed in packaging and we know very well that they are basically non-biodegradable. The environmentalists have rightly raised their serious concerns about the use of non-biodegradable packaging materials as they are simply unsustainable. The utmost necessity of biodegradable packaging has also paved the way for active food packaging materials. Within and across the terms "synthetic" and "natural" the list of biodegradable materials is very exhaustive. However, we can classify biodegradable polymers from the point of view of potential food packaging materials (Fig. 1). We intend to discuss one of the most promising naturally occurring biodegradable polymers obtained from exo-skeleton of crustacean shells as a natural material, named chitin (an acetyl glucosamine), which is incidentally the most abundant polysaccharide after cellulose. Chitosan (glucosamine) is the deacetylated derivative of chitin. The presence of amino group in chitosan makes it much more versatile reactant than even cellulose. In the present short review an account of some recent studies carried out by us as well as by other authors on the synthesis and characterization of modified chitosan films/coatings for active food packaging is presented [1–14].

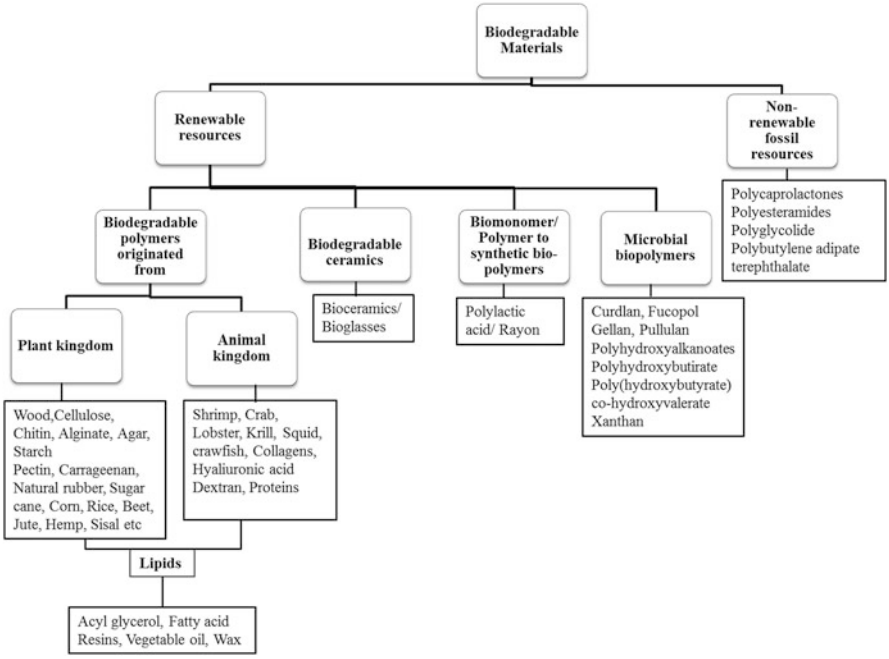

Fig. 1 Classification of biodegradable polymers for food packaging

2 Desirable Properties of Bio-Based Packaging Films

Natural biopolymers have long been limited in their use as main components of packaging material because of their poor physical properties, which are deemed inferior to conventional synthetic and petroleum-derived products. Improving the barrier and mechanical properties and antimicrobial properties has been a major challenge that prompted research on modification of these biopolymer matrices [15]. To address these problems, various researchers adopted an approach which has been to incorporate naturally derived biodegradable products into synthetic polymers [16, 17]. However, these composite materials of nanoclays, glass, carbon fibers, and carbon nanotubes with synthetic polymers of both aliphatic and aromatic nature such as polyesters, polyesteramides, polyvinyl alcohols, and polystyrene, etc., pose another problem which is toxicity. Along with their non-biodegradability and endangering ingestion by animals, plants, and marine species they eventually get absorbed into the food chain [18–20]. Chitosan with its many desirable properties such as biodegradability, non-toxicity, and low cost fits as the most suitable candidate among all naturally derived biomaterials to fabricate biocomposites [21].

3 Chitosan for Food Packaging

As outlined above, due to the environmental concerns the waste polysaccharides mainly from animal sources and inherent characteristics property have attracted the attention in the food packaging industry resulting chitin and chitosan containing bionanocomposites show attractive properties that make them suitable for application as active food packaging materials, viz. films and coatings [22].

3.1 Preparative Methods of Modified Chitosan

High moisture sensitivity of chitosan restricts its direct application in food packaging. This limitation can be overcome if it is blended with other hydrophobic substances. Thus, reinforcing chitosan with other polymer/biopolymer, small functional molecules, and non-toxic metal nanoparticles, etc., can lead to synthesis/fabrication of novel composites with enhanced physical properties, such as water resistance without loss in biodegradability. Here we described the preparation and properties of some novel chitosan-based bioactive films, viz. Quercetin-starch based Chitosan-gelatin (Ch-ge-Q), Chitosan-ZnO NPs loaded gallic acid (Ch-ZnO@gal), and Chitosan-PVA based NiNPs composite (Ch-PVA-NiNPs) films.

3.1.1 Preparation of Ch-ge-Q

Quercetin-starch based complex (Q) with Ch-ge was prepared with some modification as described elsewhere [23]. Q was designated as (1) containing OH linkages. Ch-ge conjugates (2) were formed by electrostatic interaction (hydrogen bonding). The Ch-ge-Q (3) film was prepared by solution casting method. The schematic presentation for preparation of Ch-ge-Q (3) is shown in Fig. 2.

3.1.2 Preparation of Ch-ZnO@gal

Preparation of ZnO NPs is a well-known method today [24]. Preparation of Ch-ZnO@gal with different ZnO@gal ratios was made by solution casting method. The systematic preparation of Ch-ZnO@gal film is shown in Fig. 3.

The size of the nanoparticles lied between 15 and 30 nm (Particle size analyzer (DLS) measurement as well as XRD data also well agreement with the results [25]). ZnO@gal nanoparticles diameter measured around 19.2 nm. The FTIR spectra confirmed the blending in Ch-ZnO@gal films.

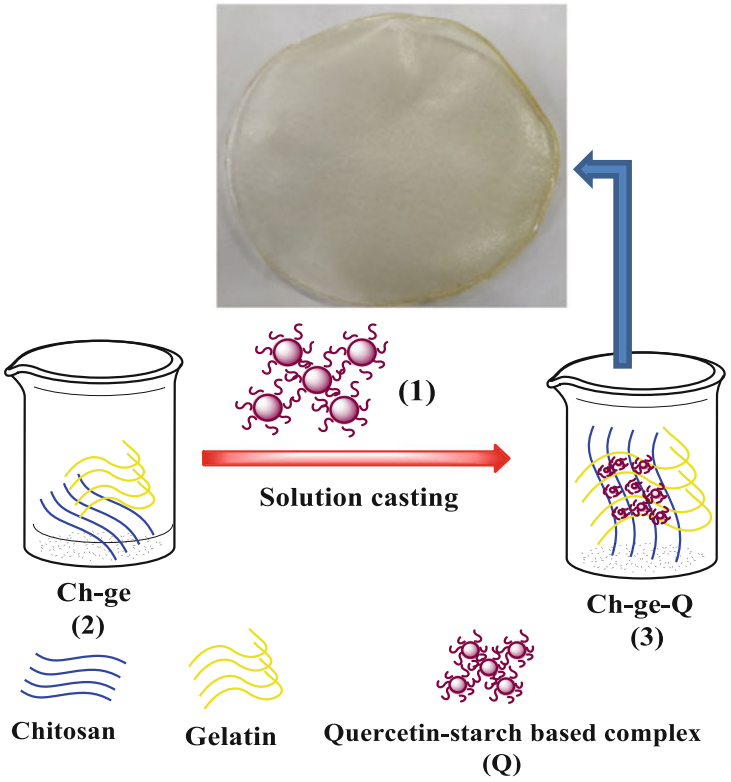

Fig. 2 Schematic presentation for preparation of Ch-ge-Q (3) from Q (1) and Ch-ge (2)

3.1.3 Preparation of Ch-PVA-NiNPs

Ni nanoparticles (NiNPs) were prepared using rose petals extract [26]. DLS of the dispersion of the Ni nanoparticles showed that NiNPs exhibit narrow size distribution and the resulting mean size was lying between 90 and 110 nm. Preparation of Ch-PVA-NiNPs film was made by PVA and chitosan solution with the addition of Ni nanoparticles by solution casting method (Fig. 4).

3.2 Structure–Property Relationships

Various synthetic methods for modification of chitosan have been employed in the past so as to make it suitable for wrapping/coating food. Herein an attempt has been made to establish a relationship between the molecular architecture, i.e. amino functionality at C2 position and its significance with the properties needed for food packaging applications [27–29]. These outcomes of the results established

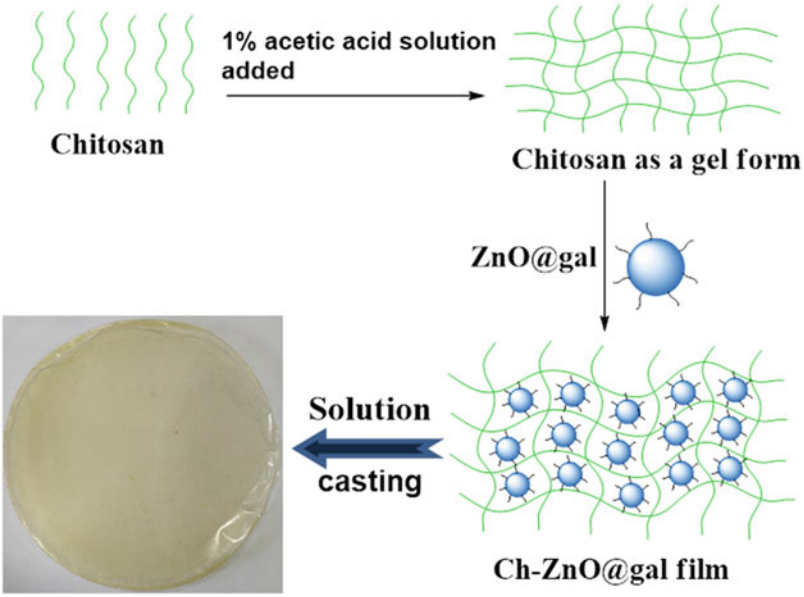

Fig. 3 Preparation of Ch-ZnO@gal film

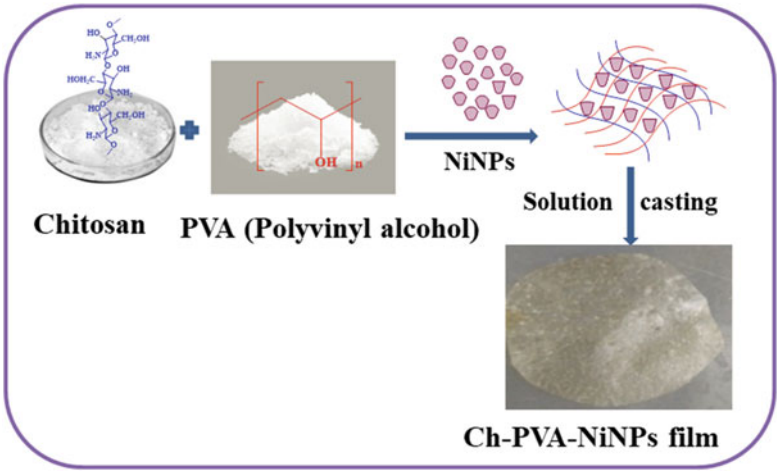

Fig. 4 Preparation of Ch-PVA-NiNPs by solution casting method

correlations for utilization of chitosan in food packaging industries (Fig. 5). This work especially deals with this aspect attempts to fine-tune the molecular structure of the resulting chitosan films/coatings in such a way that a systematic relationship

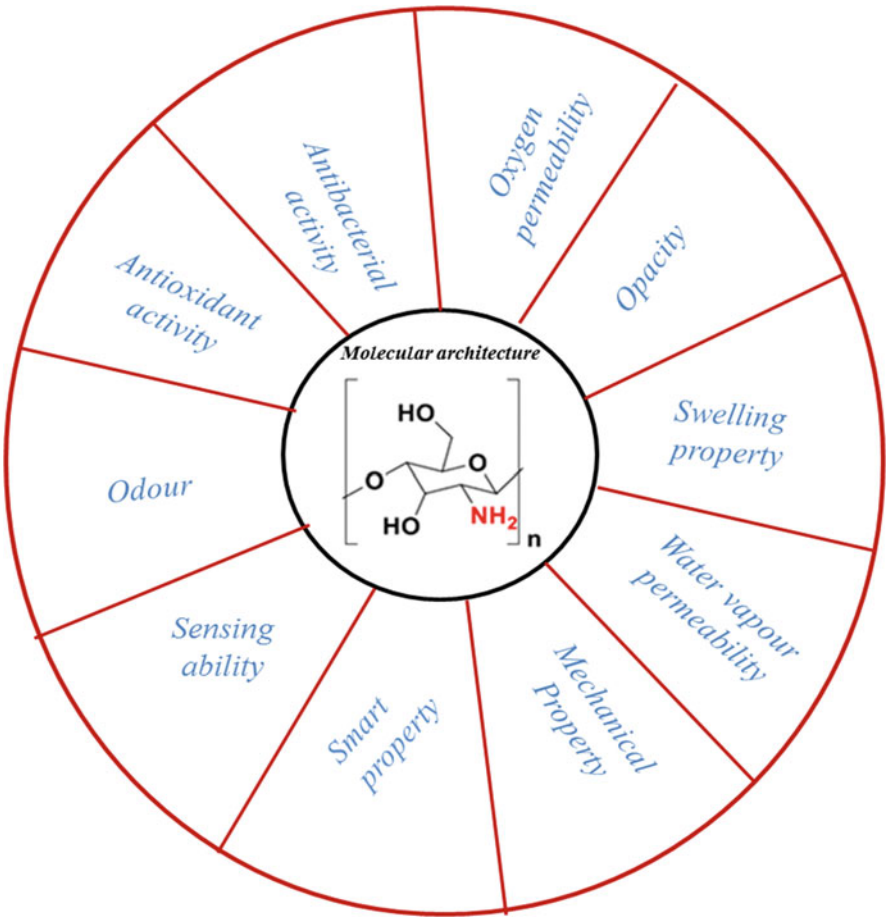

Fig. 5 Structure–property relationship of chitosan based film/coating for food packaging material

between their structure and desirable properties (WVP, mechanical, OP, sensing ability, odor, opacity, swelling, smart sensing, functional, etc.) can be deciphered.

Mechanical properties play an important role for packaging films/coatings when foods are being transported, stored without losing its acceptability. In this way mechanical property acts as a barrier and thus maintaining the food product quality and its freshness. The mechanical as well as barrier properties based on selection of chitosan in which mode like pure or modified chitosan, films, or coating forms for effective food packaging.

It has already been described in preparative method section that modification is possible in various ways like reinforcing of nanoparticles (NPs) with biopolymers/other synthetic polymers/small molecules, etc., with chitosan for enhancing the shelf-life of foods [30, 31].

3.2.1 Mechanical Properties of Ch-ge-Q Film

As per the finding of the composite film the mechanical properties were affected significantly (Fig. 6). The higher tensile strength (TS) of Ch-ge-Q (17.11 ± 0.3464 MPa) in comparison to Ch-ge film (16.10 ± 0.1414) showed better compatibility of Ch-ge and Q. The structural change in Ch-ge-Q film as well as more molecular interaction between Ch-ge and Q leads to establish good TS in the film [33]. The structure of the polymer matrix in the films makes a good correlation with property like density and distribution of interaction like intra and intermolecular nature which finally is responsible for better mechanical properties of films [34]. Further the active components of gelatin/Q mixed with chitosan film significantly reduced EAB of Ch-ge-Q films due to formation of the microspores and cavities in the films and as a result of interdiction, the flexibility of the films observed. This property clearly indicates the branched structure of the Ch-ge-Q film. The UV irradiated films as per pre-requisite assumption show lower TS and higher % EAB.

3.2.2 Mechanical Properties of Ch-ZnO@gal

The improved TS as well as EAB values were observed in ZnO@gal loaded chitosan films in comparison to pristine chitosan films (Fig. 7). Further with the increase of ZnO@gal ratio in Ch-ZnO@gal films TS and EAB both values were increased and Ch-ZnO@gal3 film was the highest with 70 mg loading of ZnO@gal. ZnO NPs effective space fillers in the chitosan matrix modified its molecular architecture and thus Ch-ZnO@gal property remodeled to strong interfacial interactions with large surface area. Shankar et al. and some other studies also suggested that addition of ZnO NPs greatly improved the mechanical property of bionanocomposite films [36–38].

3.2.3 Mechanical Properties of Ch-PVA-NiNPs

The mixing of NiNPs into Ch-PVA films increased TS and EAB values. Same as ZnO NPs, NiNPs behave like effective space fillers in the composite matrix [39]. Thus molecular architecture complied to the property due to large surface area of NPs and strong interfacial interaction with Ch-PVA molecules. The mechanical properties of different chitosan films as described above are compared in Table 1.

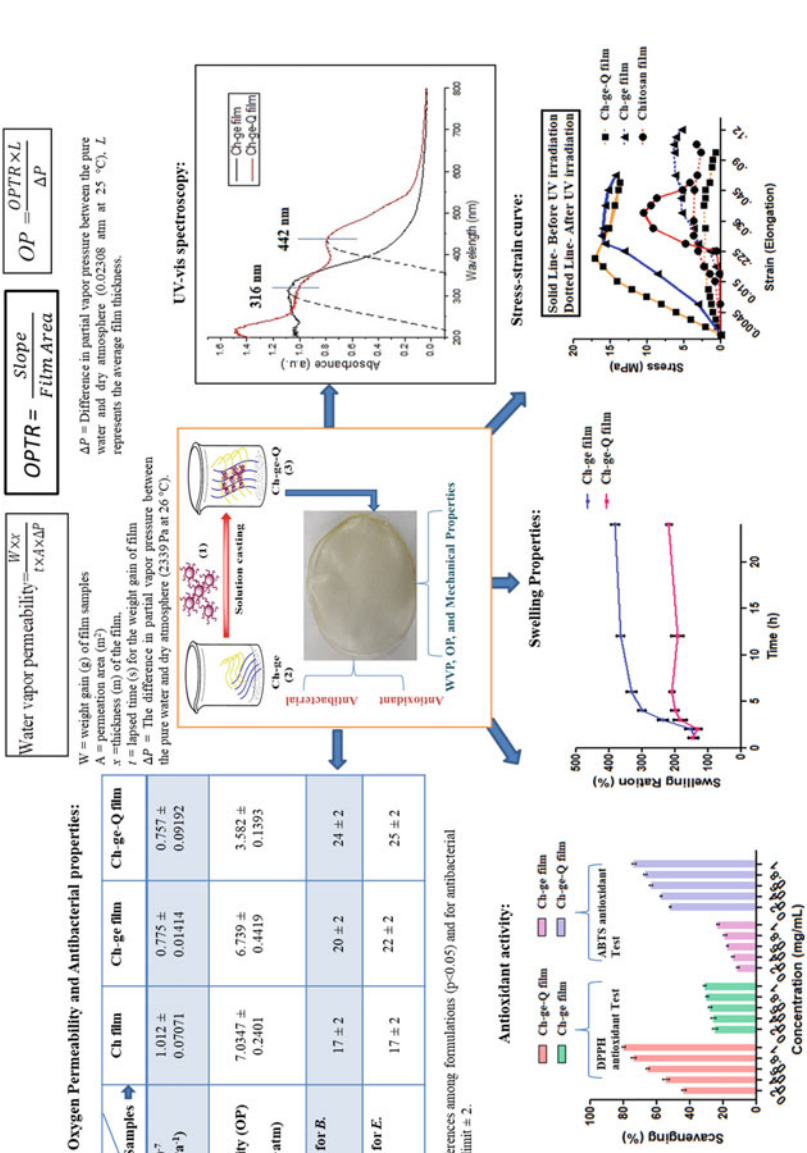

Fig. 6 A pictorial presentation for structure–property relationship of Ch-ge-Q (3) film. Some figure/table modified from Ref. [32] with permission

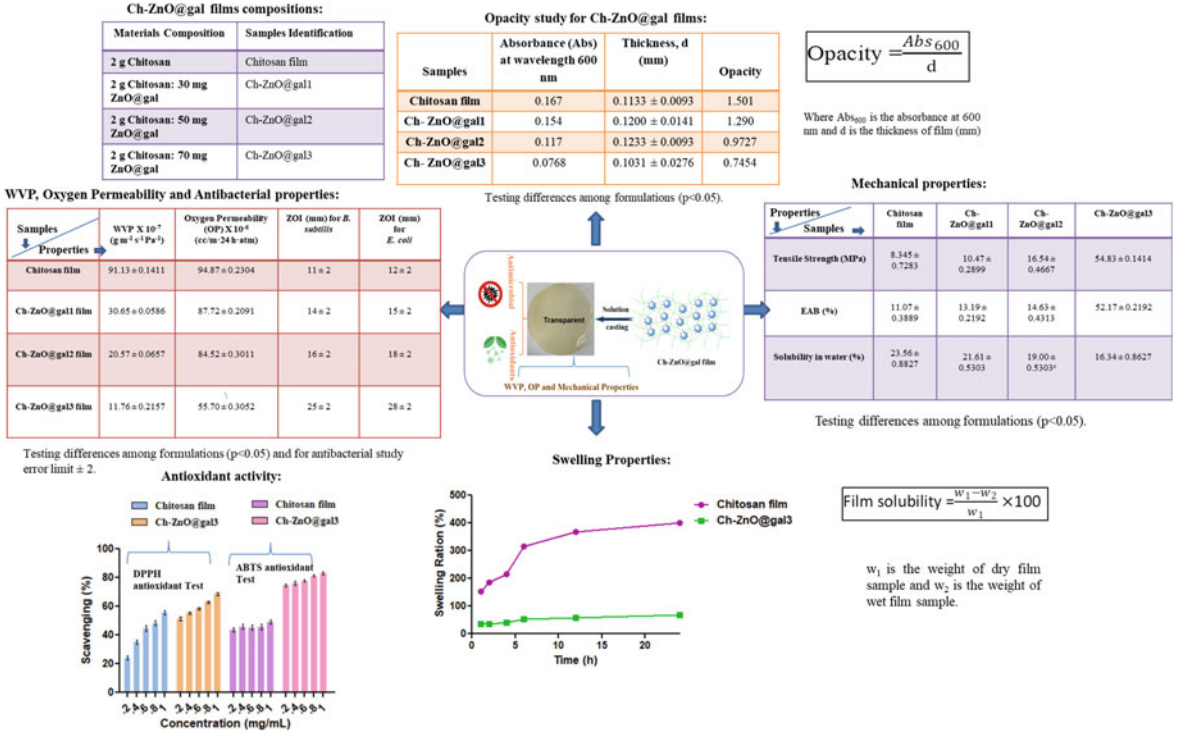

Fig. 7 A pictorial presentation for structure–property relationship of Ch-ZnO@gal films. Some figure/table modified from Ref. [35] with permission

Table 1 Mechanical properties of different chitosan films

Mechanical property	Ch-ZnO@gal3	Ch-ge-Q	Ch-PVA-NiNPs
Thickness (mm)	0.1031 ± 0.0276	0.1233 ± 0.0093	0.1343 ±. 0055
Tensile strength (MPa)	54.83 ± 0.1414	17.11 ± 0.3464 (8.345 ± 0.3134)[a]	12.64 ± 0.88062
EAB (%)	52.17 ± 0.2192	5.100 ± 0.3162 (11.37 ± 0.07071)[a]	14.71 ± 0.3001

[a]After UV irradiation

3.3 UV–Vis Spectroscopy

UV-shielding films in terms of barrier properties focused much on food packaging due to generation of free radicals by UV irradiation which can adversely affect food quality. Thus filtering of UV light to protect foods in the form of films is very essential. The evaluations of UV-protection of films are done by scanning of UV light in 200–700 nm regions.

3.3.1 UV–Vis Spectroscopy of Ch-ZnO@gal

The light transmission of Ch-ZnO@gal films in 200–800 nm UV–vis wavelength was used for the barrier properties of the films. The homogeneously dispersed ZnO@gal NPs in chitosan significantly reduced the transmission of light vis-a-vis the light barrier property of the composite film improved by blocking both of transmission and scattering light [40]. The nanocomposite with change in molecular structure the transmission light was more reduced than pure chitosan film. The opacity is usually decided for transparency of the films (Fig. 7). Ch-ZnO@gal films showed better opacity as well as lower transparency. Like mechanical property higher ZnO@gal NPs loaded films showed more opaque light barrier property [41].

3.3.2 UV–Vis Spectroscopy of Ch-ge-Q

The film showed slightly enhanced absorption in 200–400 nm region as well as extended absorption in 400–500 nm regions (Fig. 6). The filtration of high energy UV rays by the film indicated less exposure of UV rays to protect the quality of packed foods [42].

3.3.3 UV–Vis Spectroscopy of Ch–PVA-NiNPs Film

The preparation of NiNPs from the green synthesis method was physically verified by the change of color from green to brown and this is also confirmed by a UV–

Visible spectrophotometer, at 270 nm showing a narrow spectral band attributing to surface plasmon resonance effect of the NiNPs [27, 43].

3.4 Water Vapor Permeability (WVP)

Structural and chemical properties of molecular architecture of chitosan, hydrophobic interaction of active components of films, additives concentration and its type decide the WVP to study moisture transfer across the films. The packaging film capacity is evaluated through minimization of moisture transfer between food and surrounding environment of film [44]. The lower WVP value indicates the more freshness of foods. In this way chitosan films help to decrease the transfer of water between the environment and wrapped product; consequently the product's loss of water is decreased.

3.4.1 WVP of Ch-ge-Q

The WVP value of chitosan film (1.01×10^{-7} g m^{-1} s^{-1} Pa^{-1}) is shown in Fig. 6. The amino and hydroxyl groups of chitosan backbones served as water molecules binding sites and thus decreased water loss of packed foods. The WVP phenomena occurred in adsorption-diffusion step where hydrophilic domains of Ch-ge-Q films more easily adsorbed water vapor, substantially improved the diffusion step in comparison to Ch-ge film.

3.4.2 WVP of Ch-ZnO@gal

In comparison to pristine chitosan films ($91.13 \pm 0.1411 \times 10^{-7}$ g m^{-1} s^{-1} Pa^{-1}), Ch-ZnO@gal3 films showed lower WVP values ($11.76 \pm 0.2157 \times 10^{-7}$ g m^{-1} s^{-1} Pa^{-1}). The more dispersion of ZnO@gal NPs into the films decreased WVP values. It is due to the complicated path of the water molecules pass through the molecular structure. Further, good dispersion of ZnO@gal NPs into the films restricted the void spaces of the network structure of the macromolecule and thus interaction between NPs and chitosan molecules is increased. In this way structure–property correlates the condensed macromolecular network structure with lower WVP values [45]. Some previous researchers also concluded that WVP values of biopolymer composites with ZnONPs decreased [37, 46].

3.4.3 WVP of Ch-PVA-NiNPs

Ch-PVA-Ni nanocomposite film had a lower value of the WVP compared to only chitosan film [47]. The only chitosan film had a higher WVP value is

101.13×10^{-7} g m^{-1} s^{-1} Pa^{-1}. Incorporation of NiNPs into Ch-PVA film showed lower WVP value. Water solubility, WVP, and OP of all above chitosan nanocomposite films are shown in Table 2.

3.5 Water Solubility

Like WVP of chitosan based films, water solubility is also an essential parameter for food packaging application. Food packaging films should have water resistance property.

3.5.1 Water Solubility of Ch-ge-Q

Ch-ge-Q films retained its shape in water while Ch-ge films changed its shape consequently. Hence Ch-ge-Q films have water resistance property (Fig. 6). The molecular architecture of chitosan nanocomposite's particularly the amino groups present in chitosan and gelatin make interaction with phenolic compound of the active component Q and consequent upon showed high water resistance property [48].

3.5.2 Water Solubility of Ch-ZnO@gal

The Ch-ZnO@gal films showed reduced water solubility. The presence of ZnO@gal NPs made the films denser and thus more sealing. The hydroxyl groups bounded, interface formed between chitosan and nanomaterials which disturbed the intermolecular attraction, and thus unscrambled the chitosan matrix. Investigation made by [49, 50] in chitosan nanocomposites also described similar kind of phenomena. However, water solubility of Ch-ZnO@gal films slightly decreased with the increase of ZnO@gal addition (Fig. 7).

Table 2 Water solubility, WVP, and OP of different chitosan films

Property	Ch-ZnO@gal3	Ch-ge-Q	Ch-PVA-Ni nanocomposite
Solubility in water, %	16.34 ± 0.8627	41.23 ± 0.4638	20.83 ± 0.3573
WVP $\times 10^{-7}$ (g m^{-1} h^{-1} Pa^{-1})	11.76 ± 0.2157	0.757 ± 0.09192	43.60 ± 0.7308
Oxygen permeability (OP) $\times 10^{-6}$ (cc/m·24 h·atm)	55.70 ± 0.3051	3.582 ± 0.1393	–

3.5.3 Water Solubility of Ch-PVA-NiNPs

The result of only chitosan film and Ch-PVA-NiNPs film appeared that Ch-PVA-Ni nanocomposite films have lower values of water solubility. The functioning of reduced water solubility may be the same as described for Ch-ZnO@gal films. Therefore, NiNPs into chitosan film decreased the solubility of the synthesized films.

3.6 Swelling Properties

The swelling degree as well as water solubility of bionanocomposite films is highly interconnected. Like water solubility, the swelling degree is also indicative of moisture resistance [51, 52]. Hence swelling of chitosan films can also be considered as an important property in the field of food packaging application (Fig. 5).

3.6.1 Swelling Properties of Ch-ge-Q Film

The swelling degree in 24 h of Ch-ge-Q film (220%) was lower in comparison to Ch-ge film (382%) (Fig. 6). Interaction between hydrophobic groups in Q and hydrogen bonding of chitosan is responsible for lower swelling values, at the same time less exposure of polar side-chain groups to water molecules made possible reduction of water uptake by gelatin molecules (Fig. 2). Hence molecular architecture of chitosan based nanocomposites is indicative of a good correlation with their swelling property which make them suitable for application in food packaging.

3.6.2 Swelling Degree of Ch-ZnO@gal

The Ch-ZnO@gal films showed reduced swelling in comparison to neat chitosan films (Fig. 7). The possible reason for lower swelling as described in water solubility for Ch-ZnO@gal films is same because the swelling degree, water solubility as well as WVP of bionanocomposite films are very much interconnected [49]. Here also, swelling index of Ch-ZnO@gal films slightly decreased with the increase of ZnO@gal addition (Fig. 7).

3.6.3 Swelling Degree of Ch-PVA-NiNPs

As explained above, the swelling degree of chitosan decreased after the incorporation of nanoparticles. Therefore, the incorporation of NiNPs into the chitosan-PVA film, the swelling degree of the synthesized films also decreased.

3.7 Oxygen Permeability (OP)

As described above in case of mechanical property and UV-shielding for chitosan based films, the barrier property plays an important role to find out the appropriateness of packaging materials for food application. Similarly, it is true in case of barrier property against the permeation of oxygen for chitosan based films. OP values of chitosan based films may be compared with commercial ethylene vinyl alcohol copolymer films, polyvinylidene dichloride (PVDC), and polyethylene films.

3.7.1 Oxygen Permeability (OP) of Ch-ge-Q

As shown in Table 2, the value of OP decreased in Ch-ge-Q film (3.582×10^{-6} cc/m·24 h·atm in comparison to neat chitosan film (7.0347×10^{-6} cc/m·24 h·atm) [53, 54] explained the oxygen permeability of chitosan films which was basically association of O_2 with NH_3^+. The Q component mainly transforms the molecular structure of composite film and thus resulted for decrease in permeation of oxygen value.

3.7.2 OP of Ch-ZnO@gal

The Ch-ZnO@gal films showed reduced OP values ($8.772–5.570) \times 10^{-5}$ cc/m·24 h·atm in comparison to neat chitosan film ($9.487 \pm 0.2304 \times 10^{-5}$ cc/m·24 h·atm) (Fig. 7). The possible reason for lower OP value may be explained as due to the ZnO@gal component which is responsible for molecular structure transformation of nanocomposite film. Similarly, the incorporation of NiNPs into the chitosan-PVA film, the OP value of the synthesized films also decreased [32, 53].

3.8 Antioxidant Activity

The main advantage of antioxidant active packaging is to provide sustained release of antioxidants during storage of foods. The incorporation of antioxidant compounds in the film for food packaging is done by various methods. Without having antioxidant activity of packaging films deteriorate the food quality by losing it sensorial and nutritional value. Hence antioxidant activity of food packaging films plays very important role. Here we described DPPH and ABTS assay methods used for the determination of antioxidant activity.

3.8.1 Antioxidant Activity of Ch-ge and Ch-ge-Q Film

DPPH Radical Scavenging Activity

Our laboratory used DPPH assay method for Ch-ge film and Ch-ge-Q film by absorbance value at wavelength 517 nm. Both films showed radical scavenging activity, respectively, 31.2% and 81.45% at a concentration of 1 mg/mL [55]. Obviously, the addition of Q in Ch-ge film showed better antioxidant activity (Fig. 6).

$ABTS^{\cdot+}$ Scavenging Activity

$ABTS^{\cdot+}$ method where 2, 2-azino-bis (3-ethyl benzothiazoline-6-sulfonate) was used to measure the antioxidant activity. The films decolorized due to the presence of antioxidant. In ABTS assay the stabilization of unstable free radicals was faster in comparison to DPPH assay. ABTS assay of both films were found, respectively, 18.11% and 72.2% at a concentration of 1 mg/mL (Fig. 6).

3.8.2 Antioxidant Activity of Ch-ZnO@gal

DPPH Radical Scavenging Activity

As above similar DPPH scavenging activity was made for Ch-ZnO@gal films (Fig. 7). ZnO@gal NPs in chitosan films increased DPPH radical scavenging ability significantly (64.66–68.51%) in comparison to neat chitosan films (55.56%).

$ABTS^{+}$ Scavenging Activity

Similarly, the % scavenging activity of chitosan film and Ch-ZnO@gal films were measured at a concentration of 1 mg/mL (Fig. 7). In this method also ZnO@gal NPs in chitosan films increased ABTS radical scavenging ability significantly (76.38–83.43%) in comparison to chitosan films (48.96%).

3.8.3 *DPPH* Radical Scavenging Activity *of* Ch-PVA-NiNPs

The DPPH radical scavenging activity of Ch-PVA-NiNPs film showed the reducing ability of as estimated with the absorbance value at wavelength 517 nm. When increasing the concentration of the sample films, decreased the absorbance value. The result of the DPPH radical % scavenging assay of only chitosan film is 38.00 at concentration 1 mg/m and for Ch-PVA-Ni nanocomposite film is 58.05. The

presence of NiNPs in chitosan films greatly improved DPPH radical scavenging ability [35].

3.9 Antibacterial Activity

The results of the antibacterial activity of chitosan films are generally estimated against *B. Subtilis* the gram positive bacteria and *E. coli* the gram negative bacteria with film concentration of 1% acetic acid in 0.5 mg/mL. The mechanism behind excellent antibacterial of chitosan may be the interaction occurring between negatively charged macromolecules containing groups present in proteins and nucleic acid. This phenomenon results in structural changes and distortion in the cell wall and membrane of bacteria, which causes interruption of the metabolic process and finally bacteria death [56, 57].

3.9.1 Antibacterial Activity of Ch-ge-Q

The zone of inhibition (ZOI) for prepared films was in the order of Ch-ge-Q > Ch-ge > Ch (Fig. 6) which implies Ch-ge-Q film showed better antibacterial activity in comparison to Ch-ge and neat chitosan films. ZOI (mm) for the chitosan based films is shown in Fig. 6. As per structural formation of the nanocomposite, Q acts as a potential antimicrobial agent and thus enhances the ZOI of the film The structure–property relationship helped to select the best possible materials (here Ch-ge-Q film) in food packaging for effective shelf-life of products.

3.9.2 Antibacterial Activity of Ch-ZnO@gal

The antibacterial activity of Ch-ZnO@gal films linearly increased with the addition of ZnO@gal NPS where Ch-ZnO@gal3 showed best result (Fig. 7). Reactive oxygen species (ROS) as generated by NPs and presence of Zn^{2+} ions jointly attacked the cell wall of bacteria having negatively charged and thus leakage occurred which ultimately is responsible for death of bacteria. This property of Ch-ZnO@gal films may make it suitable for active food packaging for various kinds of fruits, vegetables, etc.

3.9.3 Antibacterial Activity of Ch-PVA-NiNPs

Different mechanisms have been proposed for a synergistic increase in the antibacterial property of chitosan and metal nanoparticle composites. One of them is the damage of the bacterial cell membrane due to the depolarization of membrane due to the interaction of the cationic amino group with the negatively charged cell

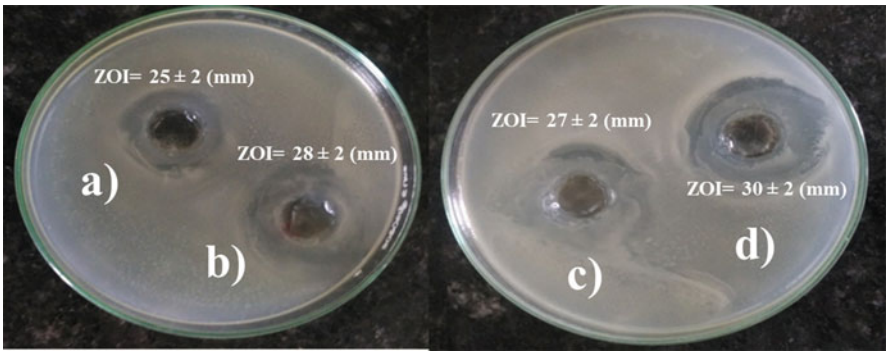

Fig. 8 Inhibition zone of (**a**) Chitosan film (**b**) Ch-PVA-Ni composite film against *B. subtilis* (**c**) Chitosan film (**d**) Ch-PVA-NiNPs film against *E. coli*

membrane of bacteria, which could result in cell leakage, loss of membrane penetrability, and eventually the cells death [32, 58]. From the results of ZOI, it was found that Ch-PVA-NiNPs films have greater activity [25] (Fig. 8).

4 Smart Packaging Property

The structure–property relationship in one way identified the smart food packaging property by selecting the active, intelligent, and green components like edible as well as biodegradable materials, plant extracts, nanomaterials of organic or inorganic nature which synergistic effect yields a most promising food packaging system. Thus smart packaging approaches an excellent direction for minimization of food loss and maximization of food quality throughout the food supply chain process. Smart packaging may be referred to green and sustainable technology in packaging systems with the advent of nanotechnology and sensing ability [59].

4.1 Nanotechnology

Through active packaging systems chitosan film/coating formed by various active components using nanotechnology bears different selective properties for protection of foods during transportation and storage as well as it enhances its shelf-life. Nanostructured coating/nanocoatings are made by ultra-thin nanoscale layers with less than 100 nm onto surfaces of foods/packaging products which helps to improve the barrier performance as well as antimicrobial aspects of both [60].

4.2 Sensing Ability

The sensing ability of foods and packages is determined by using of sensors or indicators to check the quality and integrity of packed food products [61]. Anthocyanin-based natural dye is one of the plant constituents known as flavonoids when added to coating/film, the decolorization is identified by its expiry date of using the products [62]. Yong et al. recently studied chitosan based rich purple-fleshed sweet potato extract (PSPE) and anthocyanin mixed composite to investigate the visible color changes of the films. Here the antioxidant and pH-sensing property of the films helped to monitor the quality vis-à-vis extending the shelf-life of food products [63].

Bai et al. reported carboxymethyl chitosan (CMCS)-quercetin composite films for sensing of Al^{3+} ions. The study of color index and UV–Vis spectrophotometry of composite films helped to detect Al^{3+}-sensing materials in food packaging using antioxidant and intelligent property. This kind of detection may be done in deep-fried dough sticks and steamed buns packed foods [64].

Chitosan derivative for CN^{-} recognition was studied by Nigam et al. [65]. The anion sensing property of azo-based chitosan derivatives used as biosensors for biological toxic cyanide in various food systems as a facile visual detection method. The sensing ability of chitosan derivative may be utilized for the toxicity level of food products [65].

5 Other Chitosan Food Packaging Films

There have been many reports on other kind of modified chitosan films using active components, food borne pathogens on different foods. Here in Table 3 we summarized other chitosan based antimicrobial food packaging films.

6 Other Chitosan Coating Forming Food Packaging

Chitosan has been extensively studied for edible coatings onto the surface of foods or packaging materials to functionalize them by different methods like embedding, immobilization (covalent/ non-covalent), deposition by layers, photo grafting, etc. Food coatings can cause them to become more palatable. It can improve the food quality by delaying moisture loss and enhancing product appearance. Further, chitosan based coatings exhibit good OP and CO_2 barrier properties. The presence of coatings acts as barriers for intrinsic antimicrobial, antioxidant, and color protection properties to retard microorganisms development, reduce lipid oxidation and discoloration of foods and synergistically all such kind of properties extend the shelf-life as well as effective environmental friendly packaging [101, 102]. The

Table 3 Active components used with chitosan for antimicrobial food packaging films

Active components used with chitosan	Functioning of films/Food borne pathogens	Food item used	References
Agroindustrial residue (AIR) extracts	To enhance the antibacterial property of the chitosan film and improve shelf-life of chicken product	Chicken product	[66]
Apricot kernel essential oil (AKEO)	To enhance the antibacterial properties against *B. subtilis* and *E. coli*	Sliced bread	[67]
Bamboo vinegar (BV)	To improve the antibacterial properties against *Enterobacteriaceae* spp., and *Pseudomonas* spp., lactic acid bacteria (LAB)	Ready to cook (RTC) meat product	[68]
Cinnamon essential oil (CEO)	Lactic acid bacteria, yeasts, and molds, *Enterobacteriaceae* spp., *S. aureus*	Beef patties	[69]
Crosslinked PVA	*Inhibitory effect of film using E. coli, S. aureus, and B. subtilis*	Tomato	[70]
Lemon essential oil (LEO)	Fungus, *Botrytis cinerea*	Strawberry fruits	[71]
Lysozyme (L)	To enhance the antibacterial properties against lactic acid bacteria, yeasts and molds, mesophilic, psychrotrophic	Semi-hard, unripened cheese (halloumi cheese)	[72]
Melissa officinalis essential oil (MOEO)	*E. coli*	Antimicrobial film	[73]
Nisin (N)/gallic acid	To determine the total viable count (TVC)	Pig meat (pork loin)	[74]
Nisin (N)	*Lactobacillus* spp.	Mutton meat	[75]
Nisin (N)	*Listeria monocytogenes*	Cheese	[76]
Oregano essential oil (OEO)	*L. monocytogenes*	Raw chicken meat fillets	[77]
Oregano essential oil (OEO)	Yeast and molds	Sliced bread	[78]
Plum peel extract (PPE)	To improve the antibacterial activity against gram negative bacteria *(E. coli, Salmonella)* and gram positive bacteria *(S. aureus, L. monocytogenes)*	Food packaging	[79]
Spirulina extract (SE)	To enhance antibacterial property against *E. coli, B. subtilis, L. monocytogenes, S. aureus, P. aeruginosa, S. typhimurium,* and *B. cereus*	Active food packaging	[80]
Thyme oil (TO)	Yeasts and molds and *pseudomonad*	Shiitake (*Lentinus edodes*) mushroom	[81]

(continued)

Table 3 (continued)

Active components used with chitosan	Functioning of films/Food borne pathogens	Food item used	References
Thyme essential oil (TEO)	NA	Rainbow trout fish	[82]
PVA and pectin	To study positive antimicrobial activity of the film against pathogenic bacteria	Food packaging	[83]
TiO_2 NPs	To enhance the antibacterial properties against *S. aureus, E. coli*, *C. albicans*, and *Aspergillus niger*	Food packaging	[84]
TiO_2 NPs	To enhance the antibacterial properties against *E. coli, S. aureus, Salmonella, and L. monocytogenes*	Food packaging	[79]
Cu NPs	To improve the antibacterial properties against *Alternaria solani,* and *Fusarium oxysporum*	Food packaging	[85]
CuO NPs	To enhance the antibacterial properties against *E. coli*, *Listeria monocytogenes,* and *Pseudomonas aeruginosa*	Food packaging	[86]
CuO NPs (MMTCuO) nanocomposite	To enhance the antibacterial properties against *B. cereus, S. aureus* and gram *P. aeruginosa, E. coli*	Food packaging	[87]
Gelatin/AgNPs films	To enhance red grapes shelf-life up to 14 days	Red grapes	[88]
TiO_2	To enhance antibacterial properties of film and improve shelf-life of the red grapes up to 15 days	Red grapes	[84]
TiO_2	To improve the antibacterial property and prepared film delayed the ripening process of tomatoes	Tomato fruit	[89]
ZnO nanocomposite film	At room temperature to enhance the shelf-life of okra up to 12 days	Okra or ladyfinger	[90]
AgNPs bacterial cellulose nanocrystals (BCNC)	To enhance the mechanical, physical, and antimicrobial properties of chitosan based film after the addition of silver nanoparticles and BCNC	Antimicrobial nanocomposite film	[91]
ZnONPs	To improve carrot shelf-life	Carrot pieces	[92]
Sulfur nanoparticles	To enhance antimicrobial properties of the prepared food packaging film	Antimicrobial food	[93]
Ag_2O NPs	To enhance microbial safety and extend food shelf-life	Food packaging	[94]
Nanocellulose (NC)	To decreased the LAB count in ground meat and extende shelf-life of ground meat up to 6 days	Ground meat	[95]

(continued)

Table 3 (continued)

Active components used with chitosan	Functioning of films/Food borne pathogens	Food item used	References
Carvacrol microcapsules and grape seed extract (GSE)	To increase the shelf-life of Salmon (refrigerated) up to 7 days	Salmon (*Salmo salar*)	[96]
Pink pepper residue (PPR) and peanut skin	To maintain the quality of chicken and decreasing microbial count	Chicken meat	[66]
Gelatin and grape seed extract *Ziziphora clinopodioides* essential oil	To improve the shelf-life of boneless fish up to a certain period of time	Minced trout fillet	[97]
Oregano essential oil	To enhance the shelf-life of fish tissue up to 12 days	Grass carp muscle	[98]
Kombucha tea extract (KTE)	The quality of beef maintained and shelf-life extended by 3 days	Minced beef meat	[99]
Zinc acetate dihydrate as nano ZnO antimicrobial flexible pouches	To enhance the antibacterial properties of the chitosan films and maintain the shelf-life of raw meat	Raw meat	[100]

active components used with chitosan for food coating packaging are shown in Table 4.

7 Modern Food Packaging Techniques

In the space of global digitalization modern techniques brought the advancement in packaging, where the names of a few like 3D-printing and plasma radiation can be considered for effective food packaging.

7.1 3D Printing for Food

3D printing food is now becoming more of a reality where many of the features of printing modes like multi materials additives, customized nutrition content, custom molds, desirable shape of any of complex design, requirement of no or very less human interaction, flexibility, CAD software based integration, wastages of food can be minimized as compared to other conventional methods [112, 113].

Table 4 Active components used with chitosan for food coating packaging

Active components used with chitosan	Food borne pathogens/post coating results	Food item used	References
TiO_2 NPs	To facilitate for superior antibacterial assessment	For food packaging	[103]
Oregano EO	To improve the sensory quality of chicken meat	Chicken breast	[104]
Pomegranate peel extract	To enhance the antioxidant property and also maintain the overall fruit quality	Guava (*Psidium guajava* L.) fruit	[105]
Aloe vera extract	Coating of *Aloe vera* in chitosan films improved the shelf-life of blue berry up to 5 day.	Blueberry	[106]
Coating of chitosan in guava fruit	Coating of chitosan improved the antioxidant property might be delayed the ripening property of guava	Guava fruit	[107]
Bioactive compounds and essential oils (EOs)	To improve the antibacterial property of the vegetable	Fresh cut broccoli	[108]
Different molecular weight of chitosan ($M_W = 150$ or 300 kDa)	To extend shelf-life of fruits within a week at room temperature	Papaya fruits	[109]
Chlorophyllin (Chl)	To improve the postharvest life of the fruits	Strawberries	[110]
Pullulan	To enhance the shelf-life of papaya fruits after the coating of pullulan in chitosan film	Papaya	[111]
Resveratrol powder and alginate coating	To decrease the growth of microbiological	Sea bass (*Dicentrarchus labrax*) fillets	[90]

Chitosan coated with PLA, polyethylene (PE) enhanced antimicrobial properties and thus to be used either in food

7.2 *Plasma Technology*

Cold plasma technology is one of the most novel technologies which find widespread potential as surface modification technique in the field of food packaging; this technique has recently emerged as a powerful tool for surface decontamination of both foodstuffs and food packaging materials [114, 115]. Chitosan has played versatile roles in almost all aspects of industrial and biomedical fields. The latest advancement in 3D printing and plasma technology for food and packaging will quickly be grasped by chitosan.

8 Concluding Remarks

Nowadays many of the biopolymers, chitosan has been deeply investigated for its suitability in applications for food packaging due to its characteristics increase of shelf-life of all sorts of fresh fruits, vegetables, and meat products. The addition of not only nanomaterials like NPs of silver, copper, copper oxides, zinc oxides, titanium dioxide, etc., phytochemicals (natural extracts) and modified composite substrates like numerous biopolymers, including gelatin, alginate, carrageenan, etc., and synthetic polymers also into chitosan matrices as active carrier compounds helps to improve the structure as well as property of antimicrobial, mechanical, and other barrier properties of the films/coatings, resulting in enhanced shelf-life and food safety. The use of more numbers of modified chitosan films/coating in food packaging will draw the attention of clean environment by reducing the use of conventional plastics and chemicals to support organic food and an organic climate. Here molecular architecture of chitosan and various properties relevant to food packaging relationship have been described taking three modified chitosan as prepared in our research laboratory. The advancement of food packaging research and global digitalization focused on 3D printing process and plasma techniques where chitosan may put its significant contribution in near future. In the potential expansion of further nutritious products with respect to food hygiene, the minimum presence of human interaction during food production would be less likely to contaminate the food, with high yields anticipated. The present chapter is expected to think over the large-scale manufacturers and marketing players of packaging industries to maximize the utility of chitosan based food packaging systems.

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Chitosan-Based Biosensor Fabrication and Biosensing Applications

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Contents

Abstract Recent years have seen tremendous interest among researchers in the field of biosensors for the application of medical and agricultural products, processed food, and environmental monitoring. To prepare a stable and reliable biosensor, immobilization of biological responses elements (BRE) plays a critical role. Chitosan, a natural polysaccharide with non-toxic and gellable properties, and the presence of functional groups would act as a suitable substrate material. The presence of functional groups would provide cross-linking moieties increasing the mechanical stability, immobilization of BRE and nanomaterials. Additionally, a chitosan composite/nanocomposite-based biosensor would provide enhanced conductivity and sensitivity of detecting various biological analytes such as glucose, H_2O_2, antigens, DNA, and biomolecules. This review provides a comprehensive understanding of various strategies of utilizing chitosan as a substrate for various biosensing applications.

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Keywords Biosensor · Chitosan · DNA · Electrodeposition · Enzyme · Immunosensor

1 Introduction

Over the past few decades, research in the field of biosensors has resulted in expanding the application into various areas including the medical field [1–3], food quality control [4–6], and environmental monitoring [7–9], etc. As a result of the biosensor's simplified process, very less consumption of chemical reagents and energy, a small quantity of the sample requirement, cost-efficient process with high accuracy, and small form factor are possible [10]. Typically, a biosensor is described as an analytical device to detect the soluble analyte by binding to the biological or biologically derived target, also described as biological recognition element (BRE) which would be either closely associated or immobilized in the transducer device to detect the target analyte concentration [11]. The transducer in the biosensor works to convert the interaction between the target analyte and BRE, to easily quantify the concentration of the analyte through the physicochemical way: such as optical, piezoelectric, electrochemical, electroluminescent, pyroelectric, electronic, acoustic, and so forth [10, 12]. The transduced signal is further depicted in the user-friendly interface for easy understanding through the reader device. The reader device is custom built to user requirements and is generally regarded as one of the costly parts of the device present in the biosensor.

Clark invented the first biosensor for oxygen concentration measurement in the year 1956 [13], further his group expanded the applicability of biosensors for various analytes and had discussed the process of making an "intelligent electrochemical biosensor" by including "enzyme transducers" [14]. A system was created that was sensitive to glucose level by the fact that the glucose oxidase (GOx) enzyme utilizes oxygen for converting glucose from the sample to glucono-δ-lactone [15]. The oxygen consumption was in proportion to glucose content. Since then, the biosensor field has seen rapid development in the measurement of various biological analytes such as pH, ion concentration, dopamine, and so forth. The size of the biosensor also had seen a vast size reduction over the period. In the present scenario, the biosensor could also be included in the watch for ECG and atrial fibrillation [16], in socks for gait monitoring [17], in a contact lens for ocular diagnostics [18], so forth (Fig. 1).

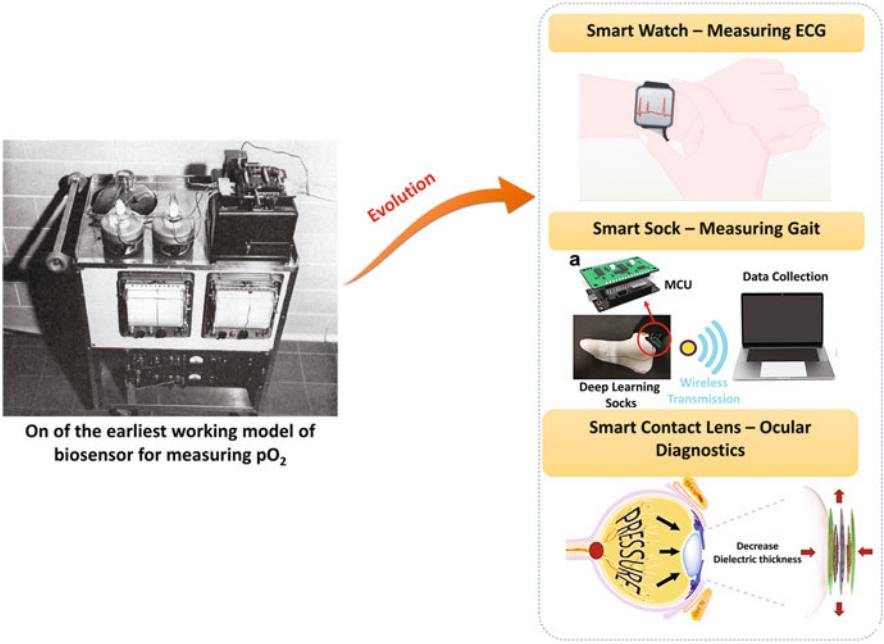

Fig. 1 Historical evolution of biosensors. Reproduced with permission from [14], Copyrights (1962). John Wiley and Sons, Reproduced with permission from [16], Copyrights (2018). Springer Nature, Reproduced under terms of the CC-BY license [17], Reproduced under terms of the CC-BY license [18]

2 Principles of Surface-Based Biosensor

In the biosensor, when the target analyte binds to the BRE, which is pinned down to the surface of the biosensor, it is regarded as a surface-based biosensor. A typical surface-based biosensor is schematically described in Fig. 2, which has the following elements [10, 19]:

1. a bio-component that reacts or attaches with the sample;
2. an interface material that is immobilized with a biological recognition element, wherein
3. the reaction takes place that results in an electrical signal in the electrical interface;
4. signal recovery and amplification circuit;
5. digital signal processing unit for analyzing the obtained signal, and
6. display unit for human-interface.

Single-stranded deoxyribonucleic acid (DNA) [20] and antibodies [21] are some of the BRE used in the biosensor. The binding of the target analyte to these BREs would result in structural changes and would result in the disturbance of its milieu. These disturbances can be transformed into an electrical signal using transducers. At

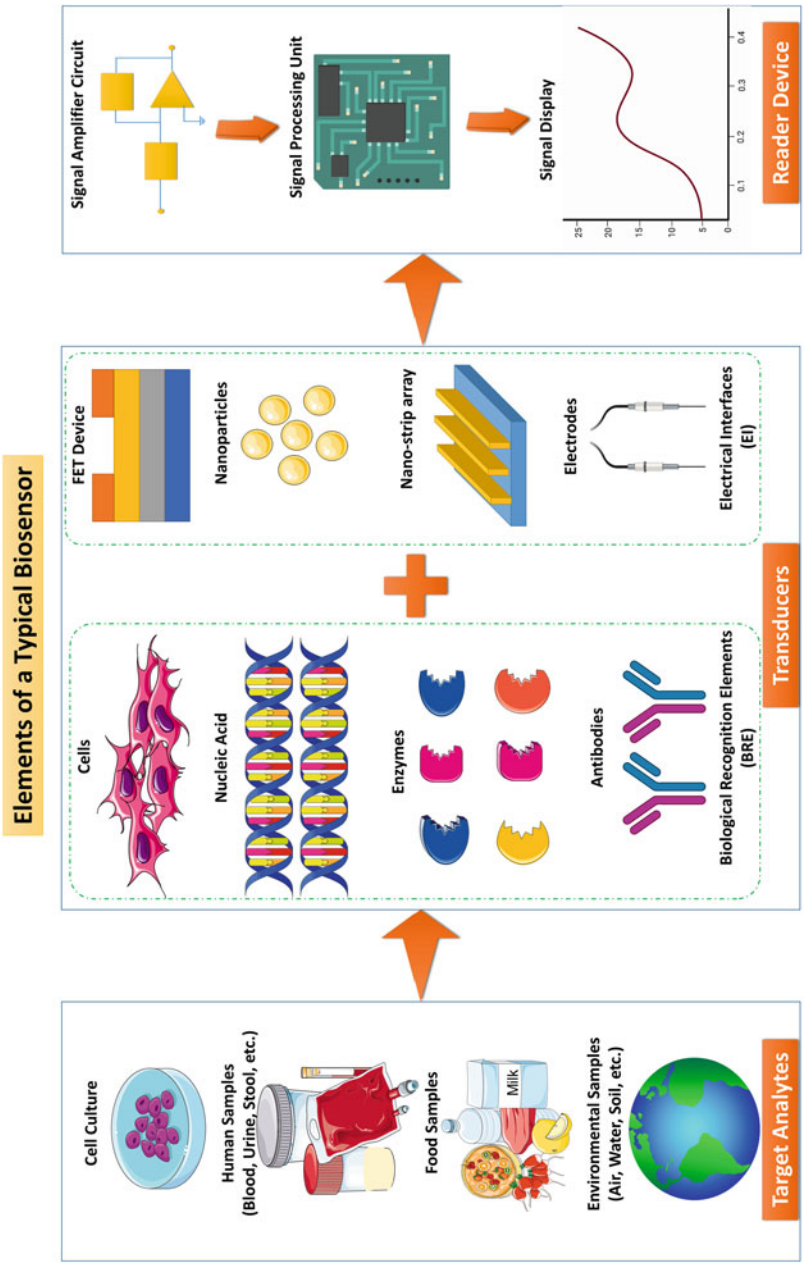

Fig. 2 Schematic representation of various components typically present in the biosensor system. Adopted from [24]

the same time, an enzyme-based biosensor utilizes catalytic activity between the target analyte and immobilized enzyme [22]. Once the target analyte is applied to the sensor, an oxidation-reduction reaction would be achieved, which would be converted into an electrical signal and analyzed using techniques such as amperometry, voltammetry, potentiometry, impedance spectroscopy, and so forth [10].

With many studies, it is being understood that biosensor's performance is associated with the innate properties of BRE in the detection of target analyte [23]. At the same time, the base/substrate material to which the BRE is immobilized also plays an important role as it could affect the binding properties of an analyte with the BRE, binding kinetics, and structure of immobilized BRE. The commonly utilized supportive matrix for the biosensor includes quartz/silicon, silicon dioxide (SiO_2), precious metal/metal oxides, carbon, biopolymers, and thin films. The general characteristics of the substrate need to be toxic-free, physio-chemically stable material, electrically conductive and high charge-carrying material [23].

The next important aspect to be seen in the biosensor is the type of immobilization of BRE to the substrate. It is of two types: i) physical adsorption and ii) chemically cross-linked or covalently bound [25]. The physical adsorption is a low-cost method of adhering BRE to the substrate; however, because of the non-covalent linkage, this method suffers from low probe coverage and non-uniform covering of BRE. In the case of covalently bound BRE, it provides reliable results; however, it needs additional steps to prepare, which adds to the cost [25]. For example, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-N-hydroxy succinimide (NHS) mediated coupling of protein molecule was attached to electrode with a linker molecule of 3-Aminopropyl ethoxy silane [26].

The next step in the biosensor that needs to be addressed is blocking. This step is essential to avoid the non-specific binding of analytes and to improve specificity and selectivity. Blocking agents such as tween 20, bovine serum albumin (BSA), casein, poly ethylene glycol (PEG), and so forth are commonly used [27]. One of the important aspects in selecting the blocking agent is that it should be compatible with the BRE and it should replace the BRE.

To improve the limit of detection (LOD) for the biosensor, the density of BRE immobilized on the surface would play an important role. Increasing the BRE density would also improve the specificity of the sensor [28]. This is a general consideration for improving the LOD, additional requirements are needed to be fulfilled, which ultimately depends on the type of biosensor used. For example, in the case of immunosensors (biosensors that utilize antibodies as BRE), it requires a specific orientation of the antibody to bind with the target analyte. This ensures the active site in the antibody is available for the capture of the antigen [29]. In the case of DNA-based biosensors, optimization of DNA concentration in the substrate is required, as single-stranded DNA is negatively charged and a higher concentration of DNA would result in repulsion and reduce the sensitivity of the biosensor [30]. In addition to the above-mentioned requirements, BRE immobilized on the substrate should be durable and preserve the active site for binding, as they are of importance in out-door usage, where the environmental factors could affect the properties of the biosensors [31]. In this regard, a chitosan-based substrate for BRE binding has

shown long-term stability [32, 33]. In the next section, the applicability of chitosan, based on its chemical and functional properties relevant to biosensor application would be discussed.

3 Role of Chitosan in Biosensor

Chitosan is an aminoglucopyran with a linear chain predominantly containing glucosamine and a lower presence of N-acetyl glucosamine in a randomly arranged manner, with β(1 → 4) linkage [33]. It is a very important derivative of naturally derived polymer, chitin. Chitin is mostly found in the shells of crustaceans such as crab, shrimp, and so forth, the cell wall of fungi, beaks of cephalopods, and insect's wings [34]. Naturally derived polymers such as chitin are highly cost-effective due to the fact that it is derived from marine food industry's "waste." Further processing of chitin, i.e., deacetylation would result in chitosan. Chitin is insoluble in most of the organic solvents. It had been found that chitin dissolves in saturated $CaCl_2.2H_2O$/Methanol, 5%LiCl-Dimethylacetamide and urea/NaOH solvent mixture [34–36]. However, chitosan is readily soluble in acids (Fig. 3). Another unique aspect of chitosan is cationic, as most of the naturally derived polymers such as hyaluronic acid, chondroitin sulfate, dextran, fucoidan, carrageenan, and so forth are anionic [37]. This unique characteristic allows chitosan to readily form a composite with other synthetic [38] or naturally derived anionic polymer [39]. Chitosan is a versatile polymer having application in tissue engineering [39–42], drug delivery [43, 44], and hemostatic applications [45–47], owing to their physio-chemical properties such as bio-compatible, bio-degradable, tuneable into various forms

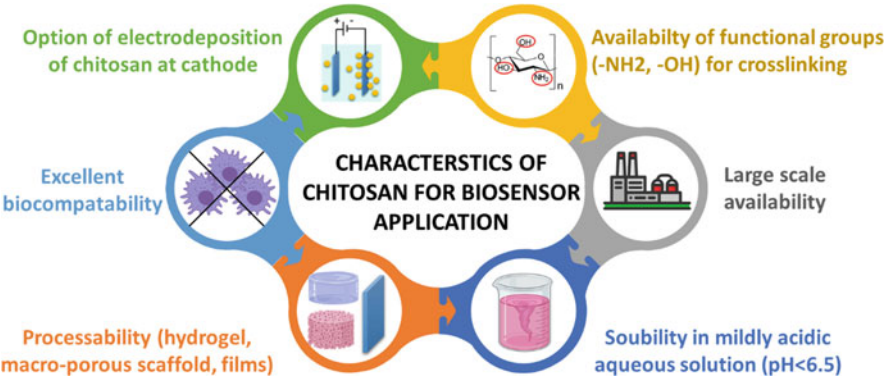

Fig. 3 Advantages of chitosan for biosensor application. The solubility of chitosan, high processability into a hydrogel, macroporous scaffolds and thin films, availability of functional groups for immobilization of BRE, electrodeposition of chitosan to the cathode, higher biocompatibility, and large-scale availability at low cost provide a new window of application for chitosan

(hydrogel, electrospun membrane, thin-film, sponges, scaffold, and so forth), and low cost.

Chitosan could be manufactured at a wide range with a degree of deacetylation and molecular weight. Chitin in the natural form has a molecular weight in MDa. However, in the large-scale extraction process, random cleavage happens in the polymer backbone. Further, the deacetylation process in a strong alkali bath would also reduce the molecular weight [48]. Currently, chitosan is marketed as powder, flakes, and small beads with high (>~500 kDa), medium (~50–500 kDa), and low (<~50 kDa) molecular weight. The solubility of chitosan could be adjusted (usually <pH 6.5) depending on the biosensor application requirement. Additionally, chitosan possesses free amine and hydroxyl groups which could be utilized in covalent conjugation with the BRE. The presence of a free $-NH_2$ group in the polymeric backbone provides an opportunity for side group attachment and covalent binding to the BRE under mild reaction conditions [49]. Chitosan could also be electrodeposited to the cathode through electrolysis of water, causing local pH [19] (Fig. 3).

The importance of substrate selection has been mentioned earlier, to improve the BRE concentration and specificity. Chitosan provides a multitude of advantages, as chitosan could be prepared into hydrogel material, which would provide a 3-dimensional architecture for the BRE binding and provides stability of the immobilized BRE in it [50]. Further, if chitosan had to be utilized in a dry environment, then it could be processed into a 3-D porous scaffold with the pore size in the order of microns [51]. The porous structure would increase the specific surface area, thereby providing more room for the BRE binding and increasing the concentration of it. This is more advantageous over the use of a planar biosensor, as increasing the BRE concentration over the unit volume would amplify the signal output. Additionally, chitosan is non-toxic, renewable polymer derived from the marine industry "waste" and it is bio-degradable [34] (Fig. 3).

4 Preparing Chitosan-Based Biosensors

4.1 Chitosan Coating on the Electrode

Chitosan solubility could be modified with a simple pH change and this provides a simpler reaction method for forming a chitosan layer over the electrode with control over its thickness. Chitosan dissolves in acidified aqueous media and regenerative into an insoluble form of chitosan above pH 7–9 [52]. This process could be utilized to coat the electrode with chitosan. The thickness of the coating could be adjusted with the concentration of the chitosan solution. But it may not be able to achieve a very thin coating. To achieve a thin and dense coating of chitosan, a spin coating technique could be utilized [53]. Alternatively, a layer-by-layer technique could help achieve thin coating, with the benefit of introducing another substrate material that could add an advantage to the biosensor [54]. One of the major shortcomings of

casting, layer-by-layer and spin coating technique is that these methods are only suitable for smooth and defined surfaced electrode, as when these methods are used to coat chitosan on to porous or complex surfaced electrode, they could result in local accumulation and uneven coating on the electrode [55].

4.1.1 Electrodeposition of Chitosan

Chitosan coating/deposition could be carried out by electrodeposition, which was first reported by Wu et al. [56]. Even though chitosan is not a conductive material, the term electrodeposition may not be the correct terminology to call, as there is no redox conversion of coat-forming material itself. It is regarded as "electrodeposition," as chitosan is deposited over the cathode through electrolysis of water. The instrumental setup and the mechanism are depicted in Fig. 4**.** During the process, consumption of protons at the cathodic end and generation of hydroxide ions at the interface cause local pH change. The local milieu in pH change is sufficient for the

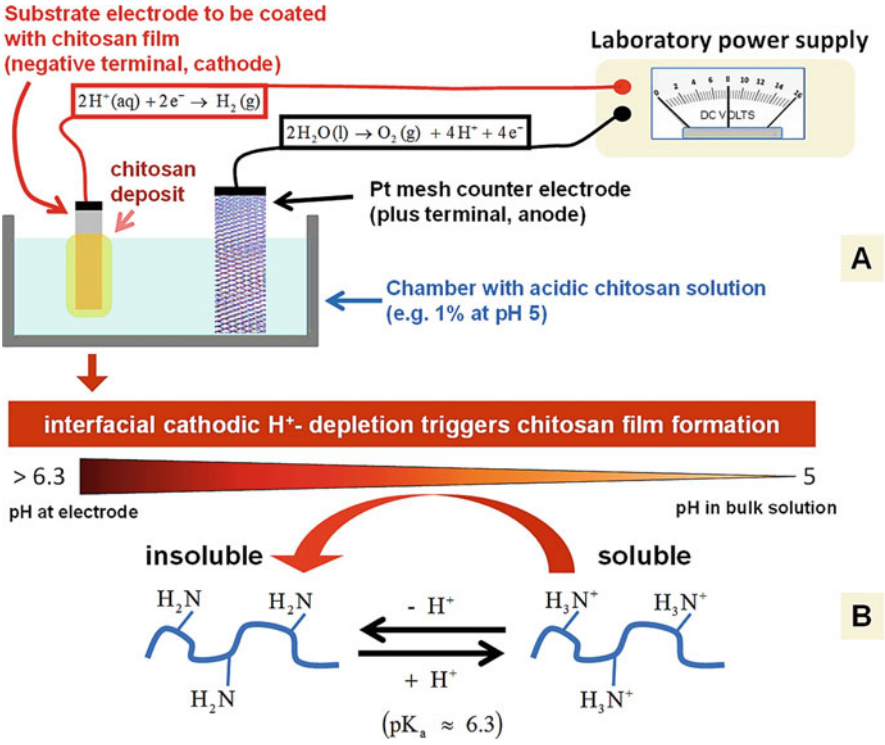

Fig. 4 Schematic representation and mechanism for electrodeposition of chitosan. Instrumental setup of electrodeposition of chitosan thin film on to cathode (**a**) and mechanistic aspect of chitosan deposition (**b**). Reproduced with permission from [19]. Copyright (2013) American Chemical Society

chitosan to undergo sol-gel transition and get deposited at the cathode. Further, to improve the stability of the deposited chitosan, intrinsic functional groups or newly introduced reactive groups could be utilized to cross-link the polymeric strands. Commonly used cross-linkers of chitosan are EDC/NHS, glutaraldehyde, glyoxal, N, N′-methylenebisacrylamide, ethylene glycol diglycidyl, genipin, sodium tripolyphosphate, and so forth [57–60]. Another interesting aspect of chitosan is that the free electron pair of nitrogen could be utilized for internal complex formation and metal binding [61].

Electrodeposition of chitosan is particularly important, as it could be deposited onto the miniature electrodes in biosensor and point of interest areas in the micro and nano arrayed electrodes that are not achievable using classical techniques such as casting and spin coating. Wu et al. [62] displayed spatio-selective deposition of chitosan in the gold band electrode arrays in a stimuli-responsive manner. The results displayed that chitosan could be electrodeposited to a 20 μm band electrode array which is separated by a distance of 20 μm. Buckhout-White et al. [63] showed that electrodeposition could be controlled to a resolution of 0.5–1 μm thick coating. The anodic deposition of chitosan was achieved by electrolysis of chloride to generate chlorine species, which further oxidizes chitosan, yielding chitosan aldehyde [64]. The aldehyde form of chitosan would covalently bind with the amino group of chitosan through Schiff base reaction. The swellable gel gets deposited in the anodic part of the sensor electrode. The chlorine species could also affect the BRE, resulting in BRE's instability. To circumvent the problem, Geng et al. [65] deposited a coordinated chitosan complex on the copper film anode. Cu^{2+} would coordinate with the chitosan molecules and then results in a blue color hydrogel, which gets deposited on to copper anode. The formed hydrogel was stable enough to peel from the electrode. This method also helped in depositing the complex structure of the electrode (Fig. 5). Islam et al. electrodeposited chitosan on the copper anode and cathode [66]. The results showed that coordinated chitosan was deposited on the copper anode and chitosan hydrogel was deposited on the cathode. Further, carbonization of the anodic hydrogel resulted in macroporous carbon/Cu composite exhibiting 15-fold enhanced electrical conductivity compared to carbon obtained from the cathodic hydrogel.

4.2 Immobilization of BRE in the Chitosan Substrate

One of the important aspects of biosensor fabrication is substrate coating on the electrode and BRE immobilization in the substrate. In the previous section, the substrate coating on the electrode was discussed. In this section, the immobilization of BRE will be discussed. BRE could be entrapped in the chitosan hydrogel during the neutralization of the chitosan solution. The porous morphology of the chitosan scaffold allows the BRE to be exposed to the surface of the chitosan substrate. Chitosan sol-gel transition wasn't affected by adding BRE to the chitosan solution [55]. Thus, by the simple addition of BRE to the chitosan solution, BRE-chitosan

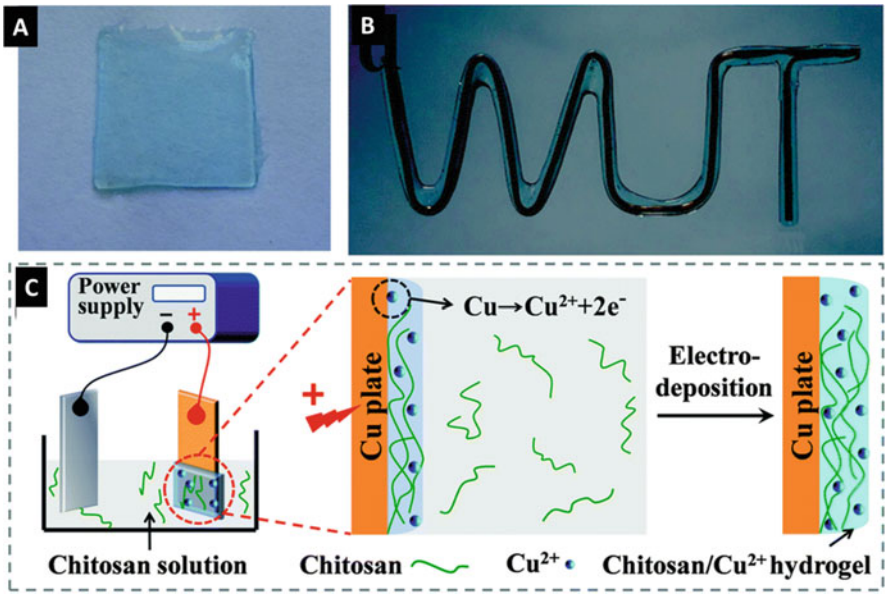

Fig. 5 Coordinate electrodeposition of chitosan. Peeled chitosan/Cu^{2+} hydrogel (**a**), complex patterning of chitosan hydrogel on the copper wire (**b**) and mechanism of Coordinate electrodeposition (**c**). Reproduced from [65]

could be coated by simple casting or by electrodeposition to the desired area of the electrode. It is expected that BRE molecules would be entrapped inside the chitosan substrate even without covalent cross-linking or by electrostatic attraction. A simple entrapment method is sufficient to hold the BRE in the chitosan substrate. One of the important requirements for this method is that BRE needs to be stable in an acidic environment. Even though entrapment is simpler and cost-effective, it suffers from poor stability of BRE, as BRE could be leaked out from the chitosan substrate during testing due to their weak interaction [67, 68].

BRE immobilization to the chitosan substrate can be achieved by covalent cross-linking. Free amino group (-NH_2) present in chitosan provides an opportunity for covalent cross-linking, which is depicted in Fig. 6. For example, glutaraldehyde could be utilized for coupling the amino group of chitosan with the amino group of BRE. Wang et al. utilized glutaraldehyde as an arm linker between chitosan/multiwalled carbon tube and ss-DNA probe [69]. Biotin-avidin is one of the strong interaction complexes, which could be utilized for the immobilization of BRE. Singh et al. fabricated chitosan/Fe_2O_3 with avidin conjugation. The biotinylated ss-DNA probe was immobilized to the substrate through the biotin–avidin interaction [70]. The phosphate group of ss-DNA could be utilized to conjugate with the amino group chitosan through EDC/NHS chemistry. In this scenario, EDC would act as the activator of the phosphate group and form a stable intermediate. NHS

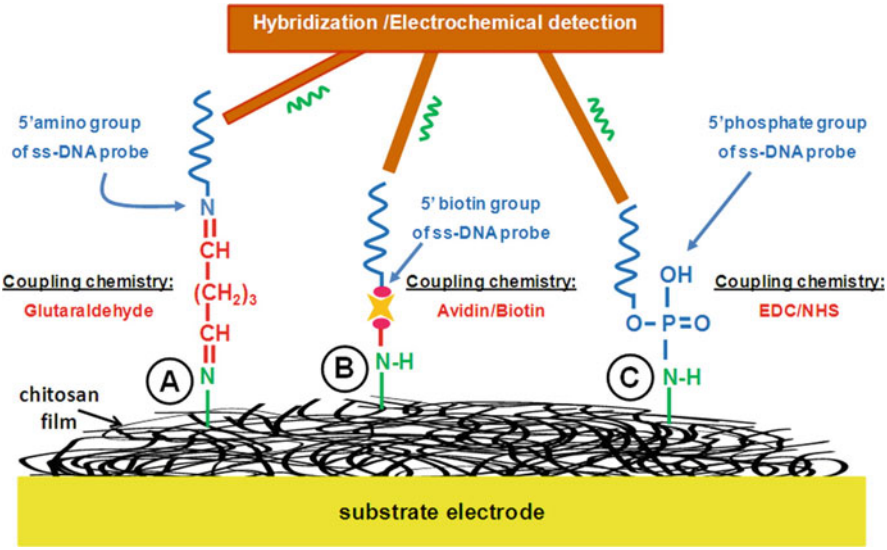

Fig. 6 Immobilization techniques of BRE to the chitosan substrate. Utilization of glutaraldehyde (**a**) for cross-linking amino group of chitosan and amine of BRE (for example: ss-DNA), avidin/biotin (**b**) based immobilization of BRE (for example: ss-DNA) and EDC/NHS (**c**) based coupling of the amino group of chitosan and phosphate group of BRE for example: ss-DNA. Reproduced with permission from [19]. Copyright (2013) American Chemical Society

activated chitosan would react with the DNA, yielding covalent linkage between phosphate and amino group [71].

In addition to the homogeneous chitosan substrate, a chitosan nanocomposite substrate is also utilized. Nanomaterials such as carbon nanotubes, graphene, metal oxides, and so forth had been added to the chitosan matrix. Carbon-based nanomaterials are hydrophobic and uniformly disperses in the chitosan matrix, providing stability to the biosensor. Nanocomposite chitosan substrate has good electrical conductivity [72] and surface flatness [73], compared to homogeneous chitosan matrix. Thus, the composite substrate opens a new window for high-performance biosensors.

5 Recent Survey of Chitosan in Biosensing Applications

5.1 Chitosan-Based DNA Biosensors

Biosensing of target analytes such as ss-DNA, short-chain oligonucleotide, and so forth from biological samples, which were regarded as clinical biomarkers for identifying the disease conditions using conventional techniques such as DNA chips and microarrays is a well-established field [74–76]. Even though these

methods are regarded as the gold standard, in certain situations, better analytical techniques are warranted. Recently, DNA biosensors have attracted interest among researchers, owing to their simple and rapid detection, yet with high sensitivity and selectivity of the target analyte and doesn't require trained personnel and extensive procedure for performing experiments [77–79]. The required parameters for biosensing of DNA samples in a clinical setting were identification and synthesis of DNA probe for detecting oligonucleotides, accurate binding and immobilization of oligonucleotide to the DNA probe, and finally detection, amplification, and processing of signal generated due to the DNA hybridization. The most commonly used electrode material in electrochemical biosensing is gold, although carbon and metal oxide are also used. DNA probe immobilization utilizes a strong binding force between Au and sulfur atoms, which produces self-assembly of DNA molecules onto the Au surface with the introduction of sulfhydryl groups (-SH) containing organic molecules [80]. The sulfhydryl-based immobilization of DNA is limited to Au surface and other chemical cross-linking techniques are required to immobilize DNA probe to the surface of the biosensor such as glass, carbon, metal oxide, or platinum electrode. Many studies have utilized chitosan in a neat form or chitosan nanocomposite for immobilization or DNA hybridization detection. Chitosan-based DNA fixation works either by chemical or physical immobilization, wherein the physical immobilization utilizes cationic group ($-NH_2$) of chitosan and anionic group ($-PO_4$) of DNA backbone. For the chemical immobilization, cross-linkers such as glutaraldehyde ($-NH_2$ group of chitosan is cross-linked with $-NH_2$ group of DNA) [69], avidin-biotin (avidin conjugated chitosan is complexed with biotinylated DNA) [70], EDC/NHS ($-NH_2$ group of chitosan is cross-linked with $-PO_4$ group of DNA) [71], and so forth are used.

The nanocomposite chitosan provides many advantages in the biosensor compared to bare chitosan, such as uniformity and evenness of chitosan, enhanced electrical conductivity, and better hybridization [81, 82]. Recently, Tiwari et al., fabricated a biosensor, wherein graphene oxide/nickel ferrite/chitosan nanocomposite was electrodeposited on to indium tin oxide (ITO) electrode and the probe DNA was immobilized on to it [83]. Differential pulse voltammetry (DPV) was utilized for studying the DNA hybridization of *Escherichia coli* with methylene blue (MB) used as a redox indicator (Fig. 7). A linear response was obtained for a range of 1 μM to 0.1 fM with LOD of 0.1 fM. Similarly, Heydarzadeh et al. fabricated a DNA biosensor with a glassy carbon electrode (GCE) modified with chitosan nanocomposite containing multiwalled carbon nanotube and Au nanoparticles [84]. Here, Au-thiol group interaction was used for immobilizing probe DNA (thiolated DNA probe was used) onto the substrate. The DPV method was utilized with MB as a redox indicator to detect the DNA at a concentration range of 90 fM to 1 μM with LOD of 7.2 fM. A label-free method for detecting *Escherichia coli* was developed by Xu et al., wherein chitosan/graphene oxide (GO/CS) nanocomposite (physical cross-inking) was immobilized onto GCE for detecting [85]. Under the DNA hybridization, the prepared biosensor showed a linear concentration of DNA at a range of 10 fM to 10 nM using electrochemical impedance spectroscopy (EIS). The LOD for the prepared biosensor was 3.584×10^{-15} M.

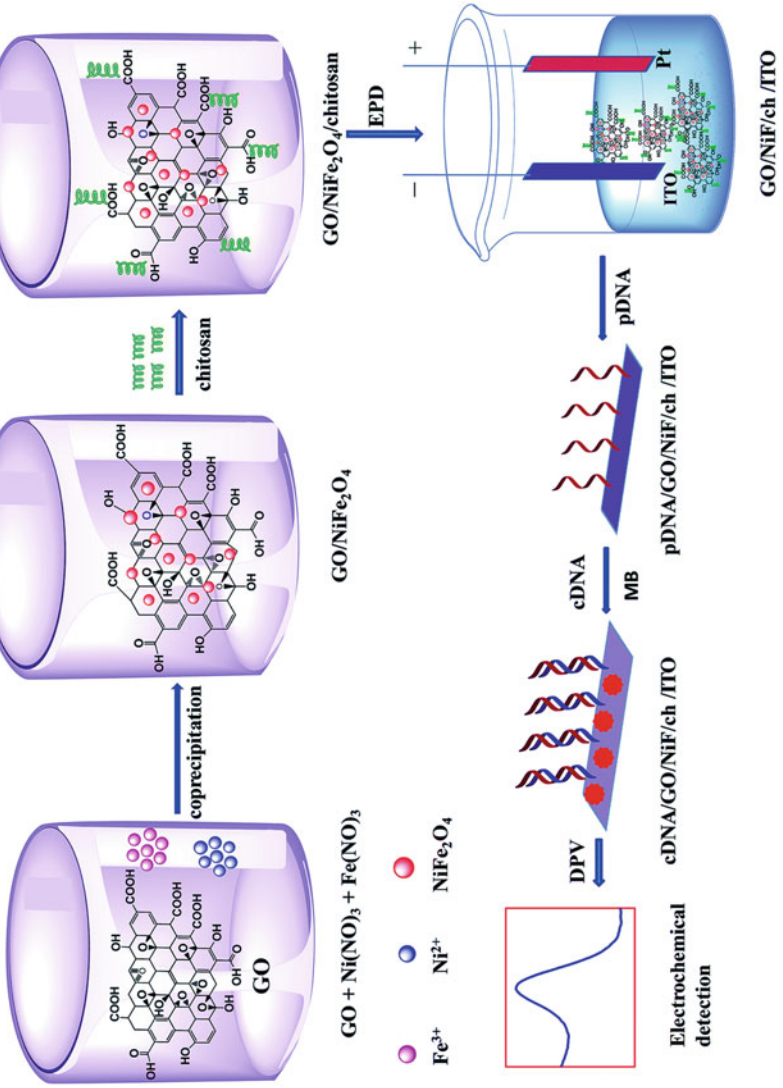

Fig. 7 Schematic illustration of preparing nickel ferrite/graphene oxide (GO)/chitosan substrate, electrodepositing on to ITO electrode for biosensing of *E. coli* using methylene blue (MB) as a redox indicator. Reproduced from [83] with permission The Royal Society of Chemistry

Further studies showed that even 1-mismatched DNA was able to distinguish complementary DNA probe. However, Xu et al. [85] reported label-free had a lower detection range compared to the Tiwari et al. [83] labeled (MB as the redox indicator) biosensor. To overcome the above-mentioned problem, Zuo et al. reported label-free biosensing of bacterial DNA with nanocomposite chitosan as a substrate, which contains iron oxide nanoparticles (Fe_3O_4)/multiwalled carbon nanotubes for detection of *Bacillus cereus* [86]. The EIS was utilized to study the DNA hybridization with a linear detection obtained for a concentration of 0.2 pM to 2 μM with a LOD of 2 pM of DNA. Similarly, Qian et al. utilized a label-free biosensing method for detecting foodborne pathogen *Clostridium perfringens* by immobilizing DNA probe on to CeO_2/chitosan-coated electrode surface through metal coordination [87]. Two morphologies of CeO_2 were prepared by adjusting the pH during the synthesis route. CeO_2 nanorods were found to have a higher surface charge compared to nanoparticles, thereby having higher DNA adsorption performance. The EIS was utilized to detect the complementary DNA with a linear detection range of 10 fM to 10 μM and LOD of 7 fM.

5.2 Chitosan-Based Immunosensors

Immunosensors take advantages of the specific recognition of antigens over their complementary antibodies. With the strong affinity-based recognition, immunosensors at the optimized parameters can detect the target antigens at attomolar concentration [88, 89] and studies have reported even sub-attomolar concentration [90]. This has led to many applications of immunosensors and added interest in the research community. One of the important requirements for the immunosensors is a higher concentration of antibody (immune-sensing component) in the electrode for good sensitivity of the target antigen [91, 92]. Accordingly, simple and effective methods such as the incorporation of chitosan in various form have increased the surface area, thereby providing more room for the antibody for detecting antigens.

Recently, Wang et al. developed an electrochemical immunosensor for detecting food contaminant T2-toxin by modifying the GCE electrode using Au nanoparticles/carboxyl acid group terminated single-walled carbon nanotubes in chitosan matrix with EDC/NHS cross-linking. Anti-T2-toxin was immobilized to the substrate and a secondary antibody conjugated with alkaline phosphatase (ALP) was used for indirect detection of antigen. Finally, hydrolysis of α-naphthyl phosphate by ALP enzyme produced the electrochemical signal. The LOD of the developed device was 0.13 ng mL^{-1}. Label-free immunosensor was developed by Sarkar et al. with a biosensing system containing ITO electrode modified with chitosan-carbon dots by drop-cast method for detection of vitamin-D_2 by conjugating anti-vitamin D_2 antibodies and bovine serum albumin (BSA) on to the substrate using EDC-NHS method [94]. With the help of DPV, the dynamic linear range of detection was 10–50 ng mL^{-1} and LOD was 1.35 ng mL^{-1}. Soares et al. reported low-cost

immunosensors made of a microfluidic device with an interdigitated electrode coated with chitosan and chondroitin sulfate by layer-by-layer (LbL) technique, which contained physically adsorbed *S. aureus* antibodies [95]. The LOD for the fabricated device containing 10 LbL was 2.83 CFU mL^{-1}. High sensitivity and selectivity of the device was attributed to the projection of EIS data to an interactive document mapping, resulting in identification of *E. coli* and *Salmonella* contamination in milk. Devarakonda et al. reported a paper-based, label-free biosensor for detection of the influenza virus. The immunosensors were fabricated by modifying the paper with spraying of hydrophobic silica nanoparticles onto it and stencil-printed electrodes [93]. The stencil-printed electrodes were modified with single-walled carbon nanotubes and chitosan with immobilization of antibodies with glutaraldehyde (Fig. 8). The fabricated device showed LOD of 113 PFU mL^{-1} by the DPV method. The LOD is sufficient for the on-site diagnosis of the disease. To improve the antibody loading and sensitivity, Choosang et al. fabricated a highly sensitive immunosensor with chitosan nanocomposite made of graphene, ionic liquid, and ferrocene with glutaraldehyde as the cross-linker [96]. The drop-casted material was freeze-dried to obtain 3-D porous chitosan nanocomposite. Further, the prostate-specific antigen (PSA) antibody and Au nanoparticles were immobilized. The developed porous substrate showed enhanced electron transfer and electrical conductivity. It showed a very high sensitivity for PSA with a detection range of 1.0×10^{-7} to 1.0×10^{-1} ng mL^{-1} with LOD of 4.8×10^{-8} ng mL^{-1}.

As most cancers express more than one cancer marker, detecting one marker is discouraged. To address this, simultaneous detection of cancer markers is warranted. One such example is Cotchim et al. developed multiplexed detection of carcinoembryonic antigen (CEA), cancer antigens 153 (CA153), and cancer antigen 125 (CA125) [97]. The multiplexed working electrode (E) of E1, E2, and E3 was prepared by depositing graphene on top of ITO. Chitosan-MB complex is drop-cast and allowed to dry to obtain cryogel. Further, anti-CEA, anti-CA153, and anti-CA125 were immobilized onto the surface of E1, E2, and E3, respectively. The LOD for all three antigens was 0.04 pg mL^{-1}.

5.3 Chitosan-Based Enzyme Biosensors

Chitosan acts as an intermediate material between enzyme and sensor, as it provides immobilization surface for the enzyme and accessibility to an active region of the enzyme for substrate and co-factor binding, due to its porous structure. Compared to immunosensors, relaxed enzyme orientation is required and enzymes are relatively larger in size, making chitosan a suitable option as a substrate or dispersion material for enzyme-based biosensors. The addition of nano-component such as nanoparticles, carbon nanotubes, and graphene nanosheets has provided enhanced electrical conductivity, electron charge transfers, and surface flatness. From the first preparation of enzyme-based biosensors, several works have been carried out in this area.

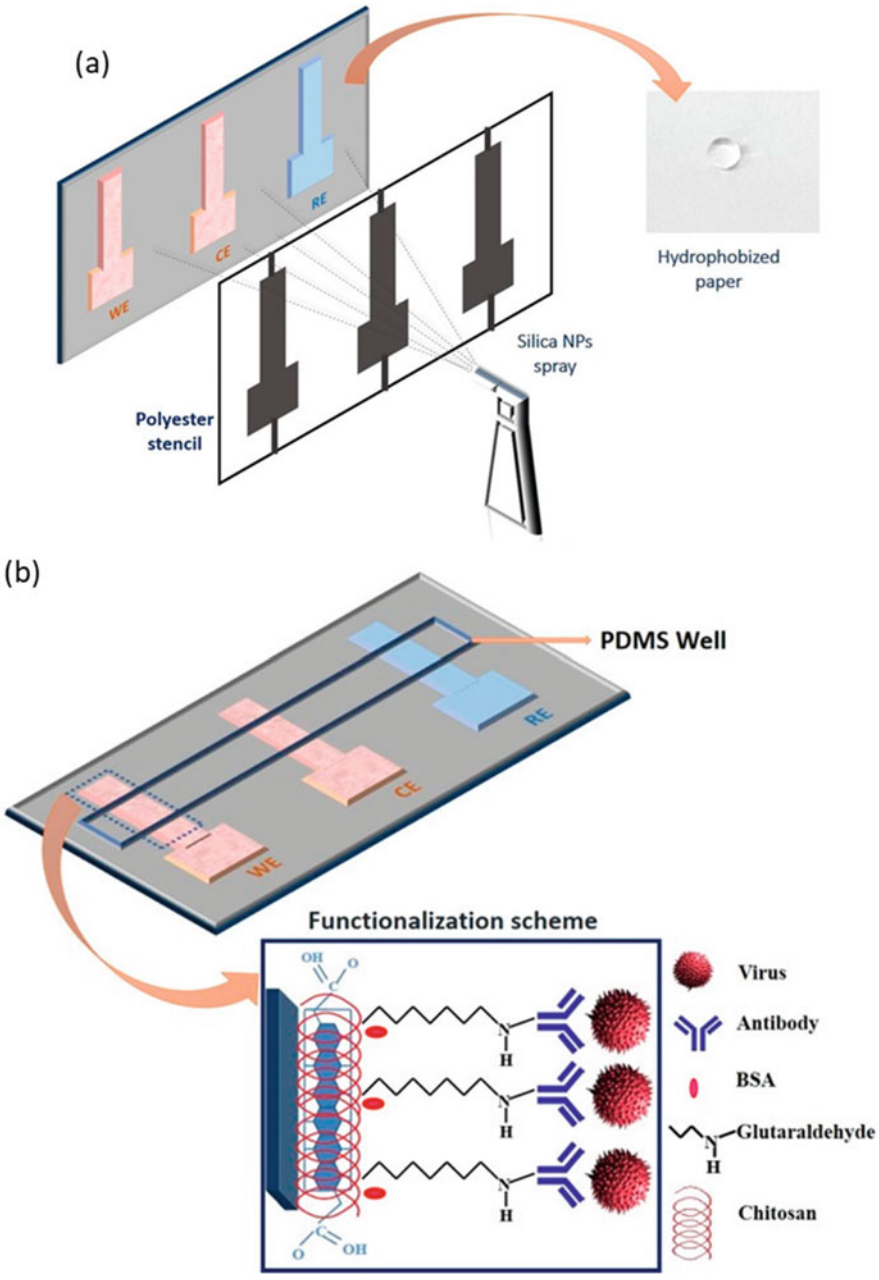

Fig. 8 Schematic representation of spraying hydrophobic silica nanoparticles onto the paper electrode (**a**) and functionalization scheme of the modified electrode with single-walled carbon nanotubes and chitosan with immobilization of antibodies with glutaraldehyde. Reproduced from [93]

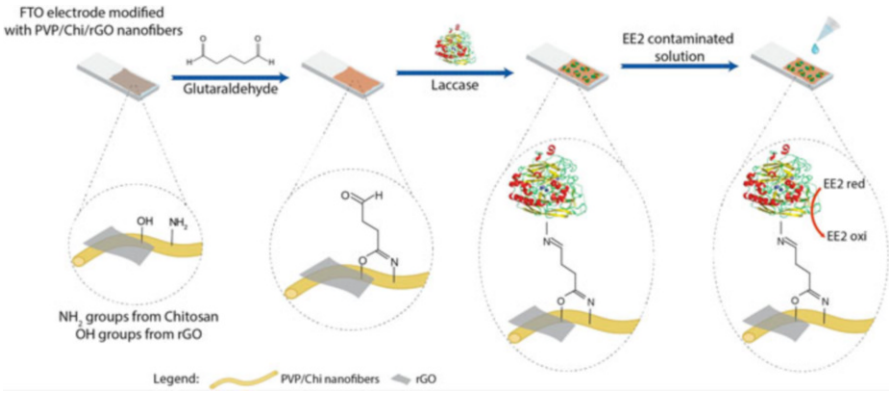

Fig. 9 Schematic representation of a modified FTO electrode with chitosan/PVP/graphene oxide nanofibers, cross-linked with glutaraldehyde. Laccase enzyme was immobilized onto the substrate for the detection of EE. Reproduced with permission from [103]. Copyrights (2018) Elsevier

Representative examples of enzyme-based chitosan biosensors are mentioned below. Lipinska et al. developed a laser processed Au-Ti electrode with chitosan with immobilized GOx for detecting glucose [98]. The sensitivity of the developed device was 23.5 μA mM^{-1} cm^{-2} with the LOD of 1.75 μM. Juska et al. developed Au-foam to increase the specific surface area and they acted as a 3-D porous matrix for deposition of multiwalled carbon nanotube and chitosan [99]. To the matrix, the dual-enzyme component of GOx and horse-radish peroxidase (HRP) was immobilized. The sensitivity of the developed system for glucose is 261.8 μA mM^{-1} cm^{-2} with LOD of 0.025 mM. Kim et al. developed biosensing of glucose molecules by preparing chitosan-poly-γ glutamic acid hydrogel containing GOx and magnetic iron oxide nanoparticles (MNPs) [100]. The device relied on the generation of H_2O_2 from the oxidation of glucose by GOx. The generated H_2O_2 would activate peroxidase-like MNPs for the conversion of chromogen to a green-colored product. Devaraj et al fabricated enzyme biosensor with GCE containing graphene oxidechitosan substrate with Au nano chains [101]. The enzyme HRP was immobilized to the substrate by physical adsorption. The developed device was used to measure hydrogen peroxide.

To further exploit the versatility of chitosan, researchers developed various architecture of chitosan, thereby increasing the specific surface area and porosity. El-Moghazy et al. developed electrospun nanofibers made of chitosan/polyvinyl alcohol with acetylcholinesterase (AChE) for detecting pirimiphos-methyl [102]. The studies showed that the electrospun system yielded a two-fold increase in electrical current compared to the cast membrane. The prepared device was used to pirimiphos-methyl with LOD of 0.2 nM. Similarly, Pavinatto et al. developed nanoarchitecture by electrospinning chitosan/poly (vinyl pyrrolidone) (PVP)/reduced graphene oxide with functionalization of laccase enzyme [103]. The nanocomposite fiber is deposited on the fluorine-doped tin oxide electrode (FTO)

(Fig. 9). The developed device was used to detect 17α-ethinylestradiol (EE) (endocrine disruptor), an endocrine hormonal analogue of 17α-estradiol. Amperometric detection of EE was studied with the LOD of 0.15 pmol L^{-1}.

6 Conclusion and Future Perspective

Chitosan is a derivative of one of the most abundant polymers, chitin. Chitosan has numerous advantages of being non-toxic, low-cost, bio-degradable, and tunable physical properties. Numerous advantages of chitosan in the biosensing field include functional groups (-NH_2 and –OH) for cross-linking and hydrogel formation with the simple adjustment of pH. In this review, we emphasized the role of chitosan in biosensing application by DNA, enzyme, and antibody-based biosensors. The clinical samples contain various substances, which could cause interferences and result in false-positive. Hence, future works should include biological samples with large testing scenarios and samples. Further, the stability of the biosensors needs to be noted, as they would be used in outside laboratory conditions. A low-cost paper-based device, which is use and throw type could be an added advantage for large population screening of diseases. The sensitivity and specificity of the developed device are very important for developing into commercial-scale products. The future outlook of the chitosan-based biosensors should be focused on developing products that can be wearable gadgets, point-of-care diagnostic kits, and also used for environmental real-time monitoring.

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Physical and Chemical Modification of Chitin/Chitosan for Functional Wound Dressings

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Contents

Abstract This chapter provides insight into the functionalization of chitin and chitosan for general and specific-purpose wound dressings, such as hemorrhage,

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infected, burn, and diabetic ulcer wounds. The understanding of different kinds of wounds, wound healing process, and factors affecting wound healing is essential for the design of well-functioning biomaterials as well as the fabrication of wound dressings. Functionalization of chitin/chitosan, including physical and chemical modification to form functional wound dressings for specific purposes, is described in this chapter.

Keywords Chitin · Chitosan · Functionalization · Modification · Regeneration · Wound dressing · Wound healing

1 Introduction

Skin is the largest organ in our body and serves its primary function as a protective barrier against external impacts. Wounds can present a cause of serious illness and mortality in everyday pathology [1, 2], and so can require complicated clinical intervention. Wounds can be categorized into acute and chronic wounds according to the duration of their healing [3, 4]. The healing of acute wounds proceeds via the normal healing pathway with both anatomical and functional restoration. Acute wounds can be caused by a traumatic loss of tissue, surgical procedure, abrasions, or even laceration (deep cuts on the skin) associated with infection [3–5]. Chronic wounds are associated with diseases, such as diabetic and pressure ulcers [3–5], and can be defined as hard to heal wounds. The current potential wound healing approaches are based on autograft, allograft, cultured epithelial autograft, and wound dressing [6]. Wound dressings function to preserve hydration within the wound in order to optimize regeneration, protect against infection, and avoid disruption of the wound base.

An ideal wound dressing should absorb exudates, maintain a moist environment at the wound interface, act as a barrier to microorganisms, and be breathable or allow gaseous exchange. It also should be non-allergenic, non-toxic, and non-adherent so as to be easily removable without trauma. Many polymeric materials have been investigated for the purpose of wound dressings, including synthetic polymers, such as polyurethane [7, 8], polycaprolactone [9, 10], poly(lactic acid) [11, 12], polyethylene glycol (PEG) [13, 14], silicone rubber [15, 16], etc., as well as natural or bio-based polymers. Increasing attention has been paid to biopolymer-based wound dressing materials because of their inherent properties, such as biocompatibility, biodegradability, hemostatic activity, moist maintaining ability, and the ability to support tissue regeneration and differentiation, leading to an accelerated wound healing [17]. Most of the currently available biodegradable polymer-based wound dressings are made from chitin/chitosan [18, 19], hyaluronic acid [20, 21], collagen [22, 23], alginate [24, 25], and so on.

Interestingly, chitin and chitosan have a high potential for wound healing applications because of their prominent properties, such as exudates absorbability,

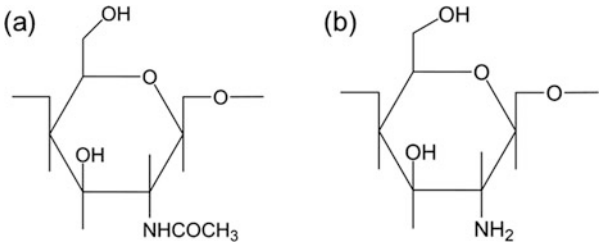

Fig. 1 Monomeric structure of (**a**) chitin and (**b**) chitosan

stimulating hemostasis, and accelerating tissue regeneration [26]. Chitin, [β-(1-4)-2-acetamido-2-deoxy-D-glucose], is the second most abundant natural polysaccharide after cellulose and occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods, mostly in crabs and shrimp shells [27]. Chitin also can be found in the cell walls of fungi and yeast [27]. Chitosan, [β-(1-4)-2-amino-2-deoxy-D-glucose] is the *N*-deacetylated derivative of chitin. Chitin can be extracted by decalcification and deproteinization, while chitosan is obtained by deacetylation of chitin. Therefore, chitin and chitosan are commonly copolymers categorized by degree of acetylation (DA) or degree of deacetylation (DD) representing the amount of *N*-acetyl glucosamine and *N*-glucosamine in their monomeric unit.

The *N*-acetyl glucosamine present in chitin and chitosan is structurally like hyaluronic acid, an extracellular matrix (ECM) component of dermal tissue that is essential for wound repair [28]. Since chitin and chitosan are biodegradable, non-toxic, and biocompatible, they also have received considerable attention in various fields of biomedical and pharmaceutical applications. The monomeric structure of chitin and chitosan is presented in Fig. 1.

Owing to their structures, chitin and chitosan are multifunctional polymers and amenable to chemical modification. Chitin has primary and secondary hydroxyl groups at the C-6 and C-3 positions, respectively, and acetamido groups at the C-2 position, while chitosan has amino groups at C-2 position. Due to the availability of the free amino groups in chitosan, it can be protonated and carries a positive charge. Considering its biodegradability, chitin and chitosan are metabolized by the human enzyme lysozyme, making it biodegradable. The biodegradation activity by lysozyme is mainly controlled by the DA or DD and also by the distribution of *N*-acetylglucosamine residues as well as molecular weight (MW) [29]. Chitin and chitosan are degraded into oligosaccharides of variable length and are non-toxic in nature. These oligosaccharides can be introduced in the metabolic pathways and degraded into *N*-glucosamine and *N*-acetylglucosamine. The amino sugar of *N*-glucosamine can be incorporated into the glycosaminoglycan (GAG) and glycoprotein pathways or otherwise excreted from the system [30]. *N*-Acetyl glucosamine also initiates fibroblast proliferation, supports an ordered collagen deposition, and stimulates an increased level of natural hyaluronic acid synthesis at the wound site, leading to faster wound healing and scar prevention [31]. Besides, chitin and chitosan can be fabricated in various forms, such as hydrogels, membranes, fibers, electrospun mats, sponges, scaffolds, and so on.

In order to design effective chitin and chitosan wound dressings, this chapter gives information on the wound healing process and factors effecting wound healing in Sect. 2, the fabrication methods of chitin and chitosan wound dressings in Sect. 3, commercially available chitin- and chitosan-based wound dressings in Sect. 4, and the physical and chemical modification of chitin and chitosan (Sect. 5) for making functional wound dressings, such as hemostatic, antimicrobial, burn, and diabetic ulcer wound dressings in Sect. 6.

2 Wound Healing Process and Factors Effecting Wound Healing

A wound is defined as a damage or destruction to the normal anatomical structure, biological feature, and function [3]. For skin, the damage can range from a simple tear of the skin surface or deep cuts into the subcutaneous tissue with damage to other surrounding tissues, such as muscles, tendons, vessels, nerves, and bone [5]. Wound healing is a complex and dynamic process starting at the moment of injury. The mechanism underlying this process consists of four continuous, overlapping, and precise programmed phases [5, 32]: (1) hemostasis and coagulation, (2) inflammation, (3) proliferation, and (4) remodeling. The normal wound healing process and biological events are schematically presented in Fig. 2. The individual phases are described below. In addition, those wound healing phases involve multiple cell types, synthesis of the extracellular matrix (ECM) proteins, and the action of mediators including growth factors and cytokines. Therefore, the bio-physiological events and major factors mediating the wound healing process are summarized in Table 1.

2.1 Hemostasis and Coagulation Phase

This phase begins immediately after injury and serves to prevent bleeding, protect the vascular system to allow it to close intact, and provide a matrix for combating invasive cells in the later phase [3, 5]. The injured vessels lightly close due to the contraction of vascular smooth muscle cells. However, the vascular smooth muscle can prevent bleeding only for a few minutes and bleeding resumes again due to the hypoxia and acidosis in the wound wall. Therefore, the coagulation mechanism is activated with platelet aggregation and clot formation to limit bleeding. The platelets and blood components come into contact with the exposed collagen and other ECM components triggering the release of clotting factors (comprised of fibronectin, fibrin, vitronectin, and thrombospondin) [33], which leads to the formation of a blood clot. Numerous proteins are contained in the α-granules of platelets: platelet-derived growth factor (PDGF), transforming growth factor (TGF), platelet factors,

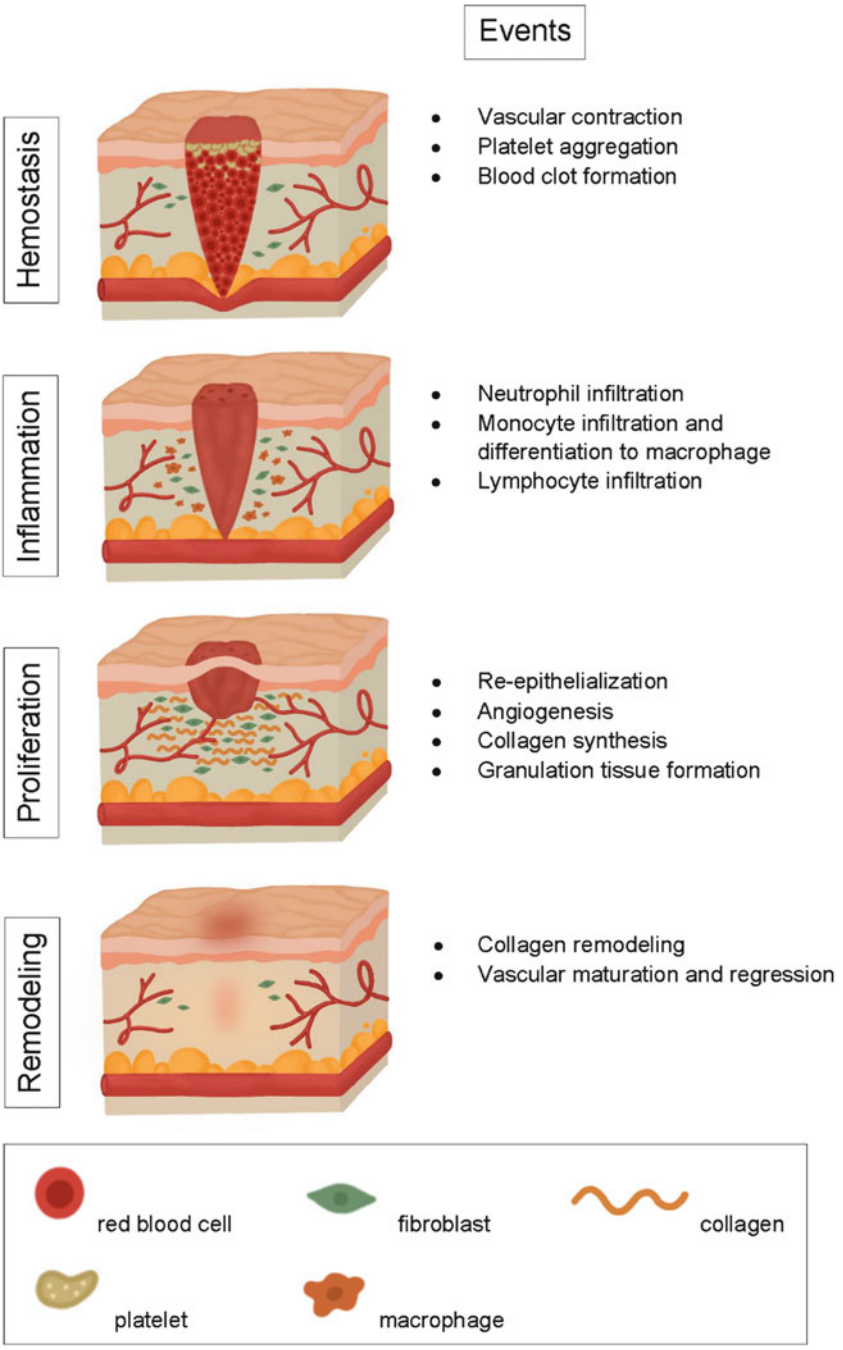

Fig. 2 Schematic illustration of the normal wound healing phases

Table 1 Bio-physiological events and major factors mediated normal wound healing

Phase	Bio-physiological events [32]	Major mediated factors
Hemostasis	1. Vascular contraction 2. Platelet aggregation 3. Blood clot formation	PDGF, TGF, IL-1, PDAF VEGF, EGF, IGF Fibronectin
Inflammation	1. Neutrophil infiltration 2. Monocyte infiltration and differentiation to macrophage 3. Lymphocyte infiltration	TGF, FGF, collagenase IL-1, IgG
Proliferation	1. Re-epithelialization 2. Angiogenesis 3. Collagen synthesis 4. GTF (ECM formation)	PDGF, TGF, FGF, VEGF
Remodeling	1. Collagen remodeling 2. Vascular maturation and regression	PDGF, TGF, FGF

interleukin (IL)-1, platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), and fibronectin [34]. These molecules promote the activities of neutrophils, macrophages, endothelial cells, and fibroblasts in later phase.

2.2 Inflammatory Phase

The second (inflammatory) phase aims to establish an immune barrier against invading microorganisms. The early inflammatory phase initiates the infiltration of the wound sites by neutrophils. Neutrophils function to prevent infection by removing foreign substances, bacteria, and tissue debris via phagocytosis. The redundant neutrophils after completing the task of phagocytosis are eliminated to the wound surface by apoptosis without potentiating the inflammatory response or damaging the tissues [35]. Initially, blood monocytes undergo differentiation with phenotypic changes to become macrophages on arrival into the wound. Macrophages appear at wound sites in the late inflammatory phase to continue the process of phagocytosis of apoptotic cells, including neutrophils [35], and so pave the way for the resolution of inflammation. The infiltrating macrophages also provide an abundant amount of potent tissue growth factors and mediators, such as TGF, collagenase, and fibroblast growth factors (FGFs) to stimulate keratinocytes, fibroblasts, and endothelial cells to proliferate, and so promote tissue regeneration [36] towards the end of this second phase and into the third (proliferative) phase.

Lymphocytes, which are the last cells to enter the wound site, arrive during the late inflammatory phase by chemotactic attraction to IL-1 and the breakdown products of immunoglobulin G (IgG) [35, 37]. The IL-1 plays a vital role in collagenase regulation, being needed for collagen production and degradation of the ECM components, and regulating wound healing [35, 37]. If the wound is

associated with various pathological alterations, including an increased protease activity and infection, the inflammation may persist for months or years and it is then classified as a chronic wound.

2.3 Proliferative Phase

The proliferative phase generally follows and overlaps with the inflammatory phase, and starts with fibroblast migration induced by the action of TGF and PDGF and deposition of a newly produced ECM, leading to the replacement of the temporary matrix composed of several proteins of fibronectin, hyaluronan, GAG, proteoglycans, and types I and III collagen [5, 32, 37]. Therefore, the granulation tissue formation (GTF) and wound contraction can be seen macroscopically in this phase of the wound healing process. Endothelial cells are responsive to numerous angiogenic factors, including FGF, PDGF, VEGF, and TGF, to regenerate epithelial tissue (re-epithelialization) and restore the vascular network [5, 6]. Keratinocytes are involved in repairing the epidermal barrier, while endothelial cells and fibroblasts are responsible for angiogenesis and GTF as part of the ECM formation [5, 6].

2.4 Remodeling Phase

The remodeling phase involves reorganization and contraction of the newly formed ECM as well as scar formation [5, 6, 37] and may last for several years. This phase is controlled by regulatory mechanisms that are designed to maintain a balance between synthesis and degradation, leading to normal healing. This process is regulated by a number of factors, including PDGF, TGF, and FGF [5].

It should be noted that exogenous and endogenous factors can modulate such events and influence the healing process. Especially, systemic disorder diseases, including diabetes, immunosuppression, and venous stasis, may disturb the process of wound healing, resulting in chronic or non-healing wounds [5]. In addition, external agents, such as the utilization of corticosteroid, inflection, smoking, alcohol consumption, stress, etc., can hinder the early closure of the wound [32, 38]. These complicating factors may also cause the appearance of keloids and hypertrophic scars [38].

Keloids and hypertrophic scars are fibroproliferative disorders of the skin that result from abnormal healing of injured or irritated skin. They can be called pathological or inflammatory scars. A hypertrophic scar occurs directly after the initial repair and develops into thick skin that does not spread beyond the original wound, whilst a keloid may occur after healing and continues to grow and spread, invading the surrounding healthy tissue. Tension on the skin around the wound results in prolonged and/or repeated inflammation and generates abnormal numbers of blood vessels as well as collagen and nerve fibers in the dermal reticular layer

[39]. The mechanobiology of the dermis and blood vessels, along with genetic and systemic factors, are possible factors that promote pathological scar development by inducing endothelial dysfunction (vascular hyperpermeability) during the inflammatory stage of wound healing. The continued presence of these factors prolongs the influx of inflammatory cells and factors, thereby leading to fibroblast dysfunction [39]. A scar is formed from the overgrowth of granulation tissue (proliferation of collagen fibers).

3 Fabrication of Chitin/Chitosan Wound Dressings in Various Forms

Chitin and chitosan have been widely used in many fields of biomedical applications, such as tissue engineering, drug carriers, and wound dressings, because of their processibility into various forms. The fabrication of chitin/chitosan wound dressings has been performed in different processes depending on the specific purpose. Chitin and chitosan can be formed into various forms, including hydrogels, membranes, scaffolds, hydrocolloids, and so on, by many methods or processing techniques. Chitin is insoluble in common organic solvents as a direct result of the strong intra- and intermolecular hydrogen bonding, while chitosan can be dissolved in dilute acid, making chitin less processable. However, chitin is more favorable than chitosan in certain applications, especially in the biomedical fields. This is because of the fact that the acetamide group present in chitin is similar to the amide linkage of protein in living tissues [40], which makes chitin more biocompatible than chitosan. This chapter describes briefly the main fabrication techniques used for making chitin-/chitosan-based wound dressings for different applications.

3.1 Hydrogels

Hydrogels are interesting for biomedical applications because of their high-water absorbability and biocompatibility. Progress in the synthesis and designing of hydrogels for wound healing has advanced. Hydrogels can absorb tissue extrudates, provide a moist environment to prevent wound dehydration, and allow transportation of oxygen [41]. The most commonly used fabrication methods for chitin- and chitosan-based hydrogels are described as follows.

3.1.1 Chitin-Based Hydrogel

Fabrication of a chitin hydrogel is difficult because of the limited solubility of chitin, making it hard to perform further processing [42]. Among many attempts, chemical

modification of chitin with various chemical reagents to substitute hydrophilic groups onto the surface of the chitin chains seems to be a promising method to enhance its water sorption ability and solubility [43]. Modification of chitin has mostly been performed in a sodium hydroxide (NaOH)/urea solvent system, and then reacted with other reagents, such as acrylamide [42], propylene oxide [44], and 2-aminoethyl chloride hydrochloride (2-AECH) [45]. Typically, chitin or chitin derivatives were cast in a container and allowed to form a gel, perhaps with the addition of some crosslinkers or physical polymerization.

3.1.2 Chitosan-Based Hydrogel

Fabrication of a hydrogel from chitosan is more attractive than that from chitin due to the *N*-deacetyl groups or amino groups of chitosan, making chitosan more hydrophilic than chitin and so it is easy to dissolve in dilute acid. In order to prepare the chitosan hydrogel, most methods have started from chitosan dissolved in acetic acid [46–48]. The addition of additives or bioactive substances into the hydrogel can improve the physiochemical and biological properties of the chitosan hydrogel for specific purposes. Some drugs or hormones, such as thyroxine [46], heparin, and bemiparin [47], have been loaded during the preparation of a chitosan hydrogel with the aim of stimulating angiogenesis and giving anticoagulant properties, respectively. In many reports, silver (Ag), in form of nanoparticles (AgNPs), was incorporated into the chitosan hydrogel in order to enhance other biological activities, such as antimicrobial and anti-inflammatory properties [48, 49]. Moreover, chitosan can be blended with other polymers, such as PEG [48] and lignin-polyvinyl alcohol (PVA) [50] to enhance its water absorbability and mechanical strength. Finally, the chitosan solution mixture was cast on a setting container and frozen overnight to stabilize the hydrogel structure, followed by neutralization. In some cases, a crosslinking agent has been applied instead of the freezing process.

In addition, a water soluble derivative of chitosan, carboxymethyl chitosan (CMCTS), was synthesized by chemical modification of chitosan with monochloroacetic acid [51]. The CMCTS can be easily dissolved in water over a wide range of concentrations [49, 52], and the CMCTS aqueous solution can be fabricated into a hydrogel under room temperature by complexing with polyelectrolytes [49] or chemically crosslinked with a crosslinker, such as genipin [52].

Hydrogels prepared from chitin, chitosan, and their derivatives can function as active substance carriers. Figure 3 illustrates the chitin and chitosan hydrogels as carriers of active substances, such as drugs, Ag, and growth factors, in order to enhance the wound healing process.

3.2 Membranes

A membrane, as a very thin film, has considerable advantages as a wound dressing, including its high surface-to-volume ratio, permeability, and breathability for

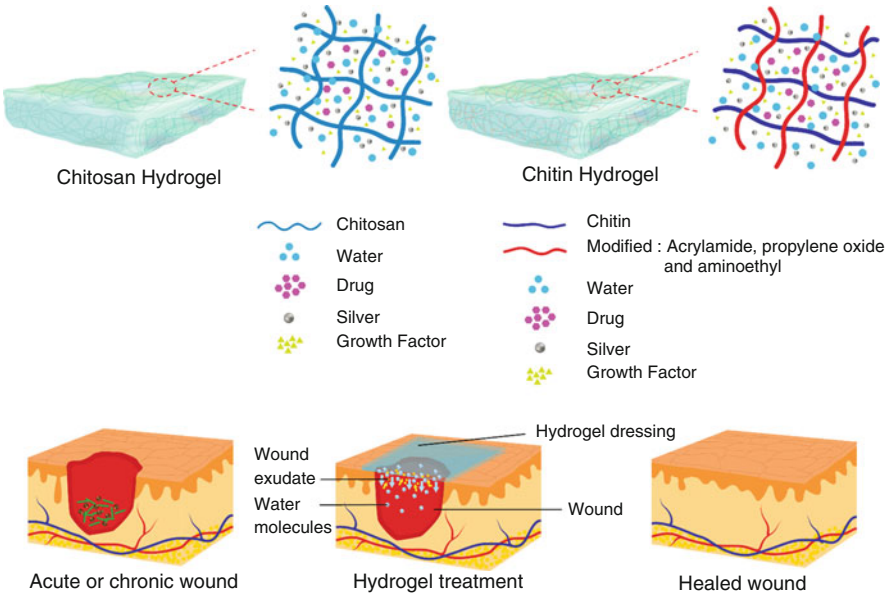

Fig. 3 Schematic illustration of using chitin or chitosan hydrogels as carriers of bioactive agents and applying the hydrogels to enhance wound healing

biological events. The application of a membrane requires the capability to control the transportation, diffusion, or separation of small molecules (such as gas and water) as well as ions. The most commonly used fabrication methods for chitin- and chitosan-based membranes are described as follows.

3.2.1 Chitin-Based Membranes

The casting process has always been used to prepare chitin membranes. The chitin is dissolved in a lithium chloride and dimethylacetamide solvent system [53, 54] and cast on a petri dish, with the dense membrane being obtained by gradual coagulation. Otherwise, after casting in a container, it was moved to soak in different kinds of solvents, washed with water, and dried to obtain the porous chitin membrane [54, 55].

3.2.2 Chitosan-Based Membranes

Currently, one of the most promising approaches for wound healing that mimics the structural similarities of the skin epidermal and dermal layers is the use of asymmetric membranes. The inner layer is a porous structure that functions as an exudate absorption matrix and supporting matrix for cell adhesion, migration, and

proliferation as well as allowing gaseous exchange and nutrients flow. Whereas, the outer layer is a dense layer mimicking the epidermis of the skin that functions as barrier to protect the wound from external threats (physical, chemical, and infection). Several techniques, such as electrospinning, bioprinting, wet-phase inversion method, dry/wet method, and supercritical carbon dioxide ($scCO_2$)-assisted phase inversion technique, have been explored to produce asymmetric membranes [56].

The fabrication of a chitosan-titanium dioxide composite membrane [57] and a membrane of gelatin/chitosan/cinnamaldehyde crosslinked with glutaraldehyde [58] have been obtained using the wet-phase inversion method. Briefly, the chitosan mixture is cast and then immersed in a non-solvent coagulant bath. The procedure is similar to the wet-phase inversion method, but the dry/wet method adds one more step of pre-evaporation (drying) of the cast membrane before immersion of the polymer matrix into the coagulation bath. This pre-evaporation stage increases the concentration of the polymeric solution and creates a dense outer top layer that acts as barrier to protect the wound against external contamination [59]. The preparation of a silver sulfadiazine (AgSD)-incorporated asymmetric chitosan membrane [59] and fabrication of a sponge-like asymmetric chitosan [60] are examples of those fabricated by the dry/wet method.

Figure 4a illustrates asymmetric chitin and chitosan membranes with the incorporation of some active substances, while Fig. 4b presents the wet-phase inversion method and dry/wet method. The $scCO_2$-assisted phase inversion method was proposed for the first time as an alternative technique to produce an asymmetrical membrane for skin wound healing. Being a green technology, it can form solvent-free membranes with short processing times and no collapse of the structure. Furthermore, $scCO_2$-assisted phase inversion allows the production of dry, clean, and ready-to-use membranes with a highly controlled morphology (by changing the pressure, temperature, and/or depressurization rate) and reduces the solvent recovery costs. The process does not require additional post-treatments or any potential organic solvents [61].

A traditionally famous method is the freeze-dried technique, in which samples are frozen and dried under vacuum to obtain porous membranes. Chitosan containing polyurethane modified with *N*-isopropyl acrylamide membrane [62] and bacterial cellulose-chitosan composite membrane [63] are examples of wound dressing membranes fabricated by the freeze-dry method.

3.3 Fiber and Electrospun Mat

Electrospinning is a simple and inexpensive method for producing nanofibers. It has become a popular fabrication method for porous fibrous materials that can be applied for tissue engineering scaffolds and wound dressings. The advantages of an electrospun mat made from ultrafine polymer fibers are its high porosity and nano-scale diameters, variable pore-size distribution, high surface to volume ratio, and, most importantly, its morphological similarity to the natural ECM in the skin

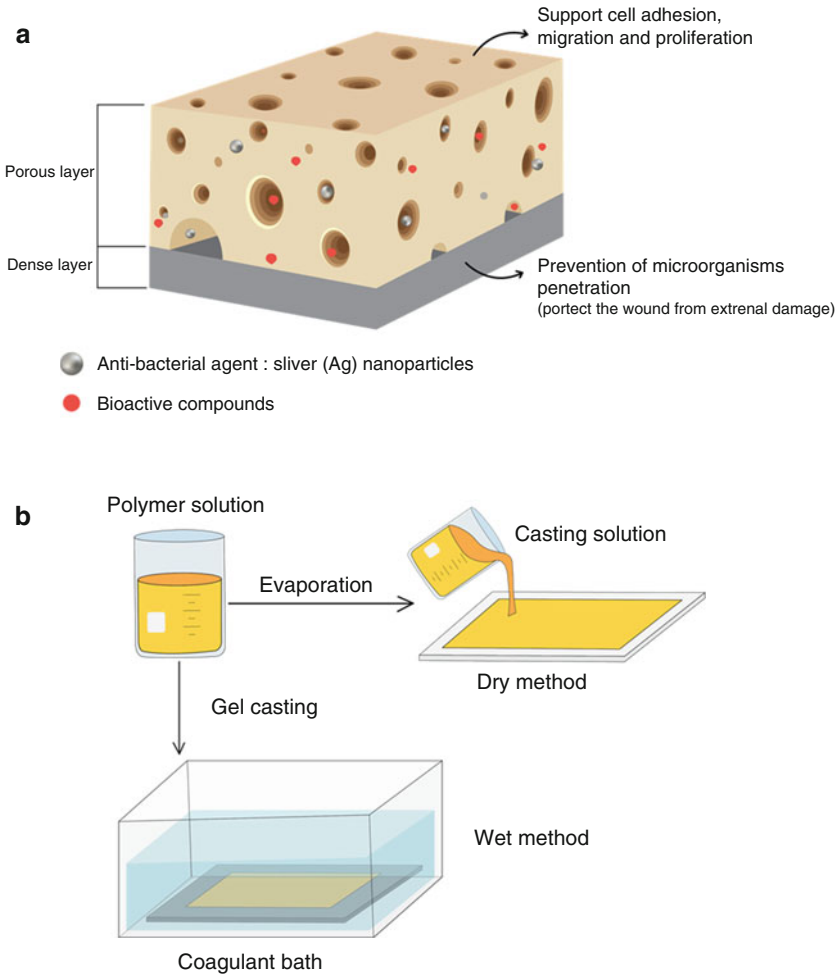

Fig. 4 Schematic illustration of (**a**) asymmetric chitin and chitosan membranes and (**b**) wet-phase inversion method and dry/wet method

[64]. The electrospun mat promotes cell attachment and proliferation. For this reason, the electrospinning technique has been used in many studies to prepare chitin and chitosan fibrous mats for wound dressing applications. However, electrospinning of natural polymers, such as chitosan and alginate, is more difficult than that of synthetic polymers. Chitosan presents a very low solubility in most organic solvents, and the use of dilute or concentrated acid solutions seems to be the most reliable option to properly dissolve it [65], since the protonation of amine groups of chitosan greatly contributes to its solubility. The spinnability of pure chitosan is challenging due to its polycationic nature and high viscosity in solution, and specific intra- and inter-molecular interactions. Indeed, formation of three-

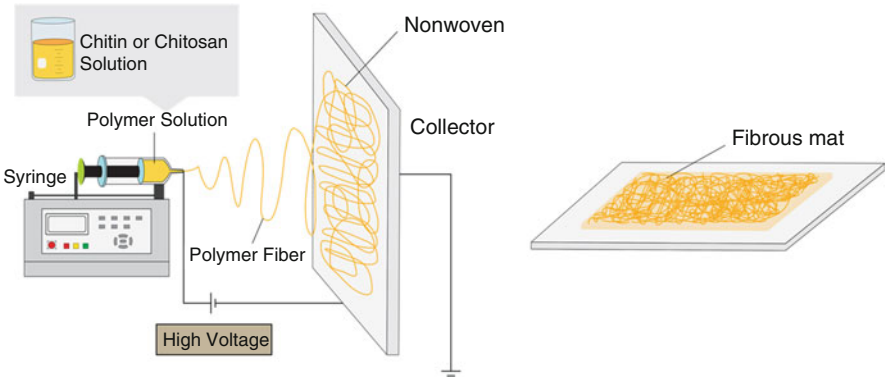

Fig. 5 Schematic illustration of the electrospinning fabrication of a chitin or chitosan fibrous mat

dimensional (3D) strong hydrogen bonds prevents the free movement of the polymeric chain segments exposed to the electrical field, making the formation of a stable jet problematic. In order to overcome these issues, blending with synthetic polymers, such as polyethylene oxide [65–67], PVA [64, 68], and poly (vinyl pyrrolidone) [69], are generally used as both a spinning enabler/enhancer and a hydrophilicity improver [65, 66]. The electrospinning fabrication of chitin and chitosan fibrous mat is represented in Fig. 5.

3.4 Sponges and Scaffolds

Sponge dressings with a porous structure exhibit a huge porosity, high strength, and high specific surface area, beneficial to meet the demands of a higher gas permeability, tunable water vapor evaporation, more wound exudate absorption, presence of good hemostatic properties, and protecting the wound from infection and dehydration [70]. While polymeric scaffolds are also a 3D porous structure that provides support for cell attachment, proliferation, and differentiation, they must possess biocompatibility, high porosity, and a good mechanical strength [17]. There are many processes to fabricate chitosan sponges, including gas foaming and particulate leaching, but the most common method is through the process of lyophilization. Lyophilization, also called freeze-drying, sublimates liquid from frozen materials, which helps to reduce damage to the material and preserves the structural and chemical integrity of proteins and natural substances. In the case of chitosan sponges, lyophilization creates pores, which in turn creates a greater surface area than dense forms of chitosan [71].

Many researchers have used the lyophilization method in their studies [72–76]. Some researchers have added the natural product *Aloe vera* extract [72, 73], metallic particles (AgNPs [73], aluminium monostearate [74], and AgSD [75]), or blended with other polymers (PVA [76]), in order to obtain an antibacterial property

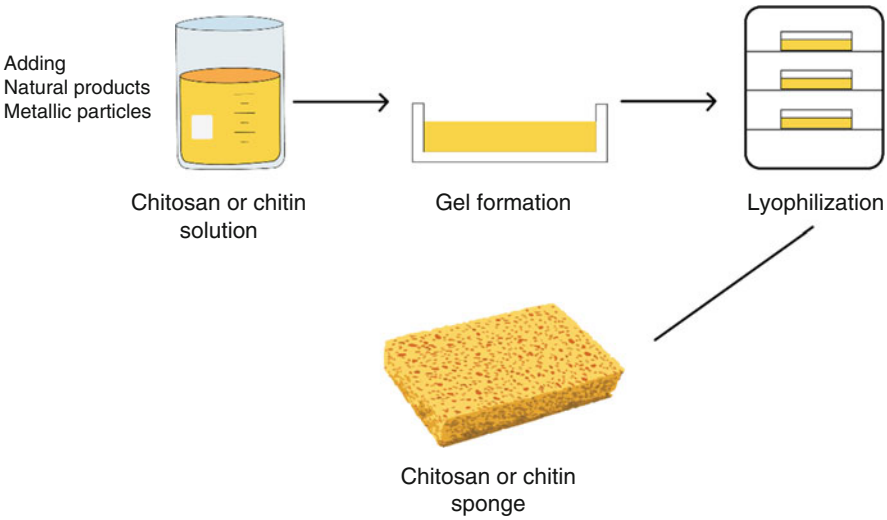

Fig. 6 Schematic illustration of chitin and chitosan sponge fabrication procedures

or improve the mechanical properties of chitosan. On the other hand, there are few studies on the fabrication of an asymmetric wettable chitosan-based sponge. A wound dressing with an asymmetric surface wettability is of great interest in wound healing. The hydrophobic surface could effectively prevent the external contamination (water, blood, and bacteria) to the dressing, while the hydrophilic surface could preserve the comfortable, moist environment to promote wound healing. One side of chitosan sponge can be modified with a thin layer of stearic acid to provide the asymmetric chitosan sponge [77].

For a chitin-based sponge, several studies have reported the use of modified chitin. Chitin was modified in form of quaternized chitin, where partial deacetylation occurred during the quaternization in a NaOH/urea solvent system. Glutaraldehyde was then used as a crosslinker of the quaternized chitin and the sponge was obtained after lyophilization [78]. The fabrication of chitin and chitosan sponges is illustrated in Fig. 6.

3.5 Hydrocolloids

Hydrocolloid dressings absorb the wound fluid and change into a jelly-like materials. Hydrocolloid dressings consist of two different structures. The inner hydrocolloid are particles that can absorb exudate to form a hydrated gel over the wound, creating a moist environment that promotes healing and protects the new tissue. The outer matrix (film, foam, or gel) not only protects the wound from bacterial contamination, and foreign debris, but it also maintains a moist environment and helps prevent

shearing [79–81]. Classical hydrocolloids always contain a gel forming agent, such as carboxymethyl cellulose (CMC), pectin, gelatin, or sodium CMC [81, 82].

Only a few fabrications of hydrocolloid wound dressing have been generated from chitin or chitosan. Tripolyphosphate, as a crosslinking agent, was added to the chitosan solution in order to obtain chitosan microbeads dispersed in the chitin solution and later transformed into a chitin hydrogel by gradual coagulation [83]. A calcium alginate hydrocolloid with chitin, chitosan, and fucoidan was reported. The dry powders of alginates, chitin, chitosan, and fucoidan were mixed and crushed. The mixed powders were then spread on filter paper and sprayed with distilled water to partially dissolve the powder into a paste. The paste on the supporter was immersed in calcium chloride solution to generate the calcium alginate hydrocolloid, which was then crosslinked with ethylene glycol diglycidyl ether [84]. Figure 7 presents the chitin and chitosan hydrocolloid dressing and its application over the wound to absorb the wound exudate as well as protect the wound from bacterial infection.

4 Commercially Available Chitin and Chitosan Wound Dressings

The ordered regeneration of wounded tissues requires the fabrication of chitin and chitosan mostly in the forms of nonwoven, fibrils, hydrocolloid, films, and sponges. With the advantages of chitin and chitosan that can be fabricated in various forms and with the variety of fabrication methods mentioned above (Sect. 3), introducing wound dressing materials based on chitin, chitosan, and their derivatives are well

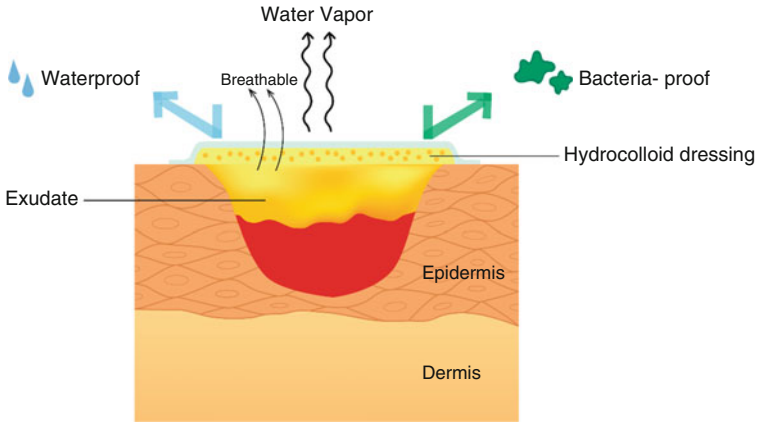

Fig. 7 Schematic illustration of the application of a chitin or chitosan hydrocolloid dressing to absorb the wound exudate and protect the wound from bacterial infection

known on the market and are produced and commercialized in many forms. The commercially available chitin- and chitosan-based wound dressings and their functional usages related to wound healing are summarized in Table 2.

5 Physical and Chemical Modification of Chitin and Chitosan for Wound Dressing in Specific Purposes

The worthwhile intrinsic properties of chitin and chitosan, including their biodegradability, biocompatibility, non-toxicity, hemostaticity, antimicrobial activity, anti-inflammatory, and antioxidant properties, make them appropriate for use as wound dressings. However, in order to develop chitin- and chitosan-based wound dressings for more specific purposes, they can be modified by means of physical or chemical modifications to add new functions, features, and to enhance some certain properties. Modification may be achieved through both covalent and noncovalent means.

There is considerable interest in providing chitin and chitosan with desired functionalities that have not been chemically modified. One investigated approach is property modification via physical modification. Physical or physicochemical modification is primarily considered because it does not require a complicated chemical reaction. Appropriate active substances are added for the specific purpose, enhancing the physicochemical properties, and might only absorb or disperse within the chitin or chitosan matrix without covalent bond formation.

Furthermore, chitosan has chemically active multiple functional groups, including the amino ($–NH_2$), and primary and secondary hydroxyl (–OH) groups, whereas chitin has the chemically active functional groups of primary and secondary hydroxyl groups. Thus, it is feasible to perform chemical modification to improve their physical and chemical properties, while their biodegradability, biocompatibility, and non-toxicity should be maintained. Furthermore, the clear definition of the positions of functional groups in their repeating units is crucial to exploit regioselectivity in the chemical modification reactions in order to perform precise and well-controlled structural modifications of chitin and chitosan derivatives.

The most favorable way for chemical modification of chitin is *O*-substitution, in which the reaction occurs at hydroxyl groups (–OH), since the acetamido group ($–NHCOCH_3$) of chitin is not chemically active unless partially deacetylated into amino groups. Whilst, the most favorable way for chemical modification of chitosan is *N*-substitution, in which the reaction occurs at the amino group ($–NH_2$) of chitosan, *O*-substitution can be performed similar to chitin. Since the amino groups have a higher reactivity than hydroxyl groups, the *O*-substitution of chitosan alone requires the protection and deprotection of the primary amino groups. The individual *N*- or *O*-substituted derivatives of chitin and chitosan provide new functional groups, giving different bulk and chemical properties belonging to well-designed molecular structure.

Table 2 Commercially available chitin- and chitosan-based wound dressings and their functional uses related to wound healing

Based materials	Trademark (company)	Forms	Functions related to wound healing process	Ref.
Chitin	Beschitin (Unitika)	Chitin nonwoven	1. Treat traumatic and surgical wounds 2. Favor early GTF, no retractive scar formation	[85]
	Chitipack S (Eisai Co. Ltd.)	Foam, sponge	1. Treat traumatic and surgical wounds 2. Favor early GTF	[85–87]
	Chitipack P (Eisai Co. Ltd.)	Chitin hydrocolloid in poly(ethylene terephthalate)	1. Treat large skin defects and those difficult to suture 2. Favor early GTF	[85, 87]
	Syvek-Patch (Marine Polymer Technologies)	Fibrils, fibrous material	1. Control bleeding at vascular access sites 2. Accelerate hemostatic process	[85, 86, 88]
Chitosan	HemCon® Bandage (HemCon Medical Technologies Inc.)	Sponge	1. Accelerate hemostatic process 2. Support antimicrobial activity 3. Use in emergency and civilian	[85, 86, 89, 90]
	TraumaStat (Ore-Medix)	Sponge	1. Treat traumatic wounds 2. Accelerate hemostatic process	[85, 86, 91]
	Chitopack C (Eisai Co. Ltd.)	Cotton-like chitosan nonwoven	1. Repair body tissue completely 2. Rebuild normal subcutaneous tissue 3. Regenerate skin regularly	[85, 87]
	Tegasorb and Tegaderm (3 M)	Chitosan hydrocolloid and film	Suitable for leg ulcers, sacral, and chronic wounds	[85, 87]

Therefore, both physical and chemical modification are the appropriate approaches to enhance the functional properties of chitin- and chitosan-based wound dressings in specific purposes.

6 Chitin and Chitosan-Based Functional Wound Dressings

Wound dressing should remove excess exudates, keep the wound interface moist, allow gaseous exchange, and act as a barrier to microorganisms. It should also fulfill the fundamental concerns of being non-toxic, non-allergenic, non-adherent, and easily removed without trauma. Beyond these fundamental requirements, functional wound dressings should be designed to provide certain other functional properties suitable for different wound types. In addition to the conducting ability of materials to promote and allow the migration and proliferation of epithelial, fibroblast, and endothelial cells, the synthesis of ECM components is also required in wound repair and skin regeneration [92]. Therefore, insight into the details of developing chitin- and chitosan-based wound dressings through their physical and chemical modification to acquire functional applications, such as the treatment hemorrhage (hemostatic wound dressings), infection (antimicrobial wound dressings), burns (wound dressings for burns), and chronic ulcers (diabetic wound dressings), are mentioned in this chapter.

6.1 Hemostatic (Blood Coagulant) Wound Dressings

Hemorrhage is a major mortality plan from traumatic injuries, which frequently happens in accidents, battlefields, and operation rooms [93]. Hemostasis is the first phase of wound healing and effective hemostasis is mandatory to stem life-threatening bleeding. Hemostatic agents derived from chitosan have been widely studied for their fast absorbing and localized hemostatic effects. Chitosan is also well known for its antimicrobial properties, where its protection against a wide range of bacteria enhances its appeal as a bandage. Several chitosan wound dressings have been developed for the rapid and effective control of bleeding. Some of them have been commercialized, such as ChitoFlex®, CELOX™, QuikClot®, and HemCon® [94].

Chitosan dressings were prepared by dissolving chitosan in dilute aqueous acetic acid, placing into a mold, lyophilized, and then neutralization with NaOH [95]. The obtained chitosan dressing presented a fast absorption rate of human whole blood in less than 5 s, owing to its homogeneous and penetrating porous structure. However, it showed a slow clotting rate when tested with human whole blood containing a normal (250,000 platelets/mL) platelet number.

Chitosan electrospun mats have been prepared to enlarge the porosity, and they showed a higher rate of blood clotting than chitosan sponges [96]. This result indicated that the thrombogenic activity of chitosan was enhanced by increasing the pore size and porosity [96]. The addition of polyphosphate to chitosan led to a more potent hemostat [97]. Polyphosphate can ionically interact with chitosan to form polyelectrolyte complexes. Both components activated coagulation by a different mechanism. The protonated amino groups of chitosan attracted negatively

charged residues on red blood cell membranes, causing strong hemagglutination [98]. Chitosan also adsorbed fibrinogen and plasma proteins, enhancing platelet aggregation [99]. In contrast, polyphosphate specifically shortened both the time lag for initial thrombin generation and the time to peak thrombin generation [100].

Although numerous studies have reported the hemostatic property of chitosan, its insolubility and weak antibacterial activity are still limitations. Surface modification of chitosan, such as carboxymethylation and quaternization, has been investigated to improve its water solubility and antibacterial activity. Accordingly, nonwoven of carboxymethyl chitosan (CMCS), *N*-succinyl chitosan (NSCS), and *N,N,N*-trimethyl chitosan (TMCS) were prepared to examine the hemostatic property. It was found that the nonwoven CMCS, NSCS, and TMCS all showed a better hemostatic property than nonwoven chitosan [101]. However, nonwoven TMCS had a lower blood absorption than the others, which might be due to the quaternary ammonium cationic group leading to hemolysis [101]. The negative charges on the surface of NSCS and CMCS could accelerate blood coagulation by activation of the intrinsic coagulation pathway along with the hemostatic mechanism of chitosan [101].

Superabsorbent polymers (SAPs) derived from chitosan and its derivatives are promising hemostatic materials with a good absorption capability and potential hemostatic ability [102]. The SAP formed from carboxymethyl chitosan grafted poly(acrylic acid) (CMCTS-g-PAA) was prepared by graft copolymerization of acrylic acid on the CMCTS [103]. The porous structure of CMCTS-g-PAA was obtained by precipitation of the hydrogel in ethanol, and was found to be non-toxic, with a high swelling capacity and with a good hemostatic performance in the treatment of the hemorrhage model in rabbits [104]. This excellent hemostatic property results from the synergistic effect of the protonated amino groups of chitosan and the strong swelling capacity of the porous materials.

During the hemostasis and wound healing, bacterial infection is also a serious issue. If there is no infection, the wound healing proceeds more smoothly. However, infection may cause a series of bacteremia reactions and greatly threaten human life. Therefore, antimicrobial wound dressings have been designed to protect wounds from infection for better wound healing.

6.2 Antimicrobial Wound Dressings

It is well known that one of the main problems in wound care treatment is wound infection. Bacterial wound infection delays the wound healing process and possibly gives rise to life-threatening complications [105]. Generally, a large amount of fluid loss and bacterial infection would lead to serious consequences in severe burns or extensive skin loss. Widely spread opinions among wound care practitioners are that Gram-positive bacteria, such as *Staphylococcus aureus* (*S. aureus*), and Gram-negative bacteria, like *Pseudomonas aeruginosa* (*P. aeruginosa*), are the primary causes of delayed healing and infection in both acute and chronic wounds [106]. Wound dressings not only stop blood loss, but also protect the wound from

bacterial infection and accelerate the wound healing process. In addition, the wound exudates between the wound and the dressing can also lead to an infection. Therefore, the application of antibacterial wound dressings with a broad activity spectrum and high bactericidal activity is one of the effective approaches for treating infected wounds.

Chitin itself does not exhibit any marked antibacterial activity [107], while chitosan exhibits antibacterial activity when the amino groups are protonated in an acidic condition, but not in a neutral (physiological) condition [108]. At a neutral pH, the antibacterial activity of chitosan increased as the MW decreased [108]. It was found that a low solubility and negative zeta potential values were determined for chitosan with a MW of more than 29.2 kDa, which may explain the loss of its antibacterial activity at pH 7.0 [108]. Therefore, the most simple physical modification of chitin and chitosan wound dressings for antibacterial activity was by the incorporation of antibacterial agents, like inorganic metal NPs and organic bioactive substances. In addition, some chemical modifications of chitin and chitosan have been developed to improve the antibacterial properties of wound dressings.

6.2.1 Physical Modification of Chitin and Chitosan for Antimicrobial Wound Dressings

Incorporation of AgNPs or Ag^+

Many research groups have been focused on incorporation of various NPs in chitin and chitosan matrices, including those of Ag, zinc oxide (ZnO), and gold [109]. Among those metal and metal oxide NPs, AgNPs are the most widely used in wound dressings and wound healing, since they exhibit significant antimicrobial activities over a wide spectrum of pathogenic and drug-resistant strains [110], as well as an anti-inflammatory activity [111]. Structurally, AgNPs are clusters of silver atoms with particle sizes ranging in diameter from 1 to 100 nm, and so provide a high surface area. They have been prepared by the reduction of silver nitrate into AgNPs using sodium citrate as the reducing agent [112]. The minimum inhibitory concentration and minimum bactericidal concentration of AgNPs are in the range of 1.56 to 6.25 μg/mL and 12.5 μg/mL, respectively, against a broad spectrum of microorganisms, including *Escherichia coli* (*E. coli*), *P. aeruginosa*, *Salmonella abony*, *Salmonella typhimurium*, *Klebsiella aerogenes*, *Proteus vulgaris*, *S. aureus*, *Bacillus subtilis*, and *Staphylococcus epidermidis* [113]. The addition of AgNPs at concentrations of 0.001, 0.003, and 0.006% (w/w) into chitin hydrogels was evaluated for their antibacterial activity against *E. coli* and *S. aureus*, whole blood clotting, and cell viability on vero (epithelial) cells [112]. Owing to the physical absorption of AgNPs within the chitin hydrogels, the AgNPs were released from the chitin hydrogels and exhibited antibacterial activity against *E. coli* and *S. aureus.* The bacterial inhibition zone increased with increasing concentrations of AgNPs. The Gram-negative bacteria (*E. coli*) were more susceptible to the AgNPs than the

Gram-positive bacteria (*S. aureus*), which might be due to the limited penetration ability of AgNPs through the thick peptidoglycan wall of Gram-positive bacteria.

The bactericidal mechanism is certainly due to the released Ag^+ ions that act as reservoirs for the Ag^+ bactericidal agent. The Ag^+ ions are known to exert several antibacterial mechanisms. One is the interaction with phosphorus and sulfur groups of the bacterial cell wall and plasma membrane proteins [114, 115]. The interaction between Ag^+ and the membrane leads to dysfunction of the proteins, causing bacterial death. The Ag^+ ions can also bind to negatively charged components of the bacterial membrane, creating holes, leading to cytoplasmic leakage, and causing cell death. Once inside the cell, the Ag^+ ions can also disturb the bacterial electron transport [116]. In addition, the bactericidal effect of silver is achieved through cell membrane disruption, followed by protein dysfunction and breakage of DNA strands, resulting in an increased level of intracellular reactive oxygen species (ROS) [116]. The mode of action of Ag^+ is similar to that of AgNPs, but with stronger antibacterial efficiency [117].

In addition, chitin hydrogels with a high concentration of AgNPs shorten the blood clotting time [113, 118], while remaining non-toxic to vero cells. Indeed, low doses of AgNPs were reported to be non-toxic both in vitro and in vivo [113]. However, in contrast, the addition of AgNPs into a chitin/silk fibroin scaffold at 0.001, 0.01, and 0.1% (w/w) revealed cytotoxicity to HFF2 (fibroblast) cells, where the higher the AgNP concentration, the lower the HFF2 cell viability, especially at 0.1% (w/w) AgNPs [118].

Hybridization of Chitosan and ZnO

The addition of AgNPs at high concentrations causes a change in color and induces cell cytotoxicity, which seriously limits their applications. An alternative way to reduce the cytotoxicity but enhance the antibacterial activity is the hybridization of chitosan with other metal oxides, such as ZnO. A 0.3% (w/v) chitosan/2 mM ZnO hybrid was developed and coated on cotton fabric by sonochemical coating to a ZnO/chitosan mass ratio of around 0.086 [119]. The physicochemical properties of the individual composite were probably due to the formation of the Zn^{2+}/chitosan complex within the hybrid coatings, following the dissolution of ZnO in the chitosan/acetic acid solution. Therefore, two possible models for hybridization of metal ions with chitosan were the pendant model, where an ion was bound to only one amino group of chitosan, and the bridge model, where an ion was bound to several amino and hydroxyl groups of one or bridging more chitosan molecules [119]. The fabrics coated with the chitosan/ZnO hybrid were tested for their antibacterial activity with a 60 min contact time and exhibited a reduction in the bacterial viability of around 98% and 99% against *S. aureus* and *E. coli*, respectively, compared to 61 and 31% for ZnO and chitosan individually, respectively, against *S. aureus* and 78 and 99% against *E. coli*, respectively.

Chitosan and ZnO have been widely reported as efficient antibacterial agents. The mode of antibacterial action of ZnO involves the oxide dissolution to Zn^{2+} and the

association of Zn^{2+} with oxidative stress in bacteria cells and generation of ROS, which subsequently oxidized the cell contents and cause cell death [120]. The mode of antimicrobial activity by chitosan has been reported to be as follows: (1) interaction with negatively charged components in microbial membranes to alter the cell permeability and (2) binding to the DNA of bacterial cells to inhibit protein synthesis [121]. The hybrid complexes of chitosan with metal ions exhibited several-fold enhanced antibacterial properties compared to the individual components.

The cytotoxicity of chitosan, ZnO, and chitosan/ZnO hybrid was determined using an indirect contact method with fibroblasts for 24 h. The fibroblast cell viability following exposure to the ZnO-coated fabric dropped to less than 5%, whilst the chitosan and the chitosan/ZnO hybrid coating did not induce considerable cytotoxicity. Therefore, the synergistic effects of chitosan and ZnO NPs resulted in an enhanced antibacterial efficiency compared to the individual chitosan or ZnO coatings, as well as avoiding the risk of adverse effects on human health [119].

6.2.2 Chemical Modification of Chitin and Chitosan for Antimicrobial Wound Dressings

Currently, the usage of broad spectrum antibiotics is generally regarded as the most effective solution to such infections. Nevertheless, the overuse of antibiotics frequently leads to the evolution and spread of multi-drug-resistant bacteria. Therefore, new antibacterial agents from natural polymeric materials are worth developing. The amino groups of chitosan are protonated (positively charged) under an acidic condition, giving chitosan an interesting inherent antibacterial property. The antibacterial activity of chitosan is greater than chitin because it has a greater number of exposed protonated amino groups that can electrostatically interact with negatively charged proteins, lipids, and carbohydrates on the surface of bacterial cells, resulting in the inhibition of bacterial growth. Owing to their own comparatively low antibacterial activities at physiological pH (unprotonated state), several studies have proposed the synthesis of new chitin and chitosan derivatives for antibacterial agents. In particular, chitin derivatives can be produced by *O*-modification, while chitosan derivatives can be produced by *O*-modification, *N*-modification, or *N,O*-modification as mentioned above (Sect. 5). Thus, chitin, chitosan, and their derivatives have become one of the desirable materials for fabrication of antibacterial wound dressings. Several research works have focused on developing new derivatives of chitin and chitosan, especially quaternary ammonium derivatives, and to fabricate them as hydrogels or apply them as a textile finishing with an outstanding inherent antibacterial activity for wound therapy.

The modification of chitin and chitosan with quaternary ammonium groups enhances their water solubility and antibacterial properties, but only a limited amount of research has been focused on developing the quaternary derivatives of chitin compared to that for chitosan. Aminoethyl chitin [122] was synthesized by the heterogeneous reaction in alkaline condition of a mixed NaOH/isopropanol solvent with 2-AECH [122]. Amino ethyl chitin hydrogels have a good antibacterial activity

against *S. aureus*, where it is hypothesized that the antibacterial activity of chitin derivatives is related to the amino groups, and so the introduction of a multi-amino chitin derivative of poly(aminoethyl) chitin (PAEMC) was prepared via grafting aminoethyl moieties onto alkaline chitin chains with an excess amount of 2-AECH [123]. The PAEMC was synthesized in the form of polymerized amino ethyl monomers and exhibited a higher antimicrobial activity against Gram-positive bacteria than against Gram-negative ones [123]. The PAEMC exerted its antibacterial activity by a membrane damage mechanism.

Poly(amino ethyl) chitosan (PAEMCS) was also synthesized by the deacetylation of PAEMC [45], and its hydrogels were fabricated under the participation of dipotassium hydrogen phosphate. The resulting hydrogels showed a higher antibacterial activity against *E. coli* and *S. aureus* than that of chitosan, implying that the increased amount of amino groups improved the antibacterial activity. However, increasing the density of amino groups in PAEMCS showed a mild cytotoxicity to the L929 mouse fibroblast cell line and human umbilical vein endothelial cells (HUVECs). Similarly, PAEMCS also exhibited a higher antimicrobial activity against Gram-positive bacteria than Gram-negative ones [45].

Hydroxypropyl trimethylammonium chitin (HPTMAC) [124] was synthesized by a heterogeneous reaction in an alkaline condition using a NaOH/isopropanol solvent with 3-chloro-2-hydroxypropyltrimethylammonium chloride [124]. In addition, HPTMAC can also be synthesized by the homogeneous reaction in other green systems of a NaOH/urea aqueous solution [125] or a potassium hydroxide/urea aqueous solution [126] with 2,3-epoxypropyltrimethylammonium chloride. The obtained quaternary derivatives exhibited antibacterial activity. For HPTMAC, it exhibited excellent antimicrobial activities against *E. coli*, *S. aureus*, *Candida albicans*, and *Rhizopus oryzae* and revealed biocompatibility and significant accelerating consequences on the healing of uninfected, *E. coli*-infected, and *S. aureus*-infected wounds. Thus, it can be applied as a novel wound dressing for skin regeneration, particularly for infected wounds [126].

Moreover, chitin betainate was modified to enhance its antibacterial activity over a wide pH range [107]. It was prepared by the acylation of chitin with carboxymethyl trimethyl ammonium chloride (CMA). Chitin betainate at 10 mg/mL exhibited complete bactericidal activity against *E. coli* within 10 min, but a 45.2% and 78% reduction in *S. aureus* viability after 10 min and 24 h exposure, respectively, whilst chitin did not exhibit any significant antibacterial activity. The bactericidal activity was concentration-dependent against both bacteria, but it was more efficient against *E. coli* than *S. aureus* [107]. A chitin betainate hydrogel was prepared for antibacterial wound dressing purposes and had good water absorption by forming an interpenetrating network with PEG.

In order to investigate the structural effect of the quaternary ammonium chitin on the antibacterial activity and specificity against *E. coli* and *S. aureus*, chitin was modified with three different quaternary ammonium groups and spacers: (1) CMA to obtain CTCMA or chitin betainate as mentioned before, (2) 3-carboxypropyl trimethyl ammonium chloride to yield CTCPA, and (3) 3-carboxypropyl-*N*-dodecyl-*N,N*-dimethylammonium chloride to give CTDDMAB [127]. The

CTCMA consisted of an *N,N,N*-trimethyl substituent with a C1 (methyl) spacer; CTCPA consisted of an *N,N,N*-trimethyl substituent with a C3 (propyl) spacer; and CTDDMAB consisted of *N*-dodecyl-*N,N*-dimethyl substituent with a C3 spacer. Thus, CTCPA had the similar quaternary substituent as CTCMA but with a longer spacer (C3) between the chitin and quaternary substituent, whilst CTDDMAB had the same C3 spacer as CTCPA but with an *N*- long alkyl substituent.

The chemical structure of these quaternary ammonium chitin derivatives showed specificity and strongly influenced the antibacterial activity against *E. coli* and *S. aureus*. The outer cell wall of *E. coli*, which is rich in negatively charged lipopolysaccharide, is susceptible to the positively charged quaternary ammonium substituents of CTCMA and CTCPA, while the thick peptidoglycan cell wall of *S. aureus* functions as a barrier against penetration of polar substances. Accordingly, CTDDMAB with its longer propyl (C3) spacer or the *N*-long alkyl substituent in its structure enhanced the penetration and antibacterial activity against *S. aureus*. Therefore, the antibacterial activity of quaternary ammonium chitins against *E. coli* required only the positively charged quaternary ammonium substituents to contact with the outermost cell wall, while that against *S. aureus* required not only the length of the spacer, but also the *N*-long alkyl substituents.

Since chitosan itself exhibits antimicrobial activity without modification, but at comparatively low antibacterial activities at neutral or physiological conditions. Therefore, research on chemical modification of chitosan has focused on enhancing its antimicrobial activities. Quaternization of chitosan can be performed by two common ways, which are substitution of quaternary ammonium groups onto the chitosan backbone and substitution of those groups as the side chains. *N,N,N*-trimethyl chitosan (TMC), which has quaternary ammonium groups on the chitosan backbone, was synthesized by first treating chitosan with formic acid and formaldehyde, followed by methylation with methyl iodide [128]. The antibacterial activity of TMC was more effective than chitosan at pH 5.5, but less effective at pH 3.5. Because the lower pH represses the ionization of trimethylated amino groups of TMC [128]. For *N*-[(2-hydroxy-3-trimethyl ammonium) propyl] chitosan (HTCC), with quaternary ammonium groups as side chains, it was synthesized by coupling glycidyltrimethylammonium chloride to chitosan in water [129, 130]. Then, the HTCC was further chemically modified to obtain double bond containing derivatives of *O*-acrylamidomethyl-HTCC, which is chemically bound with cellulose or cotton fabrics, to make a promising antibacterial fabric. For *N,N,N*-trimethyl-*O*-(2-hydroxy-3-trimethylammonium propyl)chitosan (TMHTMAPC), with quaternary ammonium groups on both the backbone and *O*-side substituent, it was synthesized by chemical modification of TMC with 3-chloro-2-hydroxypropyl trimethyl ammonium chloride. The obtained TMHTMAPC exhibited a higher antibacterial activity than TMC [131], indicating that quaternization of the more flexible side chains enhanced the interaction between the positively charged chitosan derivatives and the negatively charged bacterial cell envelope [131]. Representative schemes for the quaternization of chitin/chitosan derivatives are summarized in Fig. 8.

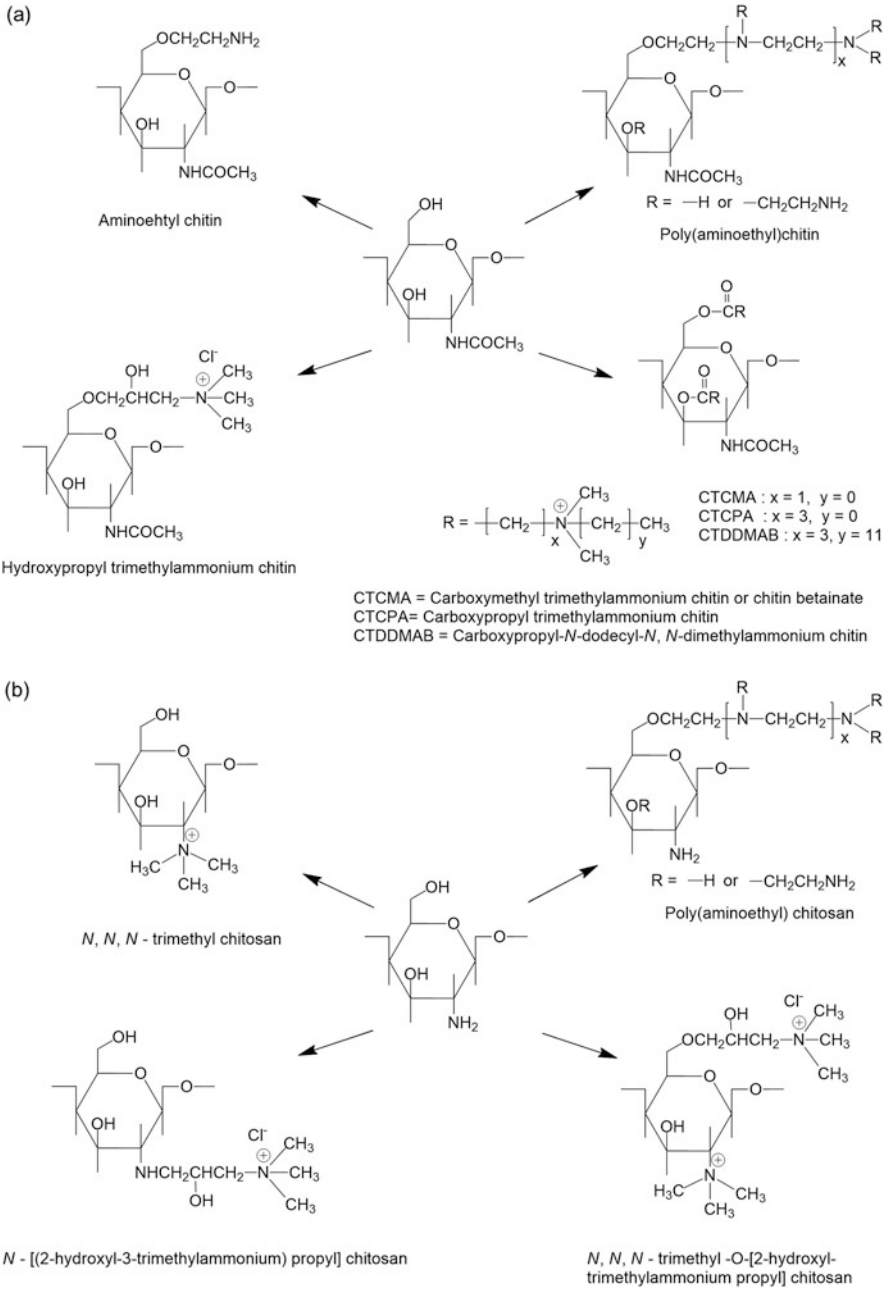

Fig. 8 Representative schemes for the quaternization of (**a**) chitin and (**b**) chitosan derivatives

Apart from the quaternary ammonium derivatives of chitin and chitosan, further information on the synthesis of chitin/chitosan derivatives and their molecular design for novel antimicrobial agents, mode of antimicrobial actions/mechanisms related to outermost membranes of microbes (bacteria, fungi, and viruses), and their compositions, as well as the effect of their MW and DA/DD on the antimicrobial activity has been presented elsewhere [132]. From the development of chitin and chitosan derivatives as antimicrobial agents, it is of great significance to fabricate them into antibacterial hydrogel wound dressings, taking advantage of their beneficial properties, including biodegradability, biocompatibility, non-toxicity, hemostatic, and antibacterial properties. Many molecularly designed chitin and chitosan derivatives appear to be promising candidates for antibacterial wound dressings.

6.3 Wound Dressings for Burn Wounds

The depth of a burn can be described as superficial, moderate partial thickness, deep partial thickness, or full thickness. A first-degree (superficial) burn is when the epidermis is injured with no significant structural damage, while a moderate second-degree (moderate partial-thickness) burn represents a superficial dermis injury. A deep second-degree (deep partial-thickness) burn is when the deep dermis is injured but the hair follicles and sweat glands remain intact, while a third-degree (full-thickness) burn is when the entire dermis is injured. Finally, a fourth-degree (full-thickness) burn is when the entire dermis is injured and it extends to the fat, muscle, and bone [133].

A superficial burn of the skin heals in a short time without a scar by epidermal resurfacing through the regeneration and migration of keratinocytes. A moderate second-degree burn heals within 1–2 weeks by epithelialization, but the skin pigmentation, somehow, changes. In contrast, a deep second-degree burn wound may not heal within 3–4 weeks by epithelialization, and it may form scars. Third- and fourth-degree burn wound healing requires tissue granulation followed by epithelialization. It often undergoes early surgical intervention [133].

Healing of a burn wound is one of the most complex healing process, causing severe discomfort and other complications. For treatment of burns of a high surface area, the patient might face a high risk of lethality and disability during the healing process. Patients are highly vulnerable to invasive microbial infections until complete re-epithelialization or recovery of the wound area has occurred. Consequently, burn wound sepsis is a major cause of mortality among these patients. Topical application of antibacterial ointment is the first choice of treatment in order to provide a moist environment together with antibacterial agents. Since chitosan itself possesses antimicrobial activity, it is particularly useful in wound treatment. The MW and DD of chitosan are structural parameters that influence its physicochemical, mechanical, and biological properties, such as its susceptibility to biodegradation by lysozyme and wound healing properties [134].

The effect of the MW and DD of chitosan on the wound healing process has been evaluated by topically applying three samples of chitosan gels with a different MW and DD on burn wounds in rats [135]. The gels were prepared at a concentration of 2% (w/v) in dilute aqueous acetic acid as CH-H (MW of 200,0000; DD of 92%), CH-M (MW of 750,000; DD of 75%), and CH-L (MW of 70,000; DD of 63%). The high MW (CH-H) gel resulted in the formation of significantly more epithelial tissue and wound contraction (around 80% within 8 days) than the wounds treated with the CH-M or CH-L gels, while the control wounds (no gel treatment) healed very slowly with a wound contraction of only around 40% after 12 days. It is evident that chitosan with a high DD and MW led to an accelerated wound healing and induced GTF or re-epithelialization in the early stages of wound healing [135]. Moreover, the incorporation of EGF at 10 μg/mL into a 2% (w/w) chitosan gel was reported to fasten the epithelialization rate and to stimulate GTF and tissue formation [136]. As known, EGF acts by binding to the EGF receptor–tyrosine kinase, thereby initiating a series of events that ultimately regulates cell proliferation [137]. Although present in small amounts, EGF exerts a powerful influence on the process of wound repair.

Wound dressings usually lead to rapid wound closure by preventing wound sepsis and excessive fluid loss through the open wounds. Therefore, suitable wound dressings should effectively prevent subsequent microbial invasion of the burn wounds as well as absorb excessive exudate. Chitosan acetate bandage (HemCon®), which is normally used as a hemostatic dressing, was applied to third-degree burns in mice infected with fatal doses of two invasive bacterial species (*P. aeruginosa* and *P. mirabilis*) [138]. The survival rates within 4 weeks of infection with *P. aeruginosa* and *P. mirabilis* were 73.3% and 66.7%, respectively, in mice treated with chitosan acetate bandages, whilst they were 13.3% and 0%, respectively, in the untreated (control) groups. The chitosan acetate bandage effectively controlled the growth of bacteria in the burn and prevented the development of systemic sepsis that led to fatality. Therefore, a chitosan acetate bandage could act as an effective topical antimicrobial dressing for infected burns. Furthermore, when *N*-carboxymethyl chitosan (NCMC), a water soluble chitosan derivative, was applied on deep second-degree burn wounds, the healing time of the wound was reduced to 25 days compared to 35 days for the untreated control group [139]. On day 25, a number of fibroblasts in the dermis could be observed in the NCMC treated groups, revealing that GTF was accelerated by the application of the NCMC. The GTF was required for the permanent closure of the wound because it filled the wound defect and prepared a route for epithelialization.

6.4 Wound Dressings for Diabetic Ulcers

Diabetes mellitus (DM), commonly known as diabetes, is a group of metabolic disorders characterized by a high blood sugar level over a long period of time (chronic hyperglycemia) caused by a low insulin level. Diabetes is due to either the pancreas not producing enough insulin or the cells of the body not responding

properly to the produced insulin. The progression of wound healing normally undergoes the four phases of hemostasis, inflammation, proliferation, and remodeling, as mentioned above (Sect. 2). However, wound healing in diabetics is complicated. A failure of completely healed diabetic foot ulcers (DFU) [140] is a consequence of chronic inflammation, peripheral neuropathy, impaired vascular function, or impaired angiogenesis [140, 141]. Moreover, fibroblasts fail to produce ECM proteins, while keratinocytes do not form an epithelium. The DFU is associated with a high risk of limb loss as a consequence of amputation and leads to a reduced survival [142]. To reduce the risk, much attention had been paid to the design and development of wound dressings that are suitable for diabetics. This chapter mentioned in detail on chitin- and chitosan-based wound dressings that have been developed through physical or chemical modification to promote the wound healing process for diabetic ulcers, which are categorized by those impaired consequences.

6.4.1 Wound Dressings Involved in Chronic Inflammation

A DFU is mostly at risk of infection due to the long existence of extensive sloughs, bacteria, and biofilms, which results in a delayed inflammatory phase. Therefore, wound dressings are often incorporated with antibacterial agents to promote their antibacterial activity, even though some polymeric materials, such as chitosan, may already have an antibacterial property. Several physical and chemical modifications of chitin and chitosan wound dressings involved in reducing chronic inflammation are mentioned in this section.

Physical Modification of Chitin and Chitosan Wound Dressings Involved in Chronic Inflammation

Incorporation of AgNPs or Ag^+

Both Ag^+ and AgNPs have been known as an antibacterial agent for a long time. Their toxicity to human cells is significantly lower than that to bacteria, which is the most favorable feature of silver [143], giving it a high availability for various applications, including wound dressing. In diabetes induced rabbits, the AgNP-loaded chitosan-PEG hydrogels were found to exhibit an excellent antibacterial activity against both Gram-positive (*S. aureus*, *B. subtilis*, and *B. pumilus*) and Gram-negative bacteria (*P. aeruginosa* and *E. coli*) [144]. Moreover, the AgNP-loaded chitosan-PEG hydrogels also offered a better wound healing capacity with a greater anti-inflammatory response and accelerated the re-epithelialization and collagen deposition compared to that with the blank hydrogel without AgNPs [144]. The wound healing potential of the AgNP-incorporated chitosan-PEG hydrogel was even faster than the blank hydrogel without AgNPs, indicating that the

combination of AgNPs and chitosan hydrogel significantly and synergistically enhanced wound healing in diabetes induced rabbits.

In addition to the anti-inflammatory function, the incorporation of growth factors into the AgNP-chitosan gel, such as EGF, can enhance keratinocyte and fibroblast proliferation, GTF, and ECM protein synthesis, and so result in promoting the efficiency of wound repair for diabetics. However, the antimicrobial activity of AgNPs may be considerably diminished after binding with proteins, such as growth factors [145]. Such unfavorable conditions likely occur even when Ag^+ and proteins are immobilized in the same matrix, whereby the usability of Ag^+ in the EGF-embedded hydrogel is seriously hindered. An alternative way is to encapsulate EGF into chitosan NPs and then disperse these into a AgNP-incorporated chitosan-PVA hydrogel, i.e. AgNPs and EGF-encapsulated chitosan hydrocolloid in chitosan-PVA hydrogel (SNP_ECHG) [145]. The SNP_ECHG was incorporated with different amounts of AgNPs (reduced from Ag^+ concentrations of 6, 12, 24, and 48 mM) and different concentrations of EGF (0.6, 6, and 60 μg/mL). Owing to the release of Ag^+, the SNP_ECHG exhibited antimicrobial activity against *S. aureus* and *S. epidermidis* when the hydrogel contained more than 24 mM Ag^+, whilst the cytotoxicity of the hydrogels to both fibroblasts and keratinocytes increased in a AgNP concentration-dependent manner. Therefore, for an effective antimicrobial activity and minimal cytotoxicity, the optimal Ag^+ dose in the hydrogel was 24 mM, while the optimal dose of EGF for promoting cell growth was around 60 μg/mL [145]. Furthermore, SNP_ECHG revealed a clear efficiency for diabetic wound healing, which was ascribed to its antimicrobial and cell growth promoting activities compared to treatment with AgNPs alone, EGF alone, or without silver and EGF [145].

Incorporation of Curcumin (Cur)

In addition to AgNPs, Cur, the main bioactive substance of turmeric, has been evaluated for its anti-inflammatory and antioxidant potential [146]. It is well known that macrophages play an important role in regulating the inflammatory response as well as in removing dead cells and cell debris from the wound. The delayed wound healing in diabetes is attributed to the prolonged inflammation, which is always induced by a large amount of inflammatory mediators secreted by macrophages. Curcumin-loaded chitosan NPs (Cur-CS-NPs) could effectively inhibit macrophage-mediated inflammation and reduce the release of inflammatory factors from the RAW264.7 mouse mononuclear macrophage leukemia cell line and attenuated local inflammation in the wound site of diabetic rats, thereby promoting the wound healing process to shift from the inflammatory phase to the proliferation and remodeling phases [147]. Furthermore, Cur-CS-NPs enhanced angiogenesis by promoting proliferation and migration of HUVECs as well as to the diabetic wound site [147]. Moreover, Cur-CS-NPs accelerated the wound closure rates, revealing that it significantly promoted wound contraction and accelerated wound healing.

Chemical Modification of Chitin and Chitosan Wound Dressings Involved in Chronic Inflammation

The addition of antimicrobial substances is straightforward to the specific purposes of developing anti-inflammatory wound dressings. However, as mentioned before, dysfunctional macrophages can induce chronic inflammation and impair tissue regeneration in diabetic wounds. Therefore, improving macrophage behaviors and functions may enhance the therapeutic effects of diabetic wound healing [148]. The incorporation of exogenous cytokines to improve macrophage behaviors is, however, subject to the instability of cytokines, their high cost, and uncertain dosage. To improve the macrophage response, the design of a functional biomaterial without the addition of cytokines is of interesting in diabetic wound healing.

Sulfated chitosan (SCS), a heparin-like substance, was crosslinked with collagen type I (Col I/SCS) and evaluated for its activity on cultured peritoneal macrophage (PM) in terms of the inflammatory response [148]. Macrophages polarize their functional phenotypes from pro-inflammatory (M1) to anti-inflammatory (M2) and provide different functions. Pro-inflammation macrophages (M1) remove pathogens and tissue debris and secrete pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β), ROS, and proteases, while the anti-inflammation macrophages (M2) secrete growth factors (e.g., FGF, EGF, and VEGF) and anti-inflammatory cytokines (e.g. IL-4 and IL-10) to resolve inflammation and stimulate tissue regeneration. It was found that Col I/SCS hydrogel reduced the expression of IL-6 and increased the expression of IL-4, IL-10, and TGF-β1 in macrophages, i.e. a Col I/SCS hydrogel promoted the secretion of anti-inflammatory cytokines and reduced the secretion of pro-inflammatory cytokines in macrophages [148], implying it could improve the shift of wound healing phases. Furthermore, the Col I/SCS hydrogel promoted the transdifferentiation of macrophages into fibroblasts, which enhanced collagen deposition and accelerated wound contraction in diabetic wounds.

6.4.2 Wound Dressings Involved in Neuroactive Substances

Incorporation of Neuropeptides

It is evident that peripheral nerves and cutaneous neurobiology contribute to diabetic wound healing [149], where a loss of peripheral sensory and autonomic nerves along with diminished neuropeptide production precedes the clinical symptoms of neuropathy [149]. Peripheral sensory neuropathy is considered to be a major contributor to the increased risk of foot amputations. Therefore, neuropeptides are an important link that directly connects neuropathy to wound healing. Neurotensin (NT) are bioactive neuropeptides that are widely distributed in the brain and in several peripheral tissues. They are involved in the activation, growth, migration, and maturation of specific skin cells, such as keratinocytes, macrophages, and mast cells, and affect new vessel formation and enhance angiogenesis during wound healing [141]. However, local administration of neuropeptides has the problem of

their short half-life and loss of bioactivity in the wound environment. Therefore, an alternative way is to load NT into chitosan-based wound dressings for their sustained delivery [150].

A water soluble chitosan derivative of 5-methyl pyrrolidinone chitosan (MPC) was synthesized and used as an NT carrier in wound dressing. A different healing profile was observed different for the non-loaded and NT-loaded MPC treated wounds in both the control (normal) and diabetic mice. Interestingly, wound reduction was evidently observed at around 20 and 40% on day 1, and at around 50 and 70% on day 10 post-wounding with MPC and NT-loaded MPC treatment in diabetic mice, respectively, indicating that an NT-loaded MPC was significantly more effective than MCP treatment alone. Moreover, granulation tissue filled the wound bed quicker in the early phase of wound healing in diabetic mice treated with NT and/or MPC. These phenomena indicated that NT-loaded MPC wound dressing improved the early wound healing in diabetics. Histopathological observation of the wound on day 10 revealed specific re-epithelialization profiles from the bottom to the top with basal cells in the epidermis covering the scar in control mice, whilst that occurred over the granulation inflammatory tissue which was undergoing repair [150].

Incorporation of Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine; Mel), the main hormone released from the pineal gland release, possesses widespread neuroprotective, antioxidant, and anti-inflammatory properties [151]. Its neuroprotective property makes it beneficial for treatment in neurological diseases, such as stroke, Alzheimer's disease, and Parkinson's disease [152]. Its antioxidant and anti-inflammatory properties, together with its neuroprotective property, make Mel an interesting potential therapeutic agent for application in wound healing related to the peripheral neuropathy of diabetic wounds. However, Mel is highly susceptible to oxidation, and so encapsulation of Mel into polymeric NPs is used to prevent its degradation. Therefore, Mel was loaded in lecithin-chitosan NPs (Mel-NP) [153]. The volume of the formulations was calculated according to the individual daily weight and locally dropped on the wound of diabetic rats at a Mel dose of 1.2 mg/kg rats/day. The wounds of the Mel-NP and NP alone treated groups were almost closed on day 7 with significant wound reduction on day 14, compared to the free Mel treated groups. In addition, NP alone stimulated fibroblast proliferation, whilst Mel-NP accelerated collagen deposition and blood vessel proliferation [153].

Another study of Mel loaded in a chitosan hydrogel also supported that Mel-loaded hydrogels promoted angiogenesis in the early stage of wound healing and reduced inflammation in the late stage [154]. The Mel-loaded hydrogel also markedly increased the expression of collagen III, α-smooth muscle actin and TGF-β1 proteins and reduced collagen I expression. These results suggested that Mel-loaded hydrogel promoted GTF and accelerated wound healing by reducing inflammation and promoting angiogenesis and collagen deposition [154].

Diabetes or hyperglycemia increases the levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, and increases oxidative stress, which mainly cause an impaired wound healing. Excessive production of ROS results in damage to cellular membranes, lipids, proteins, and DNA as well as becoming harmful to wound healing [151]. Melatonin down-regulates a variety of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, by preventing the translocation of nuclear factor-kappa B to the nucleus and its binding to DNA, as well as reducing oxidative stress [155]. Moreover, the molecular mechanisms of Mel action on diabetic wound healing had been examined in high glucose (HG) cultured keratinocytes [151]. It was found that (1) Mel reduced the mRNA expression level and release of pro-inflammatory cytokine in HG cultured keratinocytes, (2) reduced HG-induced oxidative stress in keratinocytes, (3) inhibited HG-induced activation of nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing three inflammasome, and (4) enhanced proliferation and migration as well as reduced apoptosis of keratinocytes. Therefore, Mel contributes to diabetic wound healing, including via inflammation suppression, oxidative stress attenuation, proliferation, migration promotion, and apoptosis inhibition.

6.4.3 Wound Dressings Involved in Angiogenesis

Incorporation of Bioactive Substances

Angiogenesis, the main step in the proliferative phase of wound healing, involves the formation of new blood vessels into the wound site and provides nutrition for wound healing [156]. Wound dressings can be used to deliver bioactive agents to wound sites for promoting epithelialization and treatment of severe injuries. To this purpose of promoting angiogenesis and wound healing, several research works have been reported to incorporate bioactive substances into the chitin or chitosan matrix. The analysis of non-healing acute or chronic wounds in vivo and in vitro demonstrated that those wounds were de-regulated for various growth factors, such as PDGF, VEGF, and basic (b)FGF [157]. Therefore, using exogenous growth factors and cytokines suggests a potential therapy for non-healing wounds, such as DFUs, pressure ulcers, and chronic venous leg ulcers.

Basic FGF, released by platelets, is a potent modulator for fibroblasts and the proliferation and angiogenesis of vascular endothelial cells [157]. The application of bFGF solution onto the wound, however, cannot accelerate wound healing because the bFGF is rapidly washed off at the wound site by the exudate. The incorporation of bFGF into chitosan-based wound dressings is an alternative way to stabilize and control the release of growth factors [158]. A bFGF was mixed with hydroxypropyl chitosan (HPCH) acetate, and cast as 1×1 cm^2 films with total bFGF concentrations of 0.6, 2, and 6 μg/film. The bFGF-HPCH films with different bFGF concentrations and HPCH film were placed onto full-thickness wounds excised on the back of diabetic mice. Normal saline was used in the control group. The wound sizes in the HPCH film and control groups were similar, indicating no significant acceleration of

impaired wound healing by the application of the HPCH film alone [158]. On the other hand, bFGF-HPCH films accelerated the wound healing in a bFGF dose-dependent manner. The wound sizes in the bFGF-HPCH film groups (2 μg and 6 μg/film) were significantly smaller than those in the three other groups, which indicated that the bFGF-HPCH film provided a sustained release of bFGF and accelerated wound healing in genetically diabetic mice [158].

However, the growth factors absorbed in a chitosan matrix are diffusible in vivo. The bFGF mostly caused a burst release with a low long-term accumulation at the wound sites. In order to maintain the bFGF concentration in the therapeutic range, a high dose administration is required. However, a high concentration of bFGF will lead to a vascular tumor and cancer angiogenesis [159]. To this end, bFGF immobilized onto the chitin binding domain (ChtBD) was made and named ChtBD-bFGF [160]. The ChtBD was derived from the chitinase of *Bacillus circulans* WL-12 and the ChtBD-bFGF or bFGF were absorbed onto the surface of chitin films by immersion. The amount of ChtBD-bFGF absorbed on the surface of the chitin film was determined by immunofluorescence and found to be 3.02-fold higher than that of bFGF. On the other hand, the ChtBD-bFGF showed a sustained release manner from the chitin film, whilst bFGF without ChtBD immobilization had a high burst release. The chitin films bound with ChtBD-bFGF or bFGF were subsequently evaluated for their ability in wound healing in terms of cell proliferation and promoting vascularity. It was found that the number of fibroblast cells induced by ChtBD-bFGF was twofold higher than by bFGF with a regular cell morphology, and an orderly and parallel actin filament. Furthermore, the endothelial cell-specific protein marker (CD31) was used to evaluate neovascularization at the implanted site. No obvious neovascularization was observed at the implanted site of the bFGF chitin film or chitin film alone, but was highly observed at the implanted site of the ChtBD-bFGF chitin film, indicating the promotion of angiogenesis [160].

The impaired wound healing of diabetic wounds is attributed to the low levels of endogenous growth factors, including VEGF, that normally stimulate multiple phases of wound healing. Besides, chronic diabetic wounds exhibit high levels of matrix metalloproteinases and give rise to the degradation of growth factors [161]. Therefore, attention had been paid to the incorporation of VEGF-loaded NPs to a chitosan-hyaluronic acid composite sponge, which served as a growth factor releasing wound dressing [162]. The released VEGF was sufficient to enhance tube formation in endothelial cells (HUVECs) in vitro, whilst the chitosan-hyaluronic acid sponge did not show any such property. The mechanism by which VEGF induced angiogenesis involves activation of integrins, which is essential for endothelial cell proliferation [162].

Moreover, as multiple proteins are involved in the wound healing process, it might be insufficient to use a single growth factor to accelerate wound closure in diabetic ulcers. While VEGF induces endothelial cell proliferation and migration, bFGF is a potent mitogen for fibroblasts and keratinocytes, and is involved in the proliferative phase of wound repair. It has been demonstrated that combined gene transfers of VEGF and FGF improves the reparative processes in the wounded skin of diabetic mice better than the single-agent treatment [163].

Clinical treatment of applying mixed growth factors from an autologous platelet-rich plasma gel (PRP) combined with a porous chitosan-alginate membrane to venous chronic ulcers has been reported [164]. Various proteins necessary for tissue repair and healing process are secreted by three types of granules (alpha, delta, and lambda) located inside the platelets. Alpha-granules are the most abundant platelet granule, and their degranulation releases several growth factors, such as PDGF, PDEGF, VEGF, TGF-β, EGF, IGF, and other bioactive substances, such as fibrinogen, fibronectin, osteocalcin, vitronectin, IL-1, and thrombospondin-1 [34]. The combination of various growth factors within the PRP gel and chitosan-alginate membrane gave the effective results of GTF and angiogenesis [164]. One of the most favorable results was the pain reduction in wounds from the early treatment, which was probably due to the acceleration of wound healing that resulted in a decreased analgesic intake by the patients. Owing to its high swelling capability, the exudate released was well absorbed into porous membrane and contributed significantly to the reduction in the intensity of a bad smell [164].

7 Conclusion

Chitin and chitosan both have a high potential for wound healing applications because of their prominent properties, such as exudates absorbability, stimulating hemostasis, and accelerating tissue regeneration. They can be fabricated into various forms, such as hydrogels, membranes, fibrous mats, sponges, and hydrocolloids, making chitin and chitosan promising materials for wound dressings and being commercially available. Beyond the fundamental requirements of non-toxic, non-allergenic, and non-adherent, many functional wound dressings of chitin and chitosan have been designed to provide certain other functional properties suitable for different wound types.

In order to design effective chitin and chitosan wound dressings, this chapter provided an insight information into the wound healing process and factors effecting wound healing, the fabrication methods of chitin and chitosan wound dressings, commercially available chitin- and chitosan-based wound dressings as well as the physical and chemical modification of chitin and chitosan to make more functional wound dressings, such as hemostatic, antimicrobial, burn, and diabetic ulcer wound dressings.

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Preparation of Chitin Nanofiber and Its Derivatives from Crab Shell and Their Efficient Biological Properties

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Contents

Abstract Chitin nanofibers were prepared from crab shell for the purpose of utilizing crab shells. After the series chemical treatment and wet pulverization treatment, a uniform fibrous substance having a width of about 10 nm was obtained. The reason why fine fibrous chitin can be obtained is the structure of the crab shell. A characteristic of chitin nanofibers is their high dispersibility in water. Therefore,

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processing ability is improved, and biological properties of the nanofibers can be evaluated. Chitin nanofibers whose surface is modified to chitosan can be obtained after treatment with a relatively medium concentration of sodium hydroxide. Since chitosan nanofibers have an amino group on the surface, they are positively charged in an acidic aqueous solution. Chitin and chitosan nanofibers have various physiological functions when taken or applied to the skin. Effects of oral ingestion of chitosan nanofibers on chronic kidney disease (CKD) model, non-alcoholic steatohepatitis model, and inflammatory bowel disease model were studied. Furthermore, the biological effects of chitin and chitosan nanofibers for the skin are also investigated.

Keywords Atopic dermatitis · Chitin nanofiber · Chitosan nanofiber · Chronic renal failure · Hair growth · Inflammatory bowel disease · Non-alcoholic steatohepatitis · Wound healing

1 Introduction

Tottori Prefecture has the largest landing volume of crab in Japan, and Matsuba crab (snow crab) are known as a special product. Every year, when the landing season started in November, the crab attracts many tourists. “Itsuki Boshi,” the selected premium brand of Matsuba crab, which is less than 1.5% of the total, meets the five top criteria such as size and weight. The premium crab fetched the highest price of five million yen per crab at the year’s first auction held in 2019 in Tottori, and was recognized by the Guinness World Records as the world’s most expensive crab. Sakaiminato city, located at the western end of Tottori Prefecture, is known as one of Japan’s leading red snow crab and snow crab landing bases. Leg of red snow crab is mainly used as a frozen food after taking out meat part from crab leg with shell. Therefore, in Sakaiminato city, a large amount of crab shells is generated as food residue throughout the year. Crab shells generated from marine product companies are not mixed with other food residues, so clean waste shells can be obtained. Therefore, we started the study about utilization of crab shells, which is an unused resource, by taking advantage of the land of Tottori. That is, we are making efforts to convert chitin, which is the main component of crab shells, into nanofibers and effectively use it as a functional raw material. In this book chapter, preparation of chitin nanofiber and its derivative and efficient biological properties by oral administration and application on skin are described.

2 Novel Materials from Crab Shell: Chitin Nanofibers

2.1 Preparation of Chitin Nanofibers from Crab Shell

Chitin is a long-chain polysaccharide consisting of *N*-acetylglucosamine repeating units, linked by 1,4 beta glycosidic bonds. Since cellulose is a polysaccharide consisting of glucose units, its structures are very similar to each other. Chitin is a primary component not only of the exoskeleton of crustaceans or insects, but also of the squid pens, and in the cell walls in fungi and mushroom. Therefore, these organisms mainly produce and use chitin as a structural material that supports their skeleton. The annual synthetic amount of chitin is estimated to be 1×10^{11}t [1], which is comparable to cellulose and is the most abundant biomass on the earth. However, while cellulose is stored in large quantities in nature as a tree, organisms producing chitin have a short lifespan, and when those organisms die, the chitins are rapidly enzymatically decomposed by microorganisms, so the storage amount of chitin is smaller than that of cellulose. The weight ratio of chitin contained in crab shells accounts for about 20% or more, but varies depending on the parts or species. Since the non-edible part is about 70% of the body weight of the crab, it is estimated that about 10% of the body weight is chitin. The raw material of chitin for industrial use is mainly crab and shrimp shells generated from food processing plants. In addition to chitin, the shell contains inorganic salts of $CaCO_3$ as the main component, proteins, pigments, and lipids. These coexisting substances can generally be removed by aqueous hydrochloric acid, hot aqueous sodium hydroxide, alcohol extraction, or chlorine bleaching, respectively. Since the protein called tropomyosin, which is a causative substance of crustacean allergy, is derived from crab muscle fiber, the crab shell protein is not an allergen. The phenomenon that crabs turn red when boiled is due to the release of astaxanthin, a type of carotenoid, from proteins. By a series of these extraction processes, white high-purity chitin can be obtained (Fig. 1). On the other hand, wood-derived cellulose was mainly prepared by the pulp

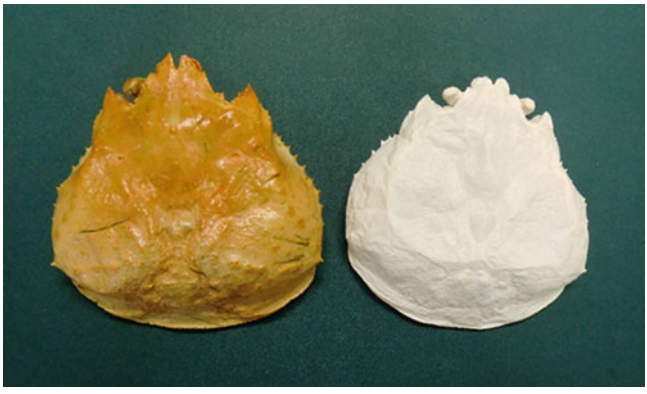

Fig. 1 Crab shell (left) and chitin purified from crab shell (right)

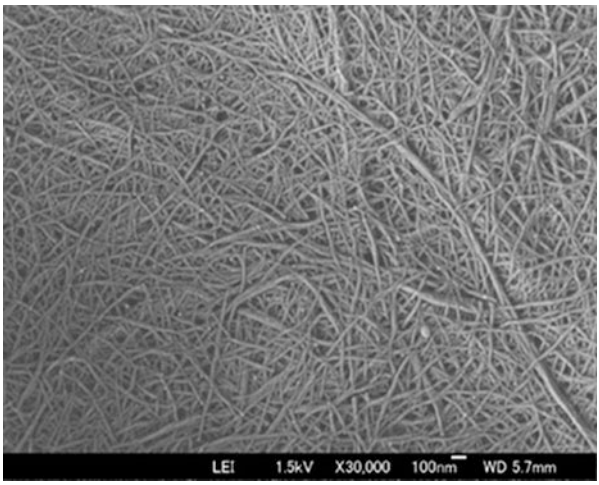

Fig. 2 Fine chitin nanofibers prepared from crab shells

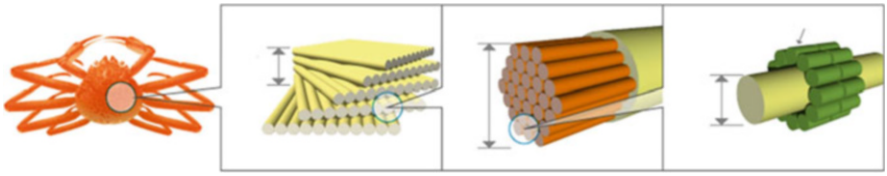

Fig. 3 Hierarchical structure of crab shell

manufacturing method, but amorphous polysaccharide called hemicellulose remains to some extent, which affects the dispersibility of cellulose nanofibers.

Chitin nanofibers are obtained by adding a large amount of water to chitin and mechanical treatment. Therefore, like cellulose nanofibers, chitin nanofibers are usually obtained as a dilute aqueous dispersion of a few percent concentration. After the wet pulverization treatment, a uniform fibrous substance having a width of about 10 nm can be observed (Fig. 2) [2].

The reason why fine fibrous chitin can be obtained by mechanical grinding is the structure of the crab shell (Fig. 3). Chitin molecules are synthesized by enzymes and self-assemble to form the so-called antiparallel chain crystalline nanofibers in which reducing ends are alternately arranged (α-chitin). Chitin nanofibers bind to proteins on their surface to form a complex. Furthermore, the complex is stacked so as to spontaneously spiral. Calcium carbonate crystallizes and fills the gap [3]. It is considered that calcium carbonate plays a role of a filler that gives elasticity to the crab shell, and protein plays a role of a nucleating agent that promotes the precipitation of calcium carbonate crystals. When calcium is removed, the shells lost their support and are easily disintegrated into chitin nanofibers by grinding.

Various wet crushers can be used in the production of nanofibers. We have confirmed that nanofibers can be obtained using a stone mill type grinder, cone mill, ball mill, high-pressure homogenizer [4], high-speed blender [5], ultrasonic

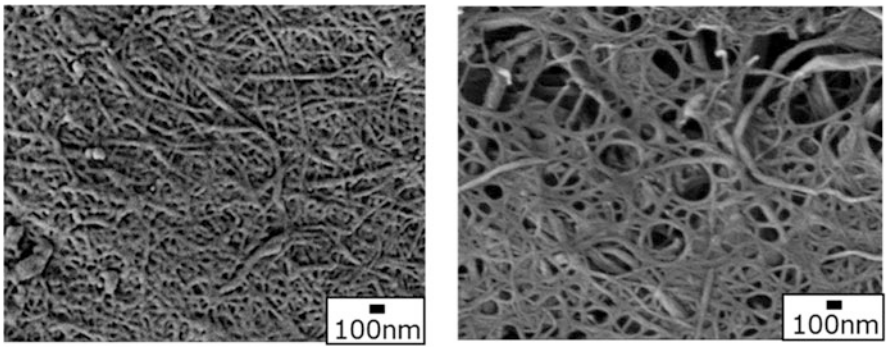

Fig. 4 Chitin nanofibers prepared from (left) cicada shells and (right) silkworm pupa

crusher, etc. Since the crushing mechanism is different for each, there are differences in the shape such as the length and thickness of the obtained nanofibers and their distribution. Care must be taken because such differences affect physical properties such as viscosity, transparency, and dispersibility. Innumerable fine fibers are dispersed in a limited space and have physical and chemical interactions between the fibers, so that they have high viscosity even at a low concentration. A characteristic of chitin nanofibers is their high dispersibility in water. The high viscosity, transparent to translucent appearance suggests that finer fibers than visible light are homogeneously dispersed [4]. Therefore, chitin nanofibers can be processed into a film [6] or sponge depending on the application. In addition, it can be blended with commercially available cosmetics or foods to make a prototype. Furthermore, it can be evaluated in vitro or in vivo to explore physiological functions. Although chitin is abundant biomass in nature, it is mainly used as an intermediate of chitosan obtained by deacetylation and glucosamine obtained by hydrolysis, and has a track record of industrial use of chitin itself as wound covering materials, sutures, and hemostatic agents. The reason why there are only some medical devices is that they dissolve only in a special solvent due to strong hydrogen bonds between molecules due to acetamido groups and hydroxyl groups and dipole interactions, and their workability is poor. Since chitin nanofibers are uniformly dispersed in water, the moldability and processability are improved. These characteristics of chitin nanofibers are important to promote the practical use of chitin. Chitin nanofibers can also be obtained from raw materials other than crab shells. This is because all-natural chitins exist as aggregates of nanofibers. For example, nanofibers are obtained from shrimp shells [7] and mushrooms [8]. Since shrimp shells are generated in large quantities as food residues likewise crab shells, chitin derived from shrimp shells is commercially available and can be used as a raw material for nanofibers. In addition, mushrooms are also cultivated in large quantities, and non-standard products that cannot be commercialized may be used as raw materials. Chitin nanofibers are also obtained from cicada shells and silkworm pupa (Fig. 4). If the characteristics of chitin nanofibers of different origins are clarified, it will be useful academically and industrially.

2.2 Preparation of Partially Deacetylated Chitin Nanofibers

Chitosan is a deacetylated derivative of chitin prepared by heating in a concentrated alkaline aqueous solution. That is, chitosan is a polysaccharide consisting of D-glucosamine units, but usually also contains *N*-acetylglucosamine units. The ratio of the two constituent units is expressed by the "deacetylation degree," but it should be noted that there is no clear definition of the deacetylation degree that distinguishes between chitin and chitosan. Chitosan is soluble in hydrochloric acid and many aqueous organic acids. This is because the amino groups are neutralized with acid to form salts. Generally, a high-concentration sodium hydroxide aqueous solution is used for producing chitosan. For example, when heat-treated with 40% sodium hydroxide, the degree of deacetylation reaches approximately 70–95%. This is because sodium hydroxide converts the hydroxyl group of chitin into an alkoxide (-ONa), swells strong chitin crystals, and promotes a deacetylation reaction (alkali chitin). On the other hand, when chitin is treated with a relatively medium concentration of sodium hydroxide, the chitin crystals do not swell, so the surface of the pulverized product is partially deacetylated, but the chitin crystals inside are retained. Therefore, chitin nanofibers whose surface is modified to chitosan can be obtained [9]. The partially deacetylated chitin nanofibers are called Chitosan Nanofibers in this chapter. Since chitosan nanofibers have an amino group on the surface, they are positively charged in an acidic aqueous solution, so that they have an electrostatic repulsive force, promote solvation with water molecules, and have an osmotic effect to dilute the salt concentration. As a result, the interaction between the nanofibers is weakened, the dispersibility is improved, and the efficiency of grinding is improved. In addition, the same properties as chitosan (antifungal [10] and diet effects [11]) have been clarified.

3 Application of Chitosan Nanofibers to Medical Fields by Oral Administration

Chitosan has been used as a supplement for reduction of the absorption of bile acids and several lipids. Recently, we also suggested that chitosan possesses an antioxidative potential and anti-lipidemic action in metabolic patients or metabolic rats [12, 13]. Further, our studies have reported the antioxidative potential as well as anti-uremic effects of chitosan in renal failure model rats or hemodialysis patients [14, 15]. However, the treatment of chitosan nanofibers has not reported in several oxidative stress related disease such as renal failure and metabolic syndrome, although chitosan nanofibers have been attracting a lot of attention. In this section, we introduce the in vivo antioxidant and related factors of chitosan nanofibers treatment established from oxidative stress related diseases using animal models.

3.1 *Effects of Chitosan Nanofibers on Chronic Kidney Diseases (CKD) Model Rats*

CKD has been a global health problem which often required kidney replacement therapy since it can become a cause of the progression of cardiovascular and end-stage renal failure [16]. The development of CKD is associated with the increment of some uremic toxins with detrimental effects, but the mechanism of this syndrome is not fully clear. For instance, in CKD patients, uremic solutes bound to proteins such as indoxyl sulfate (IS) accumulate in blood and kidney [17, 18]. In general, uremic toxins are known to damage renal tubules and endothelium by the increment of oxidative stress [19, 20]. An oral carbon adsorbent, Kremezin® is normally used for patients of pre-dialysis during the uremic stage in CKD. The adsorption of indole which is a precursor substance of IS was by Kremezin® in gut, leads to delay the progression of CKD and the initiation of dialysis treatment [21, 22]. Further, the antioxidative potential of Kremezin® in blood of CKD model rats has led to reduce CKD-related oxidative stress [23]. However, Kremezin® is usually applied for a short period of time in pre-dialysis patients. Furthermore, the dosage form using activated charcoal for Kremezin® was very difficult to take for many CKD patients, so that many patients have frequent non-compliance [24].

In our previous studies, the effects of a commercial chitosan supplement on oxidative stress and the renal petameters in CKD using CKD model rats were investigated. Oral administration of chitosan supplement for 4 weeks showed a significant reduction in renal parameters and oxidative stress, compared to the non-treated group [14]. The effects of a commercial chitosan supplement ingestion on renal factors and oxidative injury in hemodialysis patients were also investigated. Oral administration of chitosan supplement for 12 weeks showed a significant reduction of phosphate levels, IS, and oxidative stress in blood [15]. On the other hand, after the end of chitosan ingestion, an increase in blood IS concentration was confirmed. In order to take a continuous ingestion of chitosan, it is indispensable to improve the dosage form and intake method of chitosan that can be taken for a long period of time.

Recently, chitosan nanofibers, a safe naturally-occurring cationic polysaccharide, have been attracting a lot of attention in various medical fields, because of their multi-functional activities [25, 26]. The surface amino groups of chitosan nanofibers can create electrostatic interactions with an anionic charge of another component. Thus, high dispersibility would allow chitosan nanofibers to be applied for an oral adsorbent at a lower dosage than Kremezin® or non-fibered deacetylated chitin (DAC). We also showed that the binding ratio of indole to the chitosan nanofibers was almost same as Kremezin® treatment, and this ratio was higher than that for DAC in in vitro studies (Fig. 5).

Interestingly, this phenomenon could not observe the other polysaccharides such as cellulose or its nanofiber. Therefore, these results suggested that the smaller dose of chitosan nanofibers treatment in CKD model rats specifically increased the antioxidative potential and reduced CKD progression more effectively than

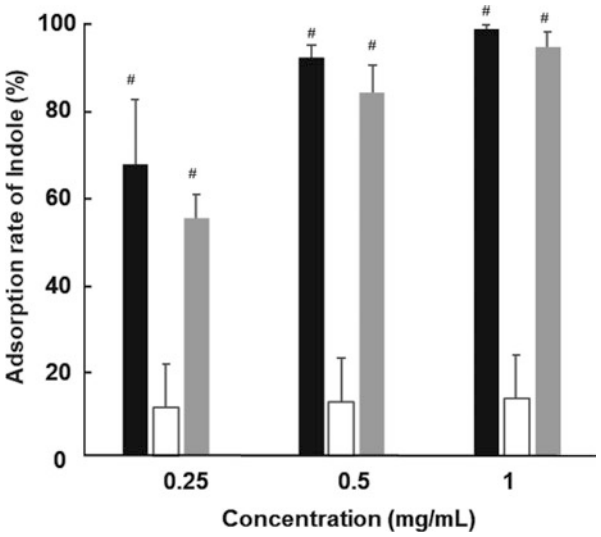

Fig. 5 Binding capacity on indole for chitosan nanofibers, deacetylated chitin, and Kremezin®. Results are shown for chitosan nanofibers (■), deacetylated chitin (□), and Kremezin® (■) [27]

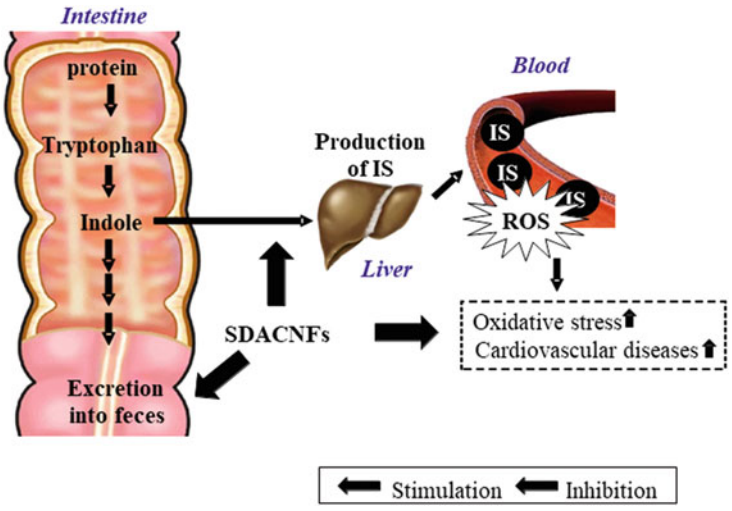

Fig. 6 The possible inhibition mechanism of chitosan nanofibers for CKD

Kremezin® or DAC [27]. Thus, oral administration of chitosan nanofibers shows a significant decrease in uremic toxins as a pro-oxidant in the gut, thereby reducing the subsequent progression of oxidative stress in blood (Fig. 6).

Further, some reports suggested that chitosan nanofibers ingestion enhances the levels of 5-HT and ATP in plasma via the intestinal flora activation [11, 28], so that the anti-lipidemic effects of chitosan nanofibers ingestion might be due to reform of the population of gut microbiota. In general, probiotics and prebiotics are believed to be powerful agents that reduce the uremic toxins in CKD rats, because gut

microbiota metabolize amino acids uremic toxins as a precursor substance. Further, some studies suggested that chitosan nanofibers as a probiotic can show renal protective effects in CKD rats by reducing the systemic inflammation [29]. Although the bio-availability of chitosan nanofibers after oral ingestion has not been fully examined, chitosan was shown to be excreted from the body within 4 h [30]. Therefore, in the future, chitosan nanofibers might be co-administrated with Kremezin® as a new strategy for antioxidative therapy, since the antioxidative mechanism of chitosan nanofibers is different from typical antioxidants such as ascorbic acids.

3.2 Effects of Chitosan Nanofibers on Non-alcoholic Steatohepatitis (NASH) Model Rats

The one of common chronic liver diseases, non-alcoholic fatty liver disease (NAFLD), has been defined by fat accumulation in the absence of high alcohol consumption [31]. NASH as a serious disease of NAFLD has been a worldwide health problem due to overeating and reduced physical exertion, but the pathophysiology of NASH is not fully elucidated. On the other hand, the major trigger of pathogenic mechanism of NASH seems to be associated with cooperative factors throughout hepatic injury, obesity, oxidative stress, and the gut microbiota [31–33]. Therefore, the inhibitory effects of NASH by treatment of chitosan nanofibers were recently examined SHRSP5/Dmcr rat as a NASH model that is similar to NASH patients for 8 weeks.

The administration of chitosan nanofibers showed a significant reduction of hepatic injury, compared to the non-treated group. The mechanism for anti-hepatic injury might be due to the reduction of certain lipids absorption in the gut by chitosan nanofibers because it has high barrier and viscosity for the mucosal membrane of the gut. For instance, the decrease in the absorption of triglycerides by chitosan nanofibers treatment for a corn oil-loading test of rats was observed in our recent studies. Thus, chitosan nanofibers have the adsorption effect of certain lipids in the gut, thereby reducing the increment of plasma and hepatic factors due to the development of NASH. In base of this hypothesis, chitosan nanofibers ingestion also showed a decrease in the levels of TNF-α and TGF-β_1 in hepatic tissue, thereby decreasing the fibrosis at 8 weeks (Fig. 7). Until recently, the progression of oxidative stress was also thought to be one of important factors of NASH. Thus, a significant reduction of oxidative stress in the systemic circulation suggests that oral administration of chitosan nanofibers causes a significant reduction of lipid substances as pro-oxidants in the gut, thereby reducing the subsequent oxidation in blood.

The change of intestinal flora is also a major risk factor for NASH [34, 35]. The intestinal flora in normal rats was dominated by major phyla such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia*. An increment of *Fusobacteria* and *Firmicutes* and a decrease in *Proteobacteria* and *Bacteroidetes* were observed in the

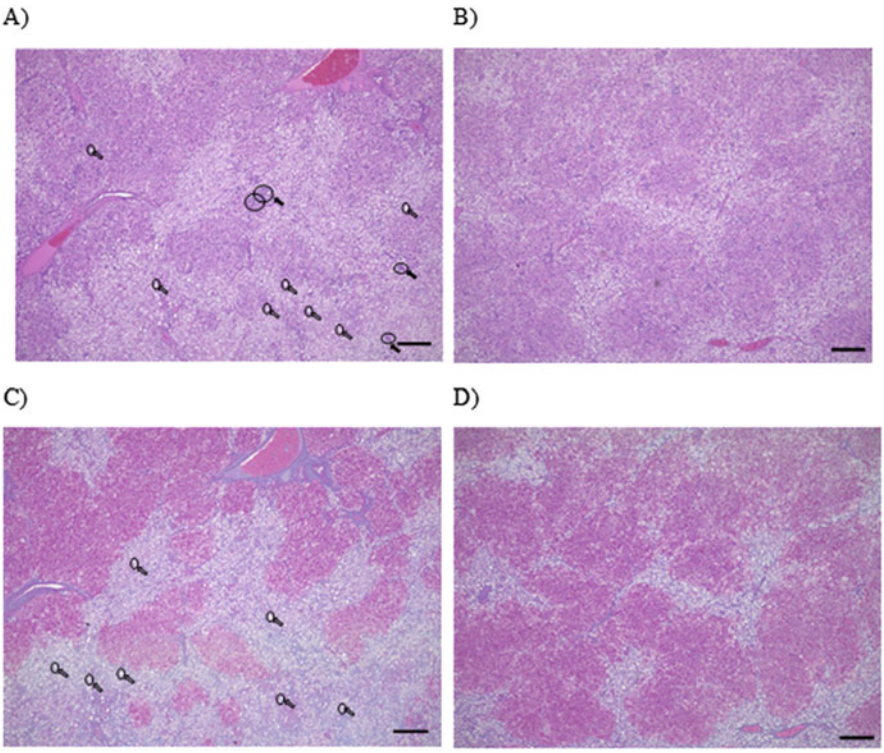

Fig. 7 Histopathological changes of livers in NASH model rats with or without chitosan nanofibers treatment. (**a**) HE (non-treatment). (**b**) HE (with SDACNFs). (**c**) AZAN (non-treatment). (**d**) AZAN (with SDACNFs) [41]

intestinal flora of the chitosan nanofibers ingestion, compared with that of the non-treatment, suggesting that chitosan nanofibers ingestion induced a lower *Bacteroidetes* to *Firmicutes* (B/F) ratio (Fig. 8a). Some reports have suggested that the B/F ratio in NASH subjects is higher than that of healthy subjects [36, 37]. In the genus level, the abundance of *Prevotella* was significantly decreased by a chitosan nanofibers ingestion (Fig. 8b, c). On another front, the genus *Blautia* was significantly enhanced by the chitosan nanofibers ingestion (Fig. 8c). In previous studies, the abundance of *Blautia* is also shown to be higher in healthy subjects than that of NASH patients. Further, the increment of *Blautia* decreases the inflammation and enhances intestinal peristalsis [37–39].

A positive relationship between Liver/Total weight ratio and the number of *Blautia* was also observed. Thus, *Blautia* might be a significant genus, which is associated with visceral fat accumulation. The genus *Prevotella* were lower in healthy individuals than in arthritis patients with high inflammatory [40]. These results suggested that the reconstruction of genus such as *Prevotella* and *Blautia* in the gut by chitosan nanofibers ingestion might induce the cooperative effects during anti-inflammatory, anti-hepatic, and antioxidant potential in NASH model. We

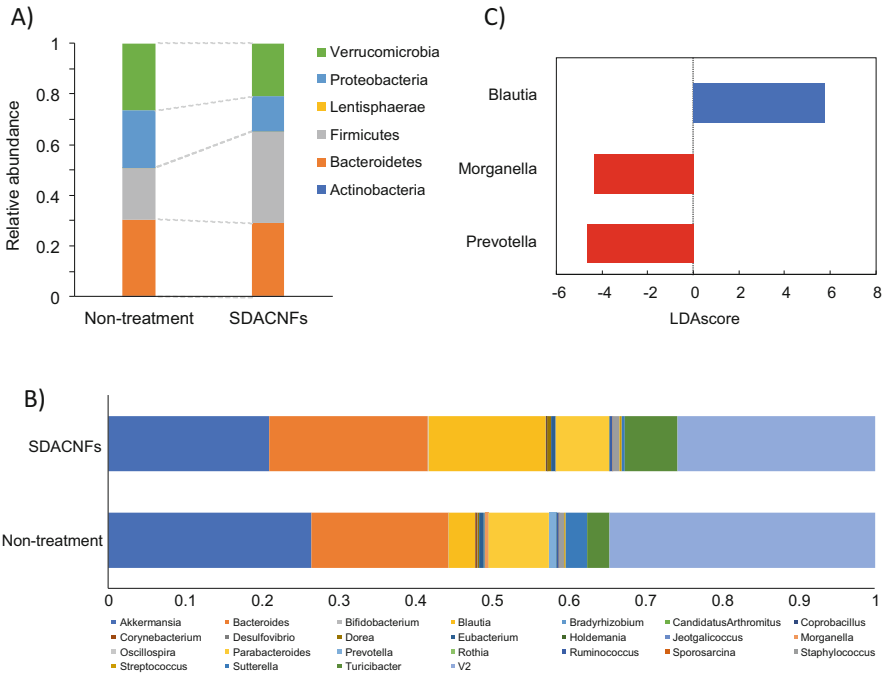

Fig. 8 Effect of chitosan nanofibers on the intestinal microbiota in NASH model. (**a**) Phylum level. (**b**) Genus level. (**c**) Significant differences in LDA scores [41]

therefore conclude that chitosan nanofibers exert anti-hepatic and antioxidant potential not only by adsorbing certain lipids but also by reconstructing the community of intestinal flora in the gut [41].

3.3 Applications for Inflammatory Bowel Disease Using the Combination Between Chitosan Nanofibers and Sulfobutyl Ether β-Cyclodextrin

The surface amino groups of chitosan nanofibers can create electrostatic interactions with an anionic charge of another component. Cyclodextrins (CD) usually form inclusion complexes with various molecules, and such compounds have been improved the pharmaceutical properties of drugs. In the various CDs, sulfobutyl ether β-CD (SBE-β-CD), as a clinical additive, was shown to form a rigid and non-fluid elastic gel, while other gels prepared from neutral CDs formed weak, rather fluid gels [42–44]. Further, we prepared the NFs-CDs elastic gel that included prednisolone (PD/NFs-CD gels) and examined the therapeutic effect of this gel on a dextran sulfate sodium (DSS)-induced colitis model mouse. As a result, the potential for using PD/NFs-CD gels for therapy in DSS-induced colitis was found to be as

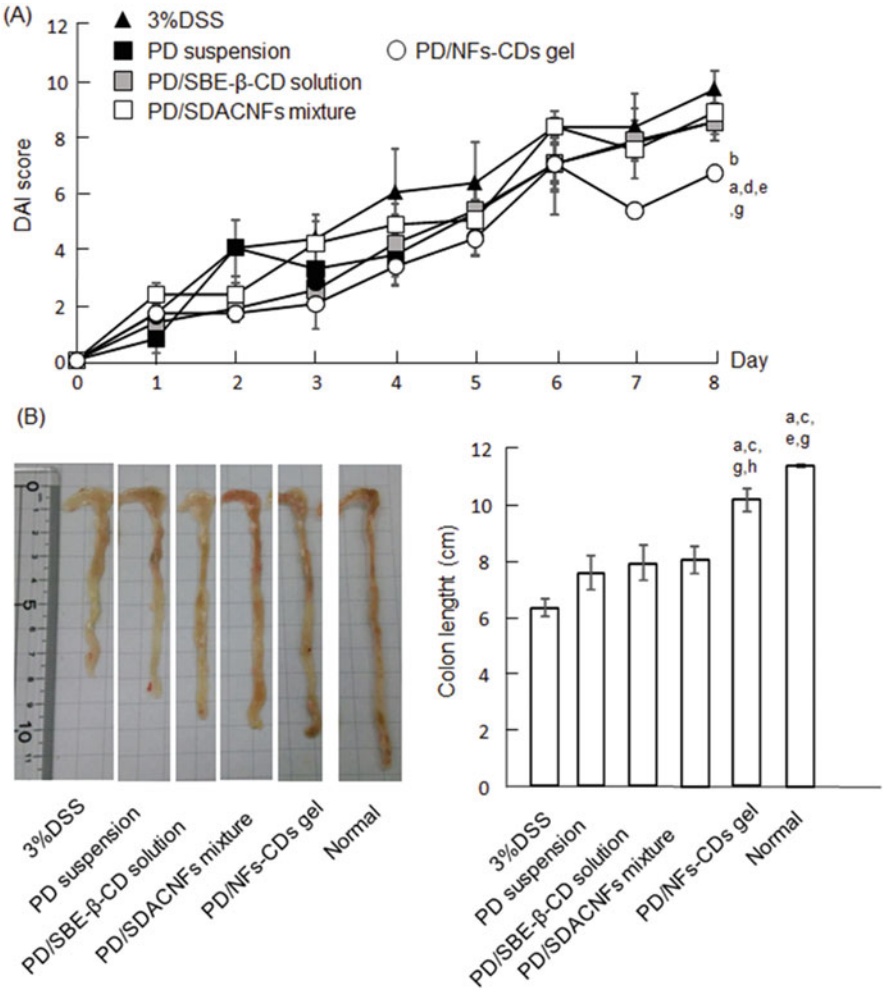

Fig. 9 Effects of PD/NFs-CD gels on biological parameters in colitis model [45]

efficient and as effective as that of a commercially available PD formulation (Fig. 9) [45]. Further, chitosan nanofibers/SBE-β-CD gels can be used to prepare homogeneous high-content gels that are able to transport poorly water-soluble drugs. Hypertension or hyperlipidemia medication is often used in treating CKD, but these materials are poorly water-soluble. If chitosan nanofibers/SBE-β-CD gels could be used to prepare homogeneous high-content gels that are capable of transporting poorly water-soluble drugs such as drugs that are used in hypertension or hyperlipidemia treatment, the drug/NFs-CDs gel has the potential for use in the therapeutic treatment of CKD patients.

4 Biological Properties of Chitin Nanofibers on Skin

Skin is the outer barrier for our body, and it prevents from external stress such as UV, bacteria, immunological cells [46]. Previously, many beneficial effects of chitin, chitosan, and its derivatives were reported for skin functions [47]. Moreover, the complex with chitin, chitosan, and its derivatives are also developed for the skin application. There are some beneficial points of chitin, chitosan, and its derivatives for the skin application. One is biological efficiencies such as direct or indirect effects for the cells, anti-microbial activities, and decomposing [48]. The other is high processability [25, 47]. Chitin, chitosan, and its derivatives can be applied such as powder, solution, gel, sponge, etc. More recently, the biological effects of chitin nanofibers and related nanofibers for the skin are also investigated. The biological activities of chitin, chitosan, and its derivatives based nanofibers are summarized.

Ito et al. evaluated the effect of skin condition by chitin nanofibers and chitin nanocrystals using an experimental model such as three-dimensional skin culture model and Franz cells [49]. The results show that chitin nanofibers and chitin nanocrystals improved the heights of the epithelial granular layer and induced the increase of granular density of the skin. Moreover, applications of chitin nanofibers and chitin nanocrystals to the skin induced a lower production of TGF-β compared to that of the control group.

Ito et al. also reported combination of chitin nanofibers with other substances provides an enhanced protective effect against UVB radiation [50]. Urocanic acid (UCA), a major UV-absorbing chromophore was used in their studies. They prepared UCA chitin nanofibers and examined its protective effect against UVB radiation. The combination of UCA and nanofibers has higher protective effects against UVB radiation compared to the UCA alone.

Izumi et al. investigated the anti-inflammatory effects of chitin nanofiber on the experimental model of atopic dermatitis (AD) [51]. Application of the chitin nanofibers suppressed hypertrophy and hyperkeratosis of the epidermis in the experimental model. Application of the chitin nanofibers suppressed the inflammatory responses by suppressing nuclear factor-kappa B, cyclooxygenase-2, and inducible nitric oxide synthase activations. Especially, chitin nanofibers suppressed skin inflammation and IgE serum levels in AD early stage.

Chemically modified chitin nanofibers also have some biological effects. Izumi et al. studied the effects of partially deacetylated chitin nanofiber (chitosan nanofiber) on wound healing process [52]. They used circular excision wound model of the rats. The results indicated that chitosan nanofibers induced the regenerations of epithelium. Chitosan nanofibers also induced the proliferation of the fibroblasts and collagen tissue. On the other hand, chitosan nanofibers did not show severe systemic inflammation in experimental wound model. The data indicated that chitosan nanofibers effectively induced the proliferation and re-modeling phases compared with chitin or chitosan nanofibers.

The effects of chitosan nanofibers and chitosan on hair growth were investigated [53]. At first, in vitro study was performed. Chitosan nanofibers and chitosan

increased the growth of human follicle dermal papilla cells on day 3 after the initiation of treatment. Moreover, chitosan nanofibers and chitosan also stimulated the fibroblast growth factor-7 (FGF-7) production. In an in vivo study, applications of the chitosan nanofibers and chitosan were investigated. Chitosan nanofibers and chitosan promoted hair growth in the dorsal skin. In both groups, the high expression levels of FGF-7 and Sonic hedgehog were observed in hair follicles.

The complex using chitosan nanofibers also have interesting bioactivities for the treatments of the skin. Tabuchi et al. developed a freeze-dried gel composed of chitosan nanofibers, reinforced with an anionic cyclodextrin, sulfobutyl ether β-cyclodextrin (SBE-β-CD) [54]. The rat wound model were used for evaluating the effects of a new biomaterial. In the groups both chitosan nanofibers gel with or without SBE-β-CD, the wound areas were decreased. Additionally, these effects were higher than that of commercially available dressings. The drug releases from the gels were also compared by using the poorly water-soluble drugs such as prednisolone (PD). The rate of release of PD from the freeze-dried chitosan nanofibers/SBE-β-CD was much quicker than that from chitosan nanofibers alone without SBE-β-CD.

Goto et al. developed pelleted preparations from chitosan nanofibers and sacran (Sac), an anionic, sulfated, carboxyl-containing polysaccharide [55]. Tetrahydrocurcumin (THC) was used as a model drug. The THC was complexed with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). As a result, its water solubility was increased. According to the rate of release of THC, Sac/chitosan nanofiber (1:1) and Sac alone pellets performed extended-release for THC.

These evidence indicate that chitin nanofiber and chitosan nanofiber have various biological activities for skin. Moreover, chitin nanofiber and chitosan nanofiber have good affinities with other polysaccharides. It is indicated that chitin nanofiber and chitosan nanofiber have good potencies as new biomaterials for the skin. However, there are some points to solve for the development as biomaterials. The action mechanism of chitin nanofiber and chitosan nanofiber is not clear. Investigations of the action mechanisms of chitin nanofiber and chitosan nanofiber are necessary to apply in the medical fields. The other is that the data are still only experimental data. The clinical examination must be performed for the evaluation of the clinical efficiencies of the chitin nanofiber and chitosan nanofiber.

5 Summary

The authors have manufactured chitin nanofibers for the purpose of utilizing a large amount of crab shells generated in the Tottori Prefecture and have clarified their various functions. Currently, products containing chitosan nanofiber as a functional raw material are on sale. Since all-natural chitins are aggregates of nanofibers, they are relatively easy to mass-produce by pulverization. On the other hand, it is difficult to explore the functions of nanofibers and identify effective applications based on social needs. As mentioned above, chitin nanofibers have various physiological

functions when taken or applied to the skin. New potential functions have been discovered because the chitin nanofibers are homogeneously dispersed in water. Since technical expertise forming a community of chitin and chitosan research ranged over various areas such as medicine, veterinary medicine, agriculture, and engineering, it is easy to collaborate interdisciplinary research. We believe that the potential functions of this new material will continue to be discovered, mainly in the fields related to healthcare.

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Chitosan–Platelet Interactions

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Contents

Abstract Since chitosan was identified as a hemostatic agent in the 1980s, "chitosan and platelets" has developed into a topic of intense interest. This chapter gives an overview of platelet biogenesis, composition, activation, and mechanisms implicated in chitosan–platelet interactions. Chitosan is a unique acid-soluble cationic glucosamine polysaccharide with tunable molecular weight, glucosamine/N-acetyl glucosamine content, and acetylation pattern. Platelets are small anuclear cells with anionic surfaces that are released to the blood stream by megakaryocytes that reside in bone marrow and the lung. Platelets are stocked with granules that contain a plethora of bioactive wound-healing and procoagulant factors. Upon activation by agonists, or adhesion to von Willebrand factor "strings" under shear stress, platelets aid in fibrin clot formation to seal off a wound and initiate wound repair. Purified platelets rapidly adhere to a variety of solid chitosan and chitin substrates but show inconsistent levels of activation in the absence of calcium. Chitosans with a positive charge state bind to platelets and potentiate alpha granule release in whole blood or recalcified platelet-rich plasma (PRP). Platelet activation kinetics were accelerated by higher chitosan deacetylation levels and molecular weight (95% vs. 80%

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deacetylated, 177 kDa vs. 102 kDa), but mis-timed platelet degranulation prior to thrombin activation led to weaker clot tensile strength. Neutral-soluble chitosans (oligomers, 50% reacetylated chitosans) do not activate platelets and hydrophobic butyryl-chitosan coatings inhibit platelet adhesion. Collective data suggest two mechanisms underlying chitosan–platelet interactions: (1) non-specific electrostatic binding of anionic platelets to positively charged chitosan surfaces, and (2) platelet binding to blood plasma factors adsorbed on chitosan or chitin surfaces. Future directions include deepening our understanding of the molecular basis for thrombocyte–chitosan interactions, and the performance of platelet-activating chitosan formulations in clinically relevant contexts where platelet physiology is altered by medications, trauma, or disease.

Keywords Alpha granule · Calcium · Chitin · Chitosan · Coagulation · Platelets

1 Chitosan Structure and Solubility

According to the Web of Science, the topic of "chitosan and platelets" started gaining attention by the early 2000s, with around 30 papers published per year on this topic. Since then, the number of publications has progressively soared to over 3,200 papers in 2020 alone, with a particular focus in the areas of hemorrhage control and wound repair. To appreciate the molecular and cellular basis of chitosan–platelet interactions, it is first important to understand that chitosan is a family of polymers with distinct chemical structure and physical forms that collectively influence the way the polymer "presents" to platelets.

Chitosan is obtained by chemical N-deacetylation of chitin, a naturally occurring polysaccharide with linearly arranged β-(O)-1-4-linked N-acetyl-D-glucosamine (GlcNAc) residues [1]. The deacetylation step can be simply achieved by autoclaving chitin particles in a 25% w/v sodium hydroxide solution [2, 3]. During this treatment, exposed acetyl groups are stripped from GlcNAc to generate glucosamine (Glc). It was proposed that GlcNAc groups buried in the nucleus of an insoluble chitin particle are protected from deacetylation, giving rise to a "block" acetylation pattern [4]. "Block" acetylation refers to consecutive clusters of GlcNAc residues (i.e., AADA, AAAA, ADAA) interspersed throughout the poly-Glc chain [5, 6]. Repeated autoclaving of chitosan under alkaline conditions can be used to reach >98% DDA, which for all practical purposes is considered fully deacetylated [2]. Because each autoclave cycle produces chain scission, chitosan M_n is inevitably diminished compared to the chitin starting material. Fully deacetylated chitosan can be reacetylated to different % DDA levels using acetic anhydride; this produces chitosans with a random acetylation pattern [2, 7]. All of these production steps create heterogeneities in each chitosan preparation. It is important to recognize that each batch of chitosan has a number-average molecular weight (M_n), degree of deacetylation (DDA), and pattern of acetylation, along with a certain level of

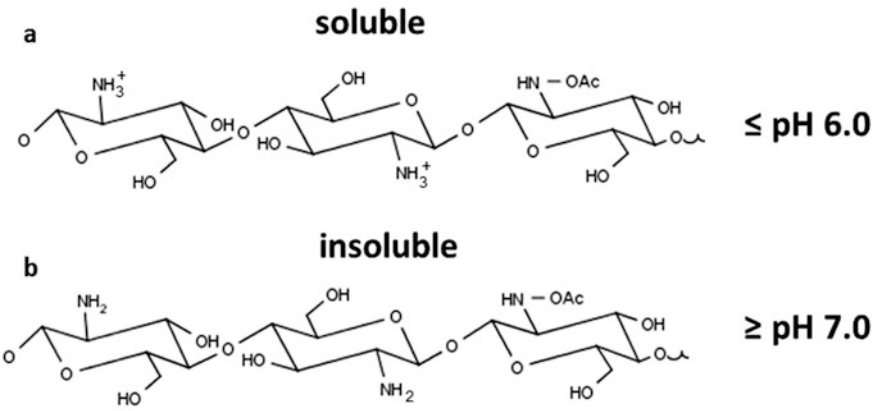

Fig. 1 For chitosan preparations with a molecular weight ≥10 kDa and 60–100% DDA, solution pH controls whether chitosan is (**a**) cationic and soluble or (**b**) neutral and mostly insoluble

polydispersity for each of these characteristics. Each of these structural features can influence the manner in which chitosan interfaces with blood plasma.

As the major structural component of exoskeletons of crustaceans and insects, chitin has evolved to remain firmly insoluble under aqueous conditions. Therefore, chitin particles and scaffolds are by nature insoluble in blood and present slightly hydrophobic poly-GlcNA surfaces to blood components [8, 9]. By contrast, chitosan powders can be dissolved in slightly acidic solutions when at least half of the Glc subunits are protonated (i.e., R-NH_3^+) [10]. In other words, chitosan will only remain soluble at a pH equal to or below the chitosan pK_0. Because all chitosans (60–100% DDA) have a pK_0 6.0–6.5 that is altogether below neutral pH [10, 11], this means that acid-soluble chitosan chains will spontaneously transition into insoluble microparticles upon mixing with neutral pH cell culture medium, blood plasma, or whole blood [12–14] (Fig. 1). When soluble chitosan chains shift to insoluble microparticles in the presence of serum, the microparticles adopt a slight negative zeta potential suggesting that chitosan-anionic serum factor complexes form spontaneously [12, 14]. Solid freeze-dried chitosan scaffolds generated from fully protonated chitosan solutions will spontaneously solubilize in whole blood or platelet-rich plasma and then disperse as microparticles in the coagulum [15–17]. By contrast, chitosan matrices treated with alkaline solutions and/or organic solvents show depressed surface energy and wettability compared to Glc and GlcNAc monomers [18]. Chitosan matrices cured in alkaline conditions have neutral amine groups and present neutral, insoluble surfaces to blood components (Fig. 1b). Chitosan neutral–solubility is enhanced by decreasing molecular weight and by very low deacetylation levels (i.e., 50% DDA) [7, 19]. Small chitosan oligomer chains (≤5 kDa) are fully soluble at neutral pH for all DDA levels [2, 20].

2 Platelets

Platelets are small anuclear disc-shaped cells released by megakaryocytes into the blood circulation [21]. Megakaryocytes are large multinuclear cells derived from a common myeloid progenitor that normally develop and reside in the bone marrow. At full maturity these cells can reach a ploidy up to 64 N [22]. Megakaryocytes are also found in the circulation and are known to populate heart and lung tissues [22–25]. According to the "pulmonary platelet production model", a significant portion of platelets could be produced in the lung [22]. It was suggested that shear forces in the lung vasculature shred apart "proplatelets" released by bone marrow megakaryocytes, while other studies using transgenic mice carrying green fluorescent protein-expressing megakaryocytes revealed that circulating megakaryocytes can lodge in pulmonary vessels and eject platelets directly into the pulmonary vasculature [22, 24, 26]. Circulating platelet levels are under the control of a humoral factor, thrombopoietin (TPO), that is released to the circulation mainly by the liver [22]. Platelet counts in normal adult peripheral blood range from around 115 to 400 $\times$ 10^6/mL. Platelet counts vary with sex and ethnicity, with higher average levels reported for Caucasians compared to Afro-Caribbean and African groups and relatively higher levels in women compared to men [27]. Activated platelets can generate small vesicles called microparticles; microparticles are sub-micron lipid vesicles released from a variety of cell types, including platelets, that wind up in the circulation [28]. Several markers are used to identify and characterize platelets, including membrane receptors P-selectin (CD62P), GPIb/IX/V (CD42), and the GPIIb/IIIa integrin complex (CD41/CD61) (Table 1).

Like red blood cells, platelets have anionic surfaces that repel their spontaneous adhesion to endothelial cells, erythrocytes, and leukocytes whose surfaces are also negatively charged [29]. The platelet glycocalyx is around 20–30 nm thick, an anionic coating of integral membrane glycoproteins, proteoglycans, glycolipids, and factors adsorbed from the blood plasma [30]. Platelets also carry blood group antigens at variable levels on several transmembrane receptors including GPIb and GPIIa and GPIIIa [31]. ABH antigens are fucosylated carbohydrate modifications of blood glycoproteins and glycolipids known to influence the risk of thrombosis in certain disease states [32–34]. In a cohort of 313 Japanese healthy volunteers, platelets showed a "high ABH antigen expressor" phenotype in 7% of the donors [31]. These data highlight donor-specific variations in the platelet glycome. In keeping with other cells, platelets show membrane asymmetry with negatively charged phospholipids hidden in the internal membrane.

Platelets can be activated through distinct mechanisms in static whole blood samples and under shear stress generated by blood flow. During whole blood coagulation initiated in vitro by the extrinsic pathway, thrombin activation initiates platelet activation, and activated platelets help propagate clot formation. Platelet activation is often monitored by the release of alpha granule contents. In a kinetic studies of tissue factor (TF)-induced clotting of recalcified citrated whole blood [35, 36], early thrombin activation (as measured by the appearance of fibrinogen

Table 1 Platelet characteristics

Membrane	**Concentration in plasma (baseline)**	**Concentration in clot serum**
Sphingosine-1 phosphate	190 pmol/mL [78]	480 pmol/mL [78]
Alpha granule factors	**Concentration in plasma (baseline)**	**Concentration in clot serum**
Platelet factor 4 (PF4/CXCL4)[a] (present in alpha granules at about 20,000× higher than plasma concentration)	84 ± 203 IU/mL [79] 12.5 ng/mL [80]	2,345 ± 158 IU/mL [79] 4,400 ng/mL [36]
β-Thromboglobulin (CXCL7; NAP2)[a]	1.75 ± 0.09 ng/mL [81] (a) 2.06 ± 0.06 ng/mL [81] (b)	9.4 ± 3.5 ng/mL [82] 206.9 ± 23.3 ng/mL [83] (a) 182.8 ± 20.1 ng/mL [83] (b) 8.4–24.2 μg/million platelets [37]
Thrombospondin-1 (TSP/THBS1)[b]	0.16 μg/mL [42]	4,900 μg/mL in platelets [42]
Fibrinogen	2–4 mg/mL [84, 85]	140 μg/million platelets [37]
Transforming growth factors β1 (TGF-β1)	0.98 ± 0.26 ng/mL [79]	23.7 ± 4.9 ng/mL [79]
Platelet derived growth factor (PDGF-bb)	0 ng/mL [71]	4.2 ± 0.9 ng/mL [71]
Osteonectin	6.9 ± 1.2 nM [35]	44.6 ± 6.7 nM [35]
Alpha granule transmembrane receptors	**Ligand**	
P-selectin (CD62P) transmembrane receptor	(Inside alpha granules of circulating platelets)	(Exposed on the outer membrane of activated platelet)
GPIIb/IIIa ($\alpha_{IIb}\beta_3$; CD41/CD61; Integrin alpha 2b, ITGA2B)	Fibrin, collagen, vitronectin, fibronectin, thrombospondin [42]	(In alpha granules and on the outer membrane, clusters upon activation)
GPIV (CD36)	Collagen type II, thrombospondin [42]	(Appears on the surface after activation)
Tubular elements	**Plasma concentration**	**Platelet concentration**
von Willebrand factor (vWF)[b]	10 μg/mL in plasma [42] 98 U/dL (type O) [86] 130 U/dL (non-O) [86]	34 μg/mL in platelets [42]
Factor V	6.6 μg/mL in plasma [85]	4.4 ng/million platelets [37]
Dense (delta) granule factors	**Plasma concentration**	**Platelet concentration**
Polyphosphate (i.e., 60 to 100 phosphate units)	N/A	0.92 ± 0.19 nmol/10^8 platelets, intragranular polyP ~130 nM [87]
Calcium	2–2.5 mM [88]	22 ± 2.8 nM in platelet cytosol [87]
ATP, ADP	N/A	23.8, 14.5 nmol/mg platelet protein [37]
Serotonin	N/A	1.5–2.5 nmol/mg platelet protein [37]
P-selectin (CD62P) transmembrane receptor [22] (ligand is PSGL-1)	N/A	(Inside alpha granules of circulating platelets, exposed upon activation)

(continued)

Table 1 (continued)

Outer membrane (resting platelets)	**Ligand** [42]	**Function**
GPIb/IX/V (CD42b/CD42a; GP1BA/GP9/GP5)	Surface-bound vWF, IIa	Adhesion
GPIIb/IIIa ($\alpha_{IIb}\beta_3$; CD41/CD61; ITGA2B or ITGAB/ITGB3)	Fibrin (mainly), collagen, vitronectin, fibronectin, thrombospondin	Aggregation, adhesion
GPIa/GPIIa ($\alpha_2\beta_1$; CD49b/CD29; VLA-2/VLA-4beta; ITGA2/ITGB1)	Collagen type I (mainly); fibronectin	Adhesion
GPIc*/IIa ($\alpha_5\beta_1$; CD49e/CD29; VLA-5; ITGA5)	Fibronectin	Adhesion
GPIc/IIa ($\alpha_6\beta_1$; CD49f/CD29; VLA-6; ITGA6)	Laminin	Adhesion
GPIV (CD36)	Collagen type II, thrombospondin (THBS)	Adhesion, aggregation

N/R not reported, *N/A* not applicable, *IIa* activated thrombin

[35] $N = 18$, from $N = 5$ healthy male and $N = 5$ healthy female human TF-activated recalcified citrated whole blood

[36] non-aspirin-using normal human donors (unspecified number of donors), whole blood +32 μg/mL corn trypsin inhibitor, 40 pmol/L TF, 80 nmol/L phosphatidylserine/phosphatidylcholine (PSPC)

[71] $N = 3$ healthy non-fasting adult female donors, unmodified peripheral blood coagulated for 30 min at 37°C

[78] $N = 6$ healthy adults, venous blood combined with 15% volume ACD (acid citrate-dextrose) 2,000 × *g* 15 min for plasma, or glass tube-induced venous blood clotted at RT for 60 min, 15% ACD added then 2,000 × *g* for 15 min

[79] $N = 12$ healthy participants in their twenties, venous blood poured into 7.5% potassium-EDTA tubes, ice for 1–2 h, or allowed to clot in unspecified tubes for 1–2 h at RT then 1,000 × *g* for 20 min 4°C, re-centrifuge 3,000 × *g* 10 min 4°C

[80] Blood with unspecified anticoagulant from $N = 217$ patients undergoing computed coronary artery angiography

[81] Either (a) $N = 42$ healthy blood donors, 57 ± 1 years old, 22% women, or (b) $N = 45$ patients with critical limb ischemia (CLI), 58 ± 2 years old 18% women or $N = 59$ patients, 57 ± 1 years old 22% women with CLI and type 2 diabetes mellitus; unspecified blood collection method, blood plasma analyzed by ELISA for NAP-2/CXCL7

[82] $N = 7$ orthopedic patients, 3 females, 4 males; mean age = 35 ± 7 years (range = 29–52) without inflammatory diseases who had undergone epidural anesthesia, serum collection method not described

[83] (a) $N = 21$ newly diagnosed patients not yet treated with depression; (b) $N = 25$ age-matched controls

[86] $N = 123$ healthy females, multi-ethnic, citrated platelet-poor plasma

[a]Heparin-binding factor

[b]Expressed in platelets and in endothelial cells

peptide A) occurred at 1 min, platelet activation (measured by release of osteonectin, or platelet factor 4, PF4/CXCL4) occurred at 2–3.5 min, and clotting time (measured by appearance of "clumps" on the sidewalls of the tubes) was observed at 4.7 min [35, 36]. It was estimated that platelets had degranulated around 50% of their alpha granule contents at clotting time [36]. In addition to PF4 and osteonectin, platelet alpha granules release a plethora of bioactive factors: platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), as well as procoagulant factors Factor V (FV), von Willebrand factor (vWF), fibrinogen, and thrombospondin [37]. Activated platelets release other factors from delta granules: adenosine nucleotide diphosphate (ADP) and adenosine triphosphate (ATP), serotonin, calcium, and polyphosphate to name a few (Table 1). Upon activation, platelets change their shape from disc-shaped to spiked spheres, adhere to surfaces, and aggregate. Activated platelets undergo cytosolic calcium spikes, release alpha and delta granule contents, express GPIIb/IIIa on the platelet surface, internalize GPIb, and lose plasma membrane asymmetry which exposes phosphatidylserine on the outer membrane [30]. Phosphatidylserine is a docking site for calcium-dependent binding of activated gla-domain coagulation factor assemblies at the platelet membrane [38]. Generation of thromboxane and thrombin at the platelet surface, along with delta granule release of serotonin and ADP, further promotes platelet activation via platelet surface receptors for each of these agonists [39].

Shear-stress induced platelet activation uses vWF to immobilize platelets at the damaged endothelial cell surface. vWF is a large multimeric glycoprotein that is present in the circulation and stored in platelet alpha granules and in Weibel-Palade bodies of endothelial cells [40]. Upon endothelial damage under blood flow-induced shear stress, vWF "strings" are ejected into the blood stream, forming an attachment site for platelets through GPIb/IX/V receptors [41]. Following platelet activation, the platelet integrin receptor GPIIb/IIIa becomes clustered which enables it to engage with subendothelial collagen fibers, fibrin, vWF and thrombospondin through an RGD sequence [30, 42]. Platelet aggregation during coagulation, and anchoring to fibrin through GPIIb/IIIa receptor interactions, is an essential step in clot retraction and hemostasis [42]. The platelet plug forms a "white thrombus" to seal a damaged blood vessel. Given the essential role for platelets in hemostasis, biomaterial features that stimulate platelet activation are clearly important for applications in hemorrhage control. In other applications involving vascular stents, however, attention has focused on formulating chitosan in a manner to inhibit platelet activation [43].

All of the studies analyzed in this chapter used "normal" or "healthy" donor platelets for chitosan interaction studies, however it is important to keep in mind that platelets from unhealthy individuals could potentially show different responses [33]. Evidence has been accumulating to suggest that platelet composition could be altered by certain disease states. Bone marrow megakaryocytes in normal individuals experience a process termed "emperipolesis" [30, 44]. Emperipolesis is a phenomenon distinct from phagocytosis and involves megakaryocyte ingestion of neutrophils, eosinophils, erythrocytes, and lymphocytes without destruction of ingested cell integrity [30]. This remarkable event was originally suggested to

serve as a mechanism for neutrophil transit from the bone marrow into the vasculature [30]. Emperipolesis is enhanced by endotoxin [45] and in experimental models of idiopathic myelofibrosis [44]. Video-epifluorescence microscopy showed that megakaryocyte uptake of neutrophils resulted in mingling of megakaryocyte-neutrophil membranes and the appearance of neutrophil membranes on circulating platelets [46]. The potential implications of these observations, notably in contexts where emperipolesis involves the trans-cellular transit of inflammatory neutrophils, remain to be identified.

3 Chitosan Interfacing with Blood Plasma

Chitosan membranes, sponges, and solid scaffolds are readily created by processes that neutralize or chemically cross-link glucosamine residues, either before or after electrospinning, surface coating, freeze-drying, precipitation or gelation [1]. These chitosan matrices and substrates are intended to remain intact after immersion in blood or blood plasma [47, 48]. Extensive modification of chitosan amine groups by chemical cross-linking reduces the density of cationic residues available for interaction with anionic blood factors. Furthermore, when solid chitosan matrices are treated with alkaline solutions or organic solvents, they strongly resist dissolving, even when hydrated in acidic aqueous solutions. In hemorrhage control, use of a solid matrix is important because chitosan solutions or powders were found to slow bleeding from capillaries but were unable to arrest arterial bleeding without additional pressure to seal off the broken blood vessel.

In certain wound-repair applications, formulations that generate chitosan microparticles are preferred [16, 49]. For example, when a liquid 80% DDA chitosan isotonic solution pH 4.5 is dispersed into unmodified whole blood and allowed to clot in a glass tube for 30 min at 37°C, the soluble chitosan chains can be observed to precipitate due to the neutral pH environment to form a hybrid biomaterial clot with chitosan microparticles interspersed in the fibrin network (Fig. 2a, b). In another approach, a freeze-dried (FD)-chitosan formulation is prepared with a fully protonated chitosan solution (pH 2.5) under controlled lyophilization conditions (inset, Fig. 2c). When this FD-chitosan scaffold is immersed in recalcified citrated platelet-poor plasma and kept at 37°C for an hour to permit fibrin clot formation (Fig. 2c), the chitosan scaffold rehydrates and disperses to form micro-hydrogel particles within the resulting fibrin clot network (Fig. 2d) [16]. Other FD-chitosan formulations intended for PRP mixing were optimized to contain ~83% DDA ~40 kDa chitosan, lyoprotectant, and calcium chloride. These FD-chitosan cakes solubilize, disperse in platelet-rich plasma (PRP), and form a micro-hydrogel particle scaffold network within the PRP fibrin clot structure [50].

As a cationic polysaccharide, chitosan is capable of forming electrostatic complexes with a variety of blood factors, most of which are anionic. Benesch and Tengvall showed that chitosan-coated surfaces develop a 10 nm thick layer of serum protein that includes serum albumin and complement C3 [51]. It was subsequently

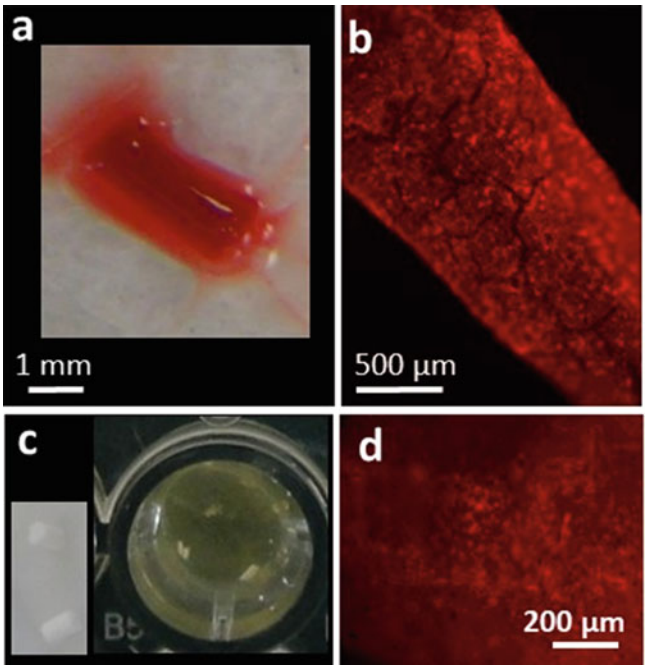

Fig. 2 In certain wound-repair applications, chitosan microparticles are preferred. Two chitosan formulations incorporated with rhodamine isothiocyanate (RITC)-chitosan tracer were used to form hybrid whole blood or recalcified platelet-poor plasma clots and imaged by epifluorescence microscopy. A 1.6% w/v chitosan/100 mM glycerol phosphate solution (RITC-chitosan 80.6% DDA, M_n 46.7 kDa) was mixed at a 1:3 v/v ratio with peripheral whole rabbit blood collected under IRB-approved protocols, and solidified ex vivo in a glass tube to form (**a**) a cylindrical hybrid clot implant with (**b**) RITC-chitosan particles interspersed in the blood clot. (**c**) FD-chitosan (1% w/v chitosan/HCl, pH 2.5, inset) was placed in recalcified citrated human platelet-poor plasma (Precision Biologics, Halifax, Canada) with glass beads to activate coagulation. The FD-chitosan scaffold spontaneously rehydrated and dispersed as micro-hydrogel particles in the fibrin clot (**d**). Panel (**d**) is reprinted from *Materialia,* Vol 9, Hoemann CD et al. Guided bone marrow stimulation for articular cartilage repair through a freeze-dried chitosan microparticle approach, 100609, 2020, with permission from Elsevier

shown that in platelet-poor plasma (PPP), chitosan microparticles form electrostatic complexes with anionic zymogens C3, C5, and Factor B without activating complement [14]. When liquid chitosan is mixed with blood, the chitosan chains form complexes with red blood cells and induce red blood cell agglutination [52]; this bioactivity is attributed to electrostatic binding between chitosan and sialic acid residues on the RBC surface [53]. Platelet activation also activates the complement cascade, therefore chitosan preparations that activate platelets in whole blood or PRP may also drive complement activation indirectly at the platelet surface [14, 54].

The blood stream is maintained in a fluid state partly through its anionic environment. Endothelial cells are coated with a 0.5 mm thick glycocalyx with dense negatively charged heparan sulfate, chondroitin sulfate, and hyaluronan that is shed

into the circulation [55]. The platelet surface carries GPIb receptors with O-linked glycosylations capped with sialic acid, which contribute significantly to the platelet's negatively charged surface [42]. vWF carbohydrate chains are also capped with sialic acid [40, 41]. Desialylation of vWF by neuraminidase leads to its clearance by the liver through the asialoglycoprotein receptor (ASGPR), also called the Ashwell receptor [41, 56]. The Ashwell receptor was also found to be responsible for clearing desialylated platelets that arise during sepsis by the action of bacterial neuraminidase [57]. Fibrinogen is also sialylated [58]. Sialyl groups in fibrinogen were suggested to serve as low-affinity calcium binding sites that favor fibrin self-assembly and branching of fibrin fibers [58]. Because platelet–vWF and platelet–fibrin interactions are mediated through integrin protein receptor interactions (GPIb:vWF; GPIIb/IIIa: fibrin), sialic acid residues in vWF, fibrin, and GPIb are free to interact with other binding partners including cationic glucosamine residues in chitosan. To summarize, the sialic acid modification of glycosylations in many blood factors, blood cell surface receptors, and platelet receptors represents a "sialome" [59] that creates a physicochemical basis for cationic chitosan complex formation. Other anionic blood proteins have a pK_a 6.0 or lower, which enables their binding to chitosan through electrostatic protein–polysaccharide interactions [14].

4 Chitosan–Platelet Interactions

One of the earliest clues that chitosan has procoagulant activity came from experiments by Malette et al., who observed that a porous DeBakey Dacron graft soaked in a 2 mg/mL chitosan solution, but not saline, prevented acute fatal bleed-out when stitched over the excised aorta of heparinized canines [60]. One day later, the chitosan coagulum was found "inside the graft, plugging the interstices" [60]. Klokkevold et al. subsequently observed that lingual hemostasis in rabbits could be accelerated by application of an 80% DDA chitosan solution in acetic acid [61]. In these early reports, the hemostatic mechanism of action was assumed to result from chitosan-induced red blood cell agglutination which creates a "red" thrombus. It was subsequently shown that chitosan-coated microtiter plates, acidic chitosan solutions and chitosan or chitin solid microparticles induce rapid platelet adhesion, aggregation, and elevated surface expression of GPIIb/IIIa in the absence of calcium [62, 63], with one report that chitosan particles could enhance the release of platelet alpha granule contents under calcium-free assay conditions [63] (Table 2). By contrast, neutral-soluble chitosans including chitosan oligomers showed no ability to induce platelet aggregation in vitro and were even slightly anticoagulant [64, 65].

Since these original reports, a variety of hemostatic chitosan devices have been developed, the majority of which are solid matrices, films, coatings, gels, filaments, or sponges that produce hemostasis through mechanisms purporting to involve red blood cell agglutination and/or platelet activation [66, 67]. These devices are intended to promote hemostasis and reside temporarily in the wound. It is important

Table 2 Studies on chitosan–platelet interactions

	Chitin/chitosan	Platelet preparation	Effect of chitin/chitosan
Calcium-free assays			
Chou [62]	Chitosan Mw 50 kDa, >90% DDA (Primex Norway). Dissolve in 2.5% citrate, mix with platelets or use to coat microtiter plates	Rabbit EDTA blood spun at 160 g, 10 min to obtain PRP, platelets washed in calcium-free Tyrode's solution with BSA. 3×10^8 platelets/mL	Rapid platelet adhesion after 5–30 min on chitosan-coated wells. Dose-dependent (10–150 μg/mL chitosan) platelet aggregation and enhanced GPIIb/IIIa expression. When 1 mM external Ca^{2+} is added, chitosan evoked a rise in platelet intracellular Ca^{2+}.
Okamoto [63]	Chitin Mw 300 kDa <10% DDA, 2.8 μm and 6.9 μm particles; chitosan, >80% DDA, Mw 80 kDa, 2.8 μm and 6.2 μm particles (Sunfive Co, Japan). Suspend in PBS pH 7.2, 30 mg/mL	18 mL whole blood from the jugular vein of an unidentified species, mixed with 2 mL 3.8% sodium citrate, 200 g 15 min spin, collect supernatant. Platelets 3×10^5 cells/mL washed in calcium-free Tyrode's buffer, 3×500g 15 min washes. For cytokine release, PRP and chitosan spun at 1,000 g 10 min, then cleared at 10,000 g for 10 min before ELISA	Platelet aggregation ratio (%) was highest at 1 mg/mL with plasma. At 0.3 mg/mL platelet aggregation ratio without or with plasma: chitin 2.8 μm: 14% vs. 19.7%; chitin 6.9 μm: 9.6% vs. 32.3%; chitosan 2.8 μm: 14.2% vs. 15.3%; chitosan 6.2 μm: 14.2% vs. 16.5%. 150% TGF-b1 and 180% PDGF-AB were released by chitosan particles vs. PBS in calcium-free PRP
Thierry [43]	Chitosan (CH) >85% DDA (Sigma), applied layer-by-layer with hyaluronic acid (HA) to NiTi disks or wires	Whole blood from healthy medication-free volunteers collected in ACD, PRP collected at 1800 rpm 15 min, then 2,200 rpm 10 min for PPP supernatant. Platelets labeled with $^{111}InCl_3$ suspended in citrate buffer at 2.5×10^6 platelets/mL. (no Ca added)	50 min incubation with LbL chitosan-HA surfaces gentle shaking, measure adhesion via gamma counter ($^{111}InCl_3$). Observe one million platelets per cm^2 adhered to NiTi, and 600,000 platelets per cm^2 adhered to $HA(CH/HA)_4$
Lin [64]	Chitosan (Koyo Chemical, Japan) nitrite-depolymerized to M_n 0.6 to 2.2 kDa, partly acetylated or fully deacetylated.	Whole venous blood from normal healthy non-aspirin volunteers, 1:9 sodium citrate, $100 \times g$ 15 min RT	Combine 50 μL chitosan +450 μL PRP. Positive control: 20 μM ADP. Oligos had an anticoagulation effect in whole blood. Turbidimetric aggregation measures showed platelet activation

(continued)

Table 2 (continued)

	Chitin/chitosan	Platelet preparation	Effect of chitin/chitosan
	Chitosan at 10% w/v in PBS pH 7.4	(PRP) then 2,000 × *g* 15 min (PPP)	by ADP and not by any chitosan oligos
Fischer [8]	Syvek Patch fibers (50 full acetylated chitin poly-GlcNAc, 3,000 kDa, ≤100 nm fibers) Clo-Sur PAD (87 ± 10% DDA) ChitoSeal (chitosan-coated polyethylene terephthalate, PET) matrix	Fresh human PRP, separated from plasma proteins by gel filtration or centrifugation. 1.5×10^8 platelets/mL in citrated PPP were combined with scaffolds for 3 min, then fixed in formalin, ESEM scanned. (Calcium was only added to PRP without chitin or chitosan)	Syvek fibers: a grape-like platelet-fiber complex with non-discoid morphology was intercalated in the thin chitin fibers Clo-Sur PAD: platelet clusters on the surface, suggested non-specific electrostatic adhesion ChitoSeal: altered platelet morphology, some spreading on the scaffold surface (non-discoid morphology)
Thatte [9]	Poly-N-acetyl glucosamine fibers or chitosan (Marine Polymer Tech., MA, USA)	Fresh human citrated anticoagulated blood diluted 1:20 v/v in HBSS (for surface PS); PRP from sodium citrated blood, purify platelets, adjust to 1.5×10^8 platelets/mL in PPP or Tyrode's buffer, incubate directly with fiber slurry or chitosan solution (polymer 1.1 mg/mL)	Platelets that were washed of plasma proteins associated after 3 min. with poly-GlcNAc fibers that were much thinner in diameter than platelets. Calcium orange dye-loaded platelets incubated with poly-GlcNAc fibers induced intracellular calcium spike in HBSS after 10 min at RT. Citrate blood in HEPES incubated with 0.35 mg/mL poly-GlcNA showed annexin V binding (exposed phosphatidylserine) vs. controls no GlcNAc
Mao [77]	Cellulose films coated with O-butyryl chitosan (OBCS) grafted with p-azidobenzoic acid. Chitosan starting material: 6.7×10^5 g/mol, 90% DDA (Lianyungang Biologicals Inc, China)	PRP of human blood supplied by Blood Center of Nanjing Red Cross, China (addition of calcium not mentioned)	After 1 h of contact, platelets adhered abundantly to cellulose membranes but not to OBCS-grafted cellulose membrane surfaces
Romani [68]	Chitosan 95/50 (HMC +, Germany) dissolved in acetic acid, salts, and sugars, heat-dried and treated in 5% w/v KOH for	Blood from $N = 4$ healthy volunteers with 3.2% citrate, 400 × *g* 10 min, then 200 × *g* 20 min at room temp. Film	Platelets adhered to chitosan films but remained discoid in shape and did not express P-selectin strongly compared to platelets adhered to glass or plastic coverslips

(continued)

Table 2 (continued)

	Chitin/chitosan	Platelet preparation	Effect of chitin/chitosan
	12 h, washed in water until neutral. Sterilize in 75% ethanol, air dry, incubate in PBS	overlaid with PRP for 1 to 2 h at 37°C. Wash with PBS to remove poorly adherent platelets, fix with formalin	
Zhao [75]	Glycidyl methacrylate functionalized quaternized chitosan (shape-memory gel)	PRP from ADC-whole blood spun 115 × *g* 10 min. Drip onto cryogel disks, incubate 37°C 1 h. (no calcium added)	Platelet adhesion and aggregation on the gel surfaces. Propose electrostatic binding of cells, fibronectin, to cationic chitosan quaternary amines
Calcium-containing assays			
Thatte [9]	Poly-N-acetyl glucosamine fibers or chitosan (Marine Polymer Tech., MA, USA). Poly-GlcNAc was used as a suspension in saline. Chitosan solution was created with unspecified pH and osmolality	Fresh human PRP from sodium citrated blood, purify platelets, adjust to 1.5×10^8 platelets/mL in PPP or Tyrode's buffer, add $CaCl_2$, incubate directly with fiber slurry or chitosan solution (polymer 1.1 mg/mL)	In recalcified PRP, poly-GlcNAc fibers incorporated into the fibrin clot (strongest gel with 0.5 mg/mL chitin fibers), observed P-selectin and αIIbβ3 on platelet surfaces. Chitosan solution failed to activate platelets. Diminished fibrin clot formation when platelets were pre-treated with anti-GPIIb/IIIa or anti-CD49f before recalcifying with 0.5 mg/mL poly-GlcNAc (this experiment was missing PRP-only controls no poly-GlcNAc)
Hoemann [71]	Chitosan 81.6% DDA, *M*n 176 kDa, PDI 1.4, <500 EU/g (BioSyntech, Canada) 1.6% w/v chitosan, 100 mM glycerol phosphate pH 6	Healthy human unmodified peripheral whole blood, combine or not with chitosan-GP, 0.5 mL clots incubated in glass tubes at 37°C for up to 4 h	Chitosan-GP/blood clots burst released 2.8 ng/mL PDGF to serum at 30 min post-clotting vs. 4 ng/mL from whole blood and LPS/blood clots
Hattori [65]	Chitosan 85.3% DDA, 888 kDa or 87.6% DDA 247 kDa (Primex, Iceland); chitosan dimers, hexamers (Seikagaku, Japan), 75–85%DDA 50–190 kDa (Sigma) and Primex-derived lower Mw (9–58 kDa) reacetylated chitosans	Male Sprague-Dawley rats, cardiac puncture whole blood in 3.13% sodium citrate, and PRP: 250 g 10 min then 3,000 g, 15 min, recalcify and combine with chitosan solutions in PBS (0.005–0.2% w/v)	Higher erythrocyte sedimentation rates (indirect measure of RBC agglutination) after adding 75–87.6% DDA chitosan, but not 33.6–50.3% DDA chitosan, oligomers, or 275 kDa chitosan. Increased PRP turbidity and disappearance of liquid phase platelets for 75–88% DDA chitosans but not oligomers
Deprés-Tremblay [17]	Chitosan (in-house) 80% DDA, *M*n 38 kDa, dissolved in	$N = 3$ male and $N = 3$ female human donors, PRP	Chitosan inhibited PRP clot retraction; chitosan found to coat platelets by ESEM, suppress

(continued)

Table 2 (continued)

	Chitin/chitosan	Platelet preparation	Effect of chitin/chitosan
	28 mM HCl, Freeze-dried: 1% w/v chitosan, 24.2 mM $CaCl_2$, 1% w/v trehalose	using ACE E-Z PRP system. 160 g, 10 min spin, then 400 g 10 min. 9.3×10^8/mL platelets	platelet aggregation, enhance surface P-selectin, and enhance PDGF and EGF release
Chevrier [15]	Chitosans (in-house), 80–85% DDA; 4–11, 28–56, 79–154 kDa, Freeze-dried: 0.56–1.5% w/v chitosan with lyoprotectant and 42.2 mM $CaCl_2$	Sodium citrate whole blood (12.9 mM citrate) from 5 human donors. 150 g 10 min then 400 g 10 min; 3× enriched for platelets	Mixture of FD-chitosan and PRP accelerated clotting time from 15 min (PRP-only) to ~2.5 min (FD-chitosan/PRP) and created a "paste-like" PRP
Dwivedi [74]	Chitosan (in-house, 80.2% DDA, M_n 36.6 kDa), Freeze-dried with lyoprotectant and $CaCl_2$	9 mL NZW rabbit blood +1 mL 3.8% citrate, 160 g, 10 min, then concentrate platelets at 400 g, 10 min. 3× enriched for platelets	Chitosan-PRP implant showed residency in a microdrilled cartilage lesion in the knee trochlea and after 8 weeks of repair stimulated twofold higher collagen type II content in cartilage repair tissues vs microdrill control defects treated with PRP-alone
Sundaram [69]	Chitosan (Source-Crab, Kyoto chemicals, Japan) Mw 100–150 kDa; 80–85% DDA	Rat whole blood in 3.2% sodium citrate. 500 g, 5 min, then 200 g, 15 min (PRP) or 2,000 g 10 min (PPP), added to 2% chitosan (Cs) with 0.25 potassium aluminum (Al) or 0.25% calcium (Ca) for 5 min at 37°C, rinse to remove unaggregated platelets	LDH (OD490) of adherent platelets used as a measure of platelet aggregation: Greatest OD490 with Cs-Al-Ca. Gel induced RBC agglutination hypothesize electrostatic interactions mediate platelet-chitosan and RBC-Si-chitosan binding. Hemostatic effect in rat liver and femoral artery hemorrhage models (effective for low pressure bleeding contexts)

to note that platelet–chitosan interaction assays have been carried out either in calcium-free (citrated) plasma or buffers, or in the presence of calcium which permits induction of thrombin and subsequent platelet activation by thrombin and a variety of other endogenous agonists.

Depending on the physical form of a chitosan device, platelets may interact with a 2-D or 3-D chitosan surface, filament, microparticle, or soluble chitosan chain. The Syvek patch is composed of solid chitin fibers, whereas Clo-sur PAD and ChitoSeal are solid patches containing chitosan. Platelets were found to adhere to all three

scaffolds, with evidence of induced P-selectin and GPIIb/IIIa expression when calcium-activated PRP was combined with a slurry of GlcNAc fibers [8, 9]. Platelets also adhered and aggregated on the smoother surfaces of chitosan-based Clo-sur PAD after 3 min, but were mainly observed adhering to exposed polyethylene terephthalate (PET) polymer surfaces on ChitoSeal [8]. In a swine splenic capsular stripping hemorrhage model, the Syvek patch produced hemostasis after three compressions compared to 8–10 for gauze, Clo-sur PAD or ChitoSeal [8]. In another study analyzing platelet adhesion under calcium-free conditions by Romani et al., alkaline-cured chitosan films supported platelet adhesion but the platelets remained discoid in shape and scarcely expressed P-selectin, while platelets adhering to glass coverslips became spiked and had strong P-selectin expression [68]. In another study by Sundaram et al., a chitosan-aluminum sulfate-calcium hydrogel was created that induced platelet aggregation, RBC agglutination, and more rapid hemostasis in rat hemorrhage models [69] (Table 2).

For wound-healing applications, some chitosan devices have been formulated to intermingle as micron-sized hydrogel particles within blood or platelet-rich plasma clots. As mentioned above, viscous isotonic chitosan solutions can be mixed with unmodified whole blood and allowed to coagulate to form a hybrid blood clot [53]. Mixture of liquid chitosan-glycerol phosphate solutions into whole blood produces a hybrid clot that, like whole blood, solidifies through the common pathway of the coagulation enzyme cascade [70]. In thromboelastography (TEG) tests, hybrid clots containing 80% DDA chitosan showed thrombin activation starting at 20 min, followed by burst platelet activation around 30 min marked by release of PF4/CXCL4 and PDGF-ββ into the serum [70, 71]. The dispersion of 80% DDA chitosan microparticles in the hybrid clot implant was found to inhibit clot retraction, protect the clot from lysis by plasmin, promote longer in situ clot residency, attract neutrophils and M2 macrophages, and exert favorable effects on osteochondral wound repair [72, 73].

Here we report the effect of chitosan structure on platelet activation in whole blood, using a library of chitosans with 80% or 95% DDA and low (80L, 95L) or high viscosity (80M, 95M) [12]. Each chitosan was prepared as a 1.6% w/v solution in 100 mM glycerol phosphate (pH 6.0) and mixed or not with unmodified human whole blood from 4 healthy donors. Samples were either harvested immediately, submitted to a TEG assay at 37°C for 160 min to assess clotting time and clot tensile strength, or cultured at 37°C for 240 min in glass tubes. As a model system, blood clots held at body temperature have the potential to reflect platelet responses that could occur in a blood clot device in vivo. At different time points ($t = 0$, 160 or 240 min), using previously described methods [70], samples were vortexed in ice cold quench buffer, centrifuged, and the supernatant analyzed by Western blotting for PF4, or ELISA assay for thrombin-antithrombin (TAT) complex (Dade) (Fig. 3).

95M chitosan (high DDA and medium viscosity) had a unique effect on inducing instant release of PF4 at $t = 0$ in all 4 donors (lane 4, Fig. 3a), prior to thrombin activation (Fig. 3b). 95M also created a rapid increase in blood viscosity that was "read" by the TEG instrument as a more rapid "clotting time" compared to all other

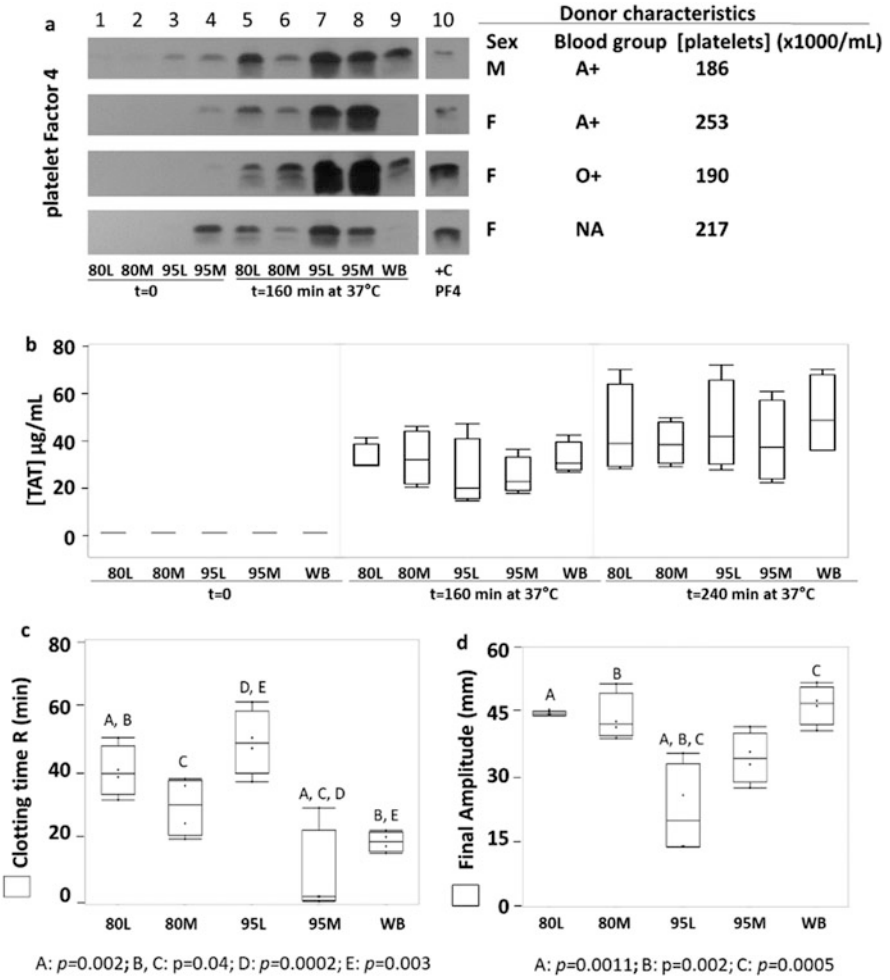

Fig. 3 Chitosan structure influenced the timing of platelet degranulation, clotting time and clot tensile strength in unmodified whole human blood. Under IRB-approved protocols, whole blood from 4 healthy human donors was mixed at 3:1 ratio with chitosan-glycerol phosphate solutions (80L, 80M, 95L, 95M) to evaluate (**a**) PF4 levels in plasma or serum using equal amounts of protein per lane and purified PF4 as a control, (**b**) TAT complex formation in plasma or serum, (**c**) clotting time (R, min), and (**d**) clot amplitude at 160 min (A, mm) by TEG assay. Chitosan (DDA and M_n) were 80L (80.2%, 108 kDa), 80M (80.6%, 144 kDa), 95L (94.6%, 102 kDa), 95M (94.6%, 177 kDa) [12]. Graphs show the median, 50% inter-quartile range, min-max, and differences due to condition were evaluated by Least Squares Means Differences with Tukey HSD post-hoc (JMP Pro 14.1.0, SAS). *R* clotting time, *NA* not available, *WB* whole blood, *TAT* thrombin-antithrombin, *PF4* platelet factor 4, *M* male, *F* female

chitosans (Fig. 3c). All chitosan-GP/blood mixtures coagulated and showed robust TAT generation at 160 and 240 min (Fig. 3b). However lower molecular weight chitosans (80L, 95L) delayed clotting time (Fig. 3c), and 95L induced a weaker clot

tensile strength compared to other clot samples (Fig. 3d). After 240 min at 37°C, PF4 was detected in serum of all chitosan-GP/blood samples, with the highest PF4 levels in 95L and 95M serum (lanes 5–8, Fig. 3a).

In this experiment, unmodified whole blood had a median 18.9 min clotting time, 47.0 mm clot tensile strength (Fig. 3c, d), robust TAT levels after 240 min (48 μg/mL, Fig. 3b), and variable serum PF4 levels at 160 min (lane 9, Fig. 3a). Donor-to-donor differences in serum PF4 levels at 160 min were not explained by selected donor characteristics (Fig. 3a). These results are consistent with previous analyses of healthy donor whole blood: 15.3 ± 4.8 min clotting time, 55.2 ± 5.7 mm maximal amplitude, ~37 μg/mL TAT, and burst appearance of PF4 at 30 min that decayed over time [70]. Altogether, these data suggested that highly deacetylated cationic chitosan chains formed complexes with platelets and red blood cells and may have formed inhibitory complexes with factors that degrade PF4. These results also suggested that platelet responses to chitosan can be influenced by the chitosan charge state at the time of platelet contact and that cationic chitosan is an effective platelet-activating biomaterial. However strong chitosan platelet–interactions by 95L were accompanied by prolonged clotting time and weaker clot tensile strength, which are considered anti-hemostatic effects.

Many experiments analyzing chitosan–platelet interactions are carried out with PRP (Table 2). PRP is generated from citrated whole blood by sequential centrifugation (200 g 15 min to pellet RBC and WBC, then 1,300 g 10 min to pellet platelets). Citrate chelates all plasma calcium and serves as an anticoagulant because calcium is required for propagation of the coagulation factor enzyme cascade leading to thrombin activation and conversion of fibrinogen to fibrin. Therefore, PRP must be recalcified to produce a PRP fibrin clot. Mixture of freeze-dried chitosan containing calcium chloride with PRP resulted in solubilization of chitosan in plasma followed by chitosan coating of platelet surfaces, hybrid chitosan/PRP clot formation, inhibition of PRP fibrin clot retraction, enhanced release of PDGF-ββ, and higher P-selectin expression [15, 17, 50]. Unlike other chitosan–platelet interactions, this FD-chitosan preparation enhanced platelet degranulation while inhibiting platelet aggregation [17]. It was suggested that chitosan inhibited clot retraction in these PRP samples by coating the platelet surfaces and preventing their aggregation [17]. FD-chitosan/PRP showed beneficial biological effects compared to PRP-alone in promoting chondrogenesis in a rabbit cartilage repair model, with greater repair tissue collagen type II content compared to PRP-alone [74]. In other applications, a shape-memory gel containing glycidyl methacrylate functionalized quaternized chitosan, with a permanent positive charge, showed platelet adhesion and aggregation on the surfaces after incubating with citrated PRP for 1 h at 37°C [75]. In the latter study, calcium was not added to the PRP preparation, suggesting the interaction was based on electrostatic interactions between anionic platelet surfaces and the cationic quaternized chitosan.

Platelet adhesion is an undesired event for vascular stents. Coating of a NiTi surface with layer-by-layer (LbL) hyaluronic acid (HA) and chitosan, with the final layer being HA was shown to reduce platelet adhesion from 1 million to 0.6 million platelets/cm^2 [43]. The persistent platelet interactions with LbL-HA(Chitosan/HA)$_4$

could be due to an irregular surface coating by HA after only 4 bi-layers that left exposed chitosan, as another study suggested that at least 6 alternating layers are needed to create a uniform anionic polyelectrolyte surface [76]. By contrast, surface coatings created with chitosans derivatized with hydrophobic butyryl functional groups and covalently attached to cellulose were shown to repel platelets and inhibit platelet adhesion and activation [77] (Table 2).

5 Conclusions and Future Perspectives

To summarize, current data suggest that chitosan–platelet interactions occur through two mechanisms. By one mechanism, electrostatic binding between the anionic platelet surface and cationic chitosan poly-glucosamine residues produces platelet adhesion that can trigger a rise in intracellular calcium, degranulation, and appearance of integrin and P-selectin receptors at the platelet surface [8, 62, 69, 75] (Fig. 4). Platelet GPIb is heavily sialylated and could potentially mediate this interaction. Neutral chitosan and chitin have little capacity to induce these responses. Although most solid matrices used as hemostatic devices have no charge (poly-GlcNAc), or are neutralized during manufacturing, recent advances have created quaternary chitosans with a permanent cationic charge state [75], which enables mechanism 1. By a second mechanism that is likely to take place in blood or PRP, abundant anionic blood plasma proteins (i.e., sialyl-fibrinogen, fibronectin, sialyl-vWF, albumin) rapidly bind to chitosan surfaces. Blood proteins are also expected to deposit on chitin surfaces. Deposition of these matricellular factors, also called "biofouling" presents a surface that permits platelet adhesion, activation, GPIIb/IIIa clustering,

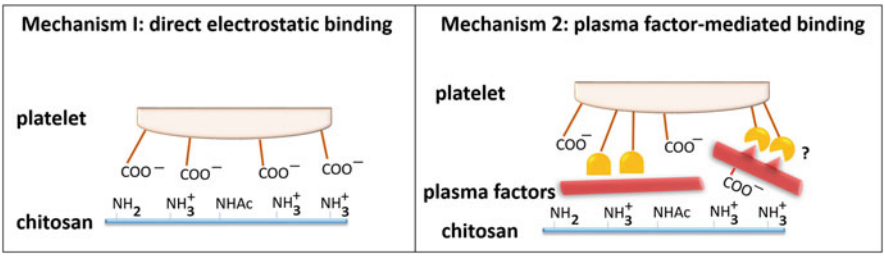

Fig. 4 Two mechanisms are proposed to explain chitosan–platelet interactions. By mechanism 1, platelet anionic surfaces form non-specific electrostatic interactions with chitosan amine groups that are initially protonated at the time of contact with whole blood or PRP. Because anionic blood proteins (including sialylated proteins such as fibrinogen) vastly outnumber platelets, most platelet interactions in PRP or blood likely occur through mechanism 2. In mechanism 2, soluble factors in blood plasma form complexes with chitosan or chitin and display binding sites, some of which may be specific high-affinity interactions via platelet surface receptors (i.e., integrin GPIIb/IIIa, GPIV, GPIc/IIa, GPIc*/IIa, GPIb/IX/V). The schematics show only one side of the platelet surface and are not to scale: platelets are on average 2 μm in diameter [30]. Note that chitosan microparticles that form in blood plasma can have a similar or smaller size compared to platelets, or a much larger size when chitosan aggregates are formed [12]

and the potential for specific receptor–ligand interactions between platelet integrins and RGD motifs in the protein coating (Fig. 4). Evidence for this second mechanism comes from slightly greater platelet aggregation induced by 2.8–6.9 μm chitin or chitosan particles in the presence of plasma proteins compared to platelets washed to remove plasma proteins [63]. Future directions on this topic will benefit from further elucidation of molecular mechanisms involved in chitosan-platelet adhesion and experiments that show the biological relevance of these interactions in whole blood where erythrocytes vastly outnumber platelets. More information is also needed on the performance of platelet-activating chitosan devices in clinical contexts, most notably where platelet physiology is altered by medications, trauma, or disease state.

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Chitosan–Stem Cell Interactions

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Contents

Abstract Chitosan, the deacetylated form of chitin, has been extensively used for tissue engineering in the form of hydrogel, scaffolds, microparticles, nanoparticles, and nanofibers. To develop composite constructs with targeted tissue regeneration, chitosan is often combined with hydroxyapatite, collagen, gelatin, hyaluronic acid, silk fibroin, etc. biomaterials. In addition to this, chitosan is often modified with varied physico-chemical properties. For regenerative application, to improve the efficacy of constructs, chitosan in various forms is combined with various stem cells, among which mesenchymal stem cells (MSCs) have been studied widely. In this review we focused on the studies that exclusively used chitosan in combination with multipotent adipose and bone marrow-derived MSCs and pluripotent-induced pluripotent stem cells (iPSCs). When cultured on chitosan, stem cells displayed greater affinity in terms of cellular adhesion, proliferation, and differentiation into functional cell types both in vitro albeit different potential. When transplanted in vivo, stem cell-laden chitosan constructs showed greater integrity into the host system, differentiated into targeted cells, and demonstrated improved repair of the damaged tissue. These studies provide great insight into the current and future potential of chitosan for regenerative applications.

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Keywords Cell-laden constructs · Chitosan–stem cell interaction · Exosomes · Stem cells

1 Introduction

Chitin is a polysaccharide obtained from the coat of the crustaceans. Various form of chitin has been generated by chemical and physical modifications. Chitosan (CS) is one such form generated by deacetylation of chitin [1–5]. The degree of deacetylation has an influence on its properties [6]. CS is mainly composed of D-glucosamine and N-acetyl-D-glucosamine that are linked randomly with β-1,4-linkage. These components in CS interact with the extracellular matrix components and modulate regenerative signals. Till date CS has been widely investigated in the form of hydrogels, scaffolds, nanoparticles, nanotubes. Depending on the application chitosan is used either alone or in combination with other biomaterials such as chitin, hyaluronic acid, hydroxyapatite (HA), collagen, silk fibroin, agarose, and/or not limited to graphene [2, 3, 7] (Fig. 1). For instance, CS in combination with HA is tested widely for bone regeneration. CS is biodegradable, biocompatible, anti-microbial [8, 9], and non-toxic which makes it an interesting biomaterial of choice for various regenerative [10, 11] biological applications. In addition to tissue regeneration, CS has been tested for anti-microbial [12] drug delivery [13]. CS has been tested for wound healing [4, 14–16], bone regeneration [17], neuronal regeneration [7], soft tissue regeneration [18], epithelial branching morphogenesis [19], skin regeneration [20–22], and with intestinal epithelial cells [23, 24].

Stem cells (SCs) are known as the rare and undifferentiated cells of a tissue with unique potential to self-renew and differentiate into functional cells. For instance, in healthy tissues, loss of functional cells is balanced by the addition of new cells by resident SCs of the tissue. SCs self-renew to maintain their own number to be

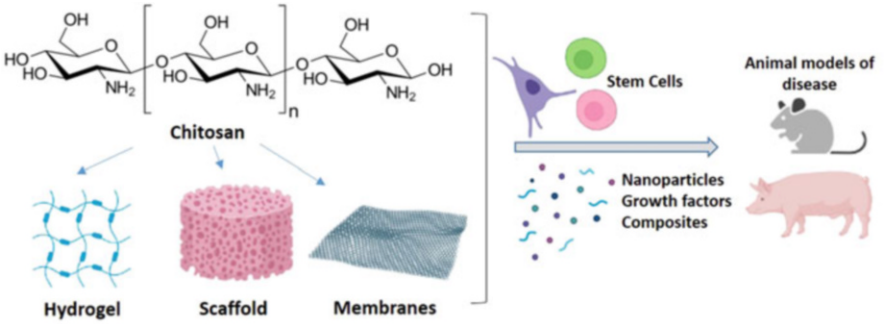

Fig. 1 Schematic showing different forms and applications of chitosan. Made with Biorender.com

supportive for tissue homeostasis for years to come. However, factors such as aging or irreversible tissue damage might diminish the number and potential of stem cells, which eventually leads to loss of tissue functionality. Based on their origin, stem cells are divided into two categories, adult SCs are obtained from adult tissues and embryonic SCs are obtained from fetal tissues; another category is the induced pluripotent SCs (iPSCs)-developed in in vitro. Adult and iPSCs are widely investigated for regenerative application. Various adult SCs, such as mesenchymal stem cells (MSCs) derived from adipose (AD-MSCs), bone marrow (BM-MSCs), and umbilical cord blood (UC-MSCs) and to some extent iPSCs were studied for their interaction with the biomaterial—chitosan.

Either injectable hydrogels, scaffolds, or grafts, the success of tissue engineering constructs depends mostly on the biomaterial of choice and cell type. The main expectation of the biomaterial to be used is to be non-toxic and support the growth of cells in vitro and in vivo. Various factors such as chemistry of components, pore size, stiffness, stability etc. are known to influence the interaction between biomaterial and cells. Therefore, to develop successful tissue engineering strategies, it is a prerequisite to understand how a biomaterial such as CS interacts with various types of cells. In this review, we focused on reports from literature that have investigated the potential of different SCs to interact with chitosan and interesting findings thereof. In the interest of regenerative application, we have focused here mainly on the interaction of various stem cells with chitosan.

2 Chitosan–Stem Cell Interactions

Mesenchymal stem or stromal cells popularly known as “medicinal signaling cells” have been widely investigated for regenerative applications for various diseases including the most recent COVID-19. According to the guidelines of International Society of Cell and Gene Therapy, MSCs are defined to express CD73, CD90, and CD105 on surface and possess multi-lineage differentiation into osteocytes, chondrocytes, and adipocytes [25]. In addition to the multi-lineage differentiation potential, MSCs are known to be immunomodulatory and regenerative. However, based on their source of origin, adipose, bone marrow, or cord blood, the MSCs possess distinct differentiation and regenerative potential [26].

2.1 Bone Marrow-Derived MSCs

Midha et al. [27] have tested the osteogenesis potential of BM-MSCs, using a highly porous, multi-material composite made of chitosan/hydroxyapatite/polycaprolactone (CHT/HA/PCL) in 3:3:2 (wt/vol). They observed that 21-day post-culture of 5×10^4 cells/composite, AD-MSCs<BM-MSCs<WJ-MSCs showed an increasing cellular activity with WJ-MSCs showing maximal activity.

When subjected to osteogenic differentiation on the composites, BM-MSCs showed significant fibroblastic cellular morphology at day 7, stellate-shaped at day 14, and showed mineralization at day 21 (increased *OCN* expression), when compared to AD-MSCs and WJ-MSCs, indicative of their successful cell–matrix interaction. In addition, BM-MSCs showed a two times increase in mRNA level expression of *Col I* expression, protein level (immunostaining) expression of COL I at day 7, β-CATENIN, OCN etc. in comparison to AD-MSCs on the composites. Composites were cultured for 7 days with undifferentiated BM-MSCs and implanted into rats with 3 mm load-bearing defect and followed over time using micro-CT and histology, acellular constructs were control. In addition to complete integration of composites at 6-week post-implant, BM-MSC laden composites showed improved bone regeneration, 21% increase in trabecular BV/TV compared to 16% in acellular composites. HE, Masson trichome staining of the bone confirmed the neobone formation in rats implanted with BM-MSC laden composites. This study concluded that in vitro screening has identified a better choice of BM-MSCs for osseointegration in vivo which resulted in improved integration and neobone formation in the bone defect model.

Yan et al. [28] have investigated the BM-MSC laden collagen–chitosan scaffold for the recovery of neuropathological injury using traumatic brain injury (TBI) model. Collagen/chitosan scaffolds of 3 × 3 × 2 mm with 80–200 μm pore size were prepared and cultured with 2×10^6/μL of (rat BM-MSCs) for 48 h. TBI was made in rats with a 10 mm diameter craniotomy adjacent to the sagittal suture and midway between lambda and bregma, and 72 h post-TBI, cell-laden scaffolds were transplanted in situ into the injured area. TBI with BM-MSC laden scaffold with and without immunosuppressor and TBI + BMSCs stereotactic injection without the scaffold and TBI without transplant were the control groups. Modified Neurological Severity Scores (mNSS) on day 1 and day 7 were significantly declined in all groups especially those with BMSC-scaffold, when compared to TBI only control. Average frequency of crossing the platform (Morris water maze test) showed a significant reduction in the transplanted groups indicative of recovery, in comparison to TBI only control up to days 35 post-transplant. Also, average escape latency (Morris water maze test) was improved in BMSC-scaffold and BMSC alone groups, compared to TBI only control. Histological evaluation post-30 days showed more GFAP positive BrU-labeled BM-MSCs at day 7 and day 14. This study concluded that BM-MSCs accelerated the functional recovery of rats after transplant.

As an alternate to cell-laden constructs, Wang et al. [29] investigated the potential of magnetic lanthanum-doped hydroxyapatite/chitosan (MLaHA/CS) scaffolds to facilitate endogenous stem cell recruitment for bone regeneration. They have used freeze-drying method to generate CS scaffolds with $SrFe_{12}O_{19}$ nanoparticles, Ca^{+2}, La^{3+}, and PO_4^{3-} ions, and nanorod bundles of LaHA were deposited on the surface and characterized for their pore size, hydrophilicity, magnetic, thermal, ion release, mechanical and biocompatible properties. They observed that when 1×10^4 rat BM-MSCs seeded/scaffold, MLaHA/CS showed higher viability (CCK-8 assay) and attracted more BM-MSCs (cell migration assay) when compared to LaHA/CS than HA/CS scaffolds. When tested for anti-inflammatory property with RAW264.7

macrophages, MLaHA/CS showed less M1 more number of M2 (CD206), anti-inflammatory and pro-regenerative macrophages in comparison to controls. In addition, MLaHA/CS showed higher osteogenic induction as indicated by increase in mRNA levels of ALP, BMP-2, OCN, RUNX-2, and activation of p-smad pathway in comparison to HA/CS and LaHA/CS scaffolds. To test the in vivo bone regenerative potential, 5 × 2 mm scaffolds were prepared and implanted into transosseous defect in the cranium of rats, calcein and alizarin red were injected 4- and 6-week post-implantation to detect the neobone formation. Micro-CT data 12-week post-implant showed new bone formation, higher BV/TV%, higher TBNs and TBTs with MLaHA/CS scaffolds. Calcitonin, alizarin red staining in the implant area was more and showed significantly enhanced generation of newly formed and mineralized bone in MLaHA/CS implant groups at 4 and 8 weeks, respectively, when compared to controls. At 12-week post-implantation, more M2 polarization was detected in MLaHA/CS scaffold group, together confirming overall improved bone regeneration with MLaHA/CS scaffolds when compared to HA/CS and LaHA/CS scaffolds.

Liu et al. [30] have studied the potential of BM-MSC incorporated, thermosensitive chitosan hydrogel to repair myocardial infarction in mice. BM-MSCs expressing Fluc and GFP were isolated from transgenic mice and 1×10^4 cells/well were seeded together with different concentrations of CS hydrogel (0.5–2 mg/mL) and found that 1 mg/mL of CS hydrogel showed an optimal proliferation of BM-MSCs based on CCK8 assay and mRNA expression of epidermal (EGF), platelet-derived (PGF), hepatocyte (HGF), and insulin (IGF) growth factors. After induction of myocardial infarction in mice, 2×10^5 BMSCs/20 μL CS hydrogel (1 mg/mL) was transplanted with 30-gauge needle into 2 position adjacent to the infarcted area, PBS was injected as control. In 30-day post-transplant echocardiography, left ventricle (LV) showed significant enlargement, anterior and posterior walls of LV were thinned. BM-MSC/CS hydrogel recipients showed a significant decrease in LVIDd and LVIDs of hearts after infarction, maintained LV contractile function with increases in FS and EF in comparison to CS only and PBS controls. CD31 (endothelial cell marker) staining at day 30 showed a significant increase in capillary density in the border region. In addition, day 5 post-transplant, a significant reduction in IL-6, TNF-a, IL-1b, IL-18, caspase-11, and caspase-1 was observed in BM-MSC/CS group, then in BM-MSC alone group and PBS only treated group. BM-MSC/CS group showed relatively less expression of caspase-1 in vascular endothelial cells ($CD31^+$) compared to the controls, indicating a reduction in pyroptosis in BM-MSC treated group. When LPS and ATP treated human umbilical vein endothelial cells (HUVECs) were treated with the conditioned media of BM-MSC/CS, they observed survival of HUVECs and decrease in pyroptosis related genes caspasae-1 and GSDMD, in comparison to the BMSCs alone controls. This study concluded the promising cardiac regenerative potential of CS hydrogel when incorporated with BM-MSCs.

2.2 Adipose-Derived MSCs (AD-MSCs)

Altman et al. [31] have investigated the effect of 75:25 silk fibroin:chitosan blend scaffold (SFCS) on seeding and in vivo delivery of human adipose-derived SCs (hASCs) on wound healing. hASCs between passages 1–8 either alone or were transfected with lentiviral eGFP and 1×10^5 cells were seeded/cm^2 of the SFCS scaffold and cultured for 24–48 h, washed with PBS and sutured into 6 mm diameter cutaneous wound, and followed for 0–10 days. Planimetric analysis on day 8 post-transplantation revealed a wound closure of 55 ± 17% in control without graft, 75 ± 11% in acellular-SFCS group and 90 ± 3% in ASC-SFCS group and by day 14 they observed a complete epithelialization in ASC-SFCS treated group. Post-2 weeks, microvascular density of wound bed biopsy specimen showed an increase in 7.5 ± 1.1 vessels/high power field in ASC-SFCS group, compared to 5.1 ± 1.0 vessels/high power field in acellular-SFCS group. At 2 weeks post, GFP$^+$ cells were observed in the dermis and cutaneous appendages and were positive for Ki67, SMA, vascular smooth muscle marker and HSP-47. At 4 weeks post, GFP$^+$ cells co-stained with cytokeratin19 (CK19) and no GFP$^+$ cells were observed 4 cm away from the site of graft. This study concluded that SFCS can support migration, proliferation, and differentiation of the seeded cells.

Liu et al. [32] have tested the rat AD-MSCs in an injectable chitosan hydrogel system to improve myocardial infarction (MI). Improper angiogenesis, inflammation, reactive oxygen species (ROS) in the MI microenvironment make it very hostile for the regenerative stem cells to home. Therefore, first they studied the role of components in CS to rescue the impaired cellular adhesion of AD-MSCs, against ROS in vitro. When seeded on culture plates, 30 μM H_2O_2 inhibited the adhesion of AD-MSCs to 63.6 ± 1.37%, which was improved to 89 ± 4.32% and 88.3 ± 4.97%, when treated with 0.5 mg/mL pf N-AC-Glu and D-Glu, the components of CS, respectively. RT-PCR data showed that ROS treatment reduced the mRNA level of integrin β1 and αV, decreased phosphorylation of FAK, Src, and p-AKt, and increased cleaved-caspase3, which were significantly restored when treated with N-AC-Glu and D-Glu. Further luciferase labeled 4×10^6 AD-MSCs in 100 μL CS hydrogel (ADSC/CS) were injected along the border of infract areas with 28-guage needle in male SD-rats and ADSC/PBS was injected as control in infract hearts. Bioluminescence imaging (BLI) at day 7 and day 14 showed a two- and four-time increase, respectively, in ADSC/Chitosan compared to ADSC/PBS, indicating cellular retention, whereas at day 28, no signal observed in ADSC/PBS whereas significant signals remained in ADSC/chitosan group, indicating retention, engraftment, and survival of ADSCs. Echocardiography data at 4 weeks showed an improved left ventricular fractional shorting (LVFS) and increased 52.92 ± 5.76% left ventricular ejection fraction (LVEF) when compared to PBS control (42.64 ± 3.8%). In addition, ADSC/chitosan treated rats showed improved hemodynamics, relatively decreased post-ischemic apoptosis (TUNEL staining), reduced infarct size, and improved arteriole density when compared to PBS control. Further the histological analysis showed luciferase-ADSCs co-staining with cTnT and

a-Sarcomeric actin, whereas some ADSCs were positive for vWAg and CD31, vascular markers, indicating differentiation of transplanted ADSCs.

Cheng et al. [33] have shown improved spheroid formation, upregulation of pluripotency genes, and increased transdifferentiation efficiency of human AD-MSCs (hASCs). Time-lapse live cell imaging showed that ASCs were motile and readily aggregated on CS coated plates and showed increase in spheroid formation with increase in cell densities from 1.5×10^3 to 5×10^4 cells/cm^2, which did not compromise cell viability when compared to cells in monolayer. ASCs in spheroids showed decrease in expression of CD29, CD90, CD105 and increase in expression of CD34, CD44, and PECAM, reduced apoptosis, increase in fibronectin, laminin when compared to those in monolayer. Quantification of mRNA levels showed a 7.7, 4.9, and 2.9 fold increase in expression of pluripotent markers Sox-2, Oct-4, and Nanog in ASC spheroids on day 3 compared to that at day 0, similar increase was observed at day 7 ASC-spheroids. When seeded back to tissue culture plates without coating, ASC-spheroids spread and show a 2.5-fold increase in activity index at day 3, a 3.2-fold and 2.7-fold increase in activity index at day 5 and day 7, respectively. When ASC spheroids were trypsinized, the single cells obtained were cultured for 14 days and showed increase in number of 71.3 ± 2.5 colony forming units (CFU), whereas monolayer derived ASCs showed 30.6 ± 8.1 CFUs. RT-PCR data showed a significant improvement in osteogenic induction (Runx2) of ASC-spheroids when compared to monolayer cells; however, no such difference observed with adipogenic induction (PPAR-γ). In addition, they observed increase in mRNA and protein levels of Nestin and albumin in ASC spheroids when cultured for 14 days in neurogenic and hepatogenic media. When dissociated and intra-muscularly transplanted into hind limbs of nude mice, ASC-spheroids showed more HNA-positive cells/injection group at day 7 and day 21 post-transplant. This data indicated a non-chemical based activation of AD-MSCs, using chitosan which improved sphere formation, stem cell marker expression, transdifferentiation in vitro and better cellular retention post-intramuscular injection in vivo.

Liu et al. [34] have isolated stromal vesicular fraction (SVF) from 9-day-old piglets and obtained AD-MSCs (pADSCs) that expressed CD29, CD44, and MHC I and negative for CD31, CD45, and MHC II and were able to differentiate into adipo, osteo, and chondrocytes. pADSCs were cultured on 1% chitosan coated tissue culture plate and differentiated into pancreatic islet-like clusters (PILC). Immunostaining data showed increased production of β-cell signature markers Pdx-1, ISL-2 and insulin was relatively more in CS coated plates at day 3. RT-PCR data showed that CS increased β-cell development gene, Pax4 and also for Pdx-1, glucokinase and insulin and maintained the expression of Glut2, Pdx-1 from day 6–day 15, whereas these levels not maintained in the absence of CS. When supplemented with 5.5 mM to 25 mM doses of glucose, increased secretion of insulin 1.0 ng/μg of protein and 1.5 ng/μg of protein was observed in the presence of CS at day 9 and day 15 to 25 mM glucose concentration, respectively. Nine days post-withdrawal of differentiation medium, PILCs on CS plated showed 1.2–2 ng/μg of protein, higher than those on regular plates (0.7–1.5 ng/μg of protein). Together

this suggested that CS improved the potential of porcine ADSCs to differentiate into pancreatic islet-like clusters, with improved functionality.

2.3 *Induced Pluripotent Stem Cells*

Induced pluripotent stem cells (iPSCs) are obtained by in vitro reprogramming of somatic cells to embryonic like pluripotent state, to be able to differentiate into multiple germ layers [35]. This makes them a great alternate to embryonic stem cells (ESCs). iPSCs can be generated from the tissue (skin or blood) of the same patient that will receive transplantation, without immune rejection. This leads to the development of personalized cell therapeutics for a broad array of diseases. Since their discovery in 2006, iPSCs have been used to generate various differentiated cell types, organoids to model diseases, etc. Till date iPSCs have been differentiated into pancreatic β-cells [36], cardiomyocytes [37], cholangiocytes [38], kidney organoids [39], lung organoids [40], cardiac organoids [41], etc. As the field of cellular therapeutics is evolving, strategies to improve the viability, functionality, and retention of cells post-transplantation gained importance. Tissue engineering strategies with biomaterials such as chitosan is one such approach. Therefore, here we focused on the studies that have used iPSCs and chitosan for regenerative applications.

Zhang et al. [42] have investigated whether ultrafine fibers' topography that mimic tendon extracellular matrix might induce tendon like differentiation of iPSC-MSCs. Ultrafine fibers (891 ± 71 nm) with 6 times more tensile strength and four-fold high Young's modulus more than random ones were prepared using SJES and CES techniques. Human iPSCs were differentiated into MSCs at 3-weeks confirmed by CD29, CD44, CD73, CD90, CD105 marker expression, adipo, osteo, and chondrogenic differentiation. Further 2×10^4 cells/cm^2 were seeded on ultrafine fiber scaffolds and cultured for 3 days. iPSC-MSCs cultured on aligned fibers showed increased expression of Mkx (tendon-related marker), and at day 14, increased mRNA levels of tendon-related genes SCX (two-fold), MKX (>ten-fold), HoxA11 (two-fold), Tnmd (two-fold), Epha4 and Bgn (>40-fold) Col1a1 (>50-fold) were observed compared to those on random fibers, in addition to decreased osteogenic differentiation (alizarin red) together confirming tenogenic differentiation of hiPSC-MSCs in vitro. Dil-labeled 14.5×10^5 hiPSC-MSCs/scaffold was transplanted into 6 mm wound created by removal of Achilles tendon in rats that were pre-treated for 24 h with cyclophosphamide. Anti-human nuclear staining showed viable cells at 2- and 4-week post-transplant with high degree of alignment along the axis of tensile load, better histology score, more collagen fiber deposition in the aligned fiber group, when compared to random fiber scaffold. Tendon ECM-related markers such as Col1a1, Col5a1, and Bgn were three-fold, five-fold, and six-fold increased 4-week post-align fiber transplant compared to random fibers. In addition, deposition of ColI and DCN, diameter of collagen fibers and stiffness were significantly higher in the aligned fiber group than the random

fiber scaffolds. Collectively this suggested ultrafine fiber induced differentiation and tendon regeneration by human iPSC-MSCs.

iPSCs from human gingival fibroblasts (hGFs) were generated by Ji et al. [43] and further tested their osteogenic differentiation potential using nanohydroxyapatite/chitosan/gelatin (HCG) scaffolds. hGFs were reprogrammed with Yamanaka factors and embryoid bodies (EBs) were generated, from which ESC like iPSC colonies were developed. Thus generated hiPSCs showed enhanced osteogenic differentiation (alizarin red) compared to hGFs. Composite scaffolds with different ratios of nHA particles, chitosan, and gelatin (HCG-111 and HCG-311) were prepared with different densities and characterized for phase separation, pore size, stiffness, adsorption, and compatibility with hiPSCs. HCG-111 showed larger pore width (51.1 ± 4.5 μm), smoother pore walls (SEM), but HCG-311 had more nHA particles on the surface and showed more adsorption with α-MEM medium. 12-week post-culture, HCG-311 scaffold was filled with cells, ECM showed an increased bone-associated gene expression (OCN and OPN), lower expression of early markers (ALP and Col1) and was less degraded than the HCG-111. hiPSC-derived 1×10^6 Stro-1 positive cells were seeded onto 8×5 mm scaffolds and transplanted subcutaneously into nude mice after 1 week of incubation. Post-12-weeks, transplanted mice showed new bone formation, larger bone formation observed with HCG-311, with increased RUNX-2 and OCN expression compared to HCG-111 scaffolds, suggesting improved neobone regeneration with hiPSCs and HCG-311 scaffolds.

Worthington et al. [44] have used surfactant (DTAB and Brij 56) templated methacrylated chitosan hydrogels (MeCTS) for neuronal differentiation of iPSCs. Post fabrication, hydrogels were washed to remove the surfactants and incubated with pluripotency media for 24 h. Mouse fibroblast derived 2×10^4 iPSCs/well were seeded and tested for cytocompatibility. Cell attachment and growth was observed on non-templated and DTAB templated MeCTS hydrogels; however, Brij56 templated hydrogels did not support the growth of iPSCs. 1-week post-culture pluripotency markers Nanog, Oct4, Sox2, Lin28, Dnmt1, and Klf4 were expressed in iPSCs cultured on non-templated and DTAB templated hydrogels. Post-2 weeks, iPSCs expressed mRNA levels of neural retina markers Pax6, Otx2, and Rx and protein levels of neuronal differentiation marker Tuj-1 on non-templated and DTAB templated MeCTS hydrogels. Functionality of these cells cultured on MeCTS hydrogels needs to be determined.

Chitosan (CS) and chitosan-hyaluronic acid (CS/HA) membranes were prepared by Chang et al. [45] and their effect on the 3D spheroid formation, differentiation potential of human iPSCs (cell lines form cell bank, Tokyo) was studied and compared with that of vitronectin (VTN) substrates. At a cell density of 5×10^4 cells/mL, 90% of hiPSC spheroids of <200 μm diameter were formed on CS and CS-HA when cultured for 2–4 days. High alkaline phosphatase (AP) activity, increase in Oct4, Nanog, Tra1–60, SSEA4 was observed in the 3D spheroids on the CS membranes. EB formation, differentiation into ectodermal (Nestin), mesodermal (α-SMA), endodermal (GATA4) lineages were showed by both VTN and CS derived hiPSCs. Teratoma formation with ecto, meso, and endoderm layers was

observed 12-weeks post in NOD/SCID mice. RNA sequencing data showed that relative to VTN-derived, the CS-derived iPSCs showed differential expression of 400 genes (DEGs), with 243 upregulated and 189 downregulated genes. The DEGs were related to response to metal ions, hypoxia, might be due to 3D spheroid formation. An increase in naive marker genes was observed when cultured on CS and CS-HA membranes, however pluripotent markers Oct4, Sox2 and Sall4 did not change significantly. When passaged, the hiPSC-3D spheroids survived up to 100 passages, with high AP activity and pluripotency markers and germ layer differentiation potential. When BMP and TGFβ signaling were inhibited, hiPSC-3D spheroids showed neural stem cell like phenotype (Sox9, Pax6, Msi1, Sox2, and Nestin). When modulated with Wnt signaling for a week, beating cardiomyocyte-like spheroids were observed after 2 weeks, with cardiomyocyte marker (Actn2, Tnnc1, and Tnnt2) gene expression and cardiac troponin T (cTnT) at 3 and or 15-weeks post-differentiation. hiPSC-3D spheroids when induced on CS membranes showed hepatocyte differentiation (Alb, Cyp3a4, Ugt1a1) with albumin and E-cadherin expression at 20 days. This data suggests that CS membranes are a promising alternate to maintain and differentiate hiPSCs in 3D spheroid form, better than the conventional VTN substrates.

2.4 Stem Cell-Derived Extracellular Vesicles

Extracellular vesicles (EVs) or previously known as exosomes are released by cells as an inter-cellular communication network that carries information in the form of proteins, lipids, nucleic acids (RNA, DNA), etc. [46]. Based on the type and state of the cells, these signals could be regenerative, destructive, immunomodulatory, etc. Stem cell-derived EVs seem to be more interesting with respect to the regenerative property and paracrine signaling of stem cells, such as MSCs. Their small size (50–200 nm) and non-immunogenic properties make the EV therapeutics a promising alternate to cellular therapeutics. However, challenges remain in clinical grade and scale of EV production from stem cells, route of delivery of EVs due to their short life span, etc.

Chitosan is widely studied for its drug delivery. In the study by Tao et al. [47] MSCs were isolated from the Synovium (SMSCs) and miR-126-3p was overexpressed, exosomes were obtained and were incorporated in chitosan hydrogel tested for their potential to repair cutaneous wounds. SMSCs were positive for CD73, CD44 and negative for CD34, CD45 and were able to differentiate into osteo, chondro, and adipocytes. 48 h SMSC conditioned media was subjected to ultracentrifugation and filtration to obtain EVs that were 30–150 nm and positive for Alix, Tsg101, CD9, CD63, and CD81 markers. miR-126 was overexpressed in SMSCs using lentivirus, and exosomes were isolated from these cells and incorporated into chitosan hydrogel and freeze dried to remove water (CS-SMSC-126-Exo) and characterized in comparison to CS hydrogel alone. Both hydrogels exhibited some weight loss during preparation, CS-SMSC-126-Exo showed higher

exothermic peaks, 10 μm macropores, nanoparticles of CS and C, O, and H according to SEM and EDS data. Immersion of CS-SMSC-126-Exo for 6 days in media showed a total of $183.08 \pm 15.44 \times 10^8$ exosome particles (ExoELISA) and Dil labeling also confirmed the release and presence of exosomes in perinuclear region of HMEC-1. Increase in miR-126 was confirmed by qRT-PCR in the SMSC-126-Exo. When added to HMEC1, SMSC-126-Exo increased the proliferation, migration, and tube formation potential of HMEC-1 cells and activated AKT and ERK1/2 pathways. In in vivo, CS-SMSC-126-Exo transplanted wounds were closed significantly faster by day 3, 7, and 14 with better healing than the controls. A significantly increased blood vessel number was observed in CS-SMSC-126-Exo transplanted wounds at day14 as observed with micro-CT. Further histological scoring confirmed re-epithelialization, thicker and mature granulation tissue, collagen deposition, development of hair follicles and sebaceous glands with CS-SMSC-126-Exo treated group. More number of newly formed vessels were identified with CD31 and α-SMA co-staining in CS-SMSC-126-Exo group at day 7 and day 14 in comparison to CS alone and non-treated groups. This study suggests that incorporation of miR-126 overexpressing SMSC-exosomes could be regenerative post-encapsulation into CS hydrogel and is a promising delivery method for EV therapeutics.

3 Summary and Future Perspectives

The goal of this review is to bring together the knowledge on different forms of chitosan and its interaction with multiple stem cell types such as adipose-derived MSCs, bone marrow-derived MSCs, induced pluripotent stem cells (Table 1). Successful regeneration and long-term functional recovery of tissue engineering constructs depends largely on their biocompatibility, stability, long-term integrity, and functionality, which in turn is influenced by the affinity between biomaterial and cells. As discussed in this review chitosan (CS) has been used in the form of hydrogel, scaffold either alone or in combination such as hydroxyapatite, collagen, gelatin, silk fibroin, etc. Studies that focused on the use of stem cells with chitosan largely focused on bone regeneration, myocardial infarct repair, and wound healing (Fig. 2). With the same type of CS hydrogel, three different cell types, AD-MSCs, BM-MSCs, and WJ-MSCs showed distinct osteogenic potential [27]. Especially with complex chemistry, the composite scaffold has broader properties, which need to be often tested with multiple cell types to identify a most compatible-functional cell type. Components such as cellular signaling modulating growth factors, cytokines, chemokines could be incorporated to enhance the endogenous stem or somatic cell stimulation, for example, like VEGF to stimulate endothelialization. Most of the studies focused on achieving recovery or successful repair of damage; however, more insight into mechanism of recovery, whether mediated by chemical or physical properties of hydrogels/scaffolds could provide better understanding of the materials' potential. This knowledge could be extrapolated to other models of

Table 1 Table showing different reports that have used chitosan and AD-MSCs, BM-MSCs, and iPSCs for regenerative application

References	Stem cell type	Stem cell source	Stem cell characterization	Modification of cells	Hydrogel/scaffold type	Application	Species tested	Model
[28]	BM-MSCs	Rat	CD29, CD45	NA	Collagen-chitosan porous scaffold	Neuropathological repair	Rat	Traumatic brain injury
[27]	BM-MSCs	Human	Characterized previously	NA	Chitosan/hydroxyapatite/PCL	Bone regeneration	Rat	Bone defect model-tibia
[29]	BM-MSCs	Rat	Characterized previously	NA	Ferrite NP incorporated hydroxyapatite/chitosan (HA/CS) scaffold	Bone regeneration	Rat	Bone calvarial defect model
[30]	BM-MSCs	Mouse	Characterized previously	Transgenic mice expressing luciferase and GFP	Chitosan hydrogel	Cardiac regeneration	Mouse	Myocardial Infraction
[31]	AD-MSCs	Human	Characterized previously	Lentiviral eGFP	Silk-fibroin-chitosan scaffold	Wound repair	Mouse	Cutaneous wound
[32]	AD-MSCs	Rat	CD31, CD45, CD90, CD29; alizarin red, oil red O staining	Lentiviral firefly-luciferase and mRFP	Chitosan hydrogel	Cardiac regeneration	Rat	Myocardial Infraction
[32]	AD-MSCs	Human	CD31, CD45, CD90, CD29; alizarin red, oil red O staining	NA	Chitosan coated plates	Stemness maintenance	In vitro only	
[34]	AD-MSCs	Porcine	CD29, CD44 positive, tri-lineage differentiation	NA	Chitosan coated plates	Insulin secretion	In vitro only	
[42]	iPSC-MSCs	Human	Nanog, Oct4, SSEA4; CD31, CD45, CD90, CD29; alizarin red, oil red O staining etc.	NA	Chitosan-based ultrafine fibers	Tendon regeneration	Rat	Achilles tendon defect

[43]	iPSCs	Human	Characterized previously	NA	Nanohydroxyapatite/ chitosan/gelatin scaffolds	Osteogenic differentiation	Mouse	Nude mice
[44]	iPSCs	Mouse	Characterized previously	NA	Surfactant templated chitosan hydrogel	Neuronal differentiation	In vitro only	
[45]	iPSCs	Human	Characterized previously	NA	Chitosan hyaluronic acid membranes	3D culture, neuronal, hepatocyte, and cardiomyocyte differentiation	In vitro only	

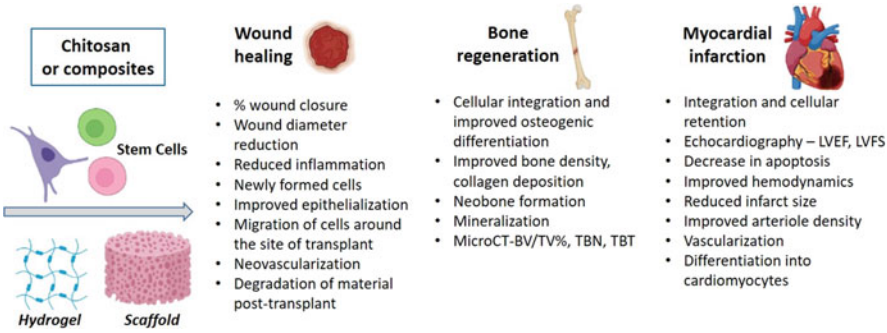

Fig. 2 Schematic showing regenerative end points that were considered with chitosan-based materials in different models of wound healing, bone regeneration, and myocardial infarction. Made with Biorender.com

regeneration. Liu et al. [32] have used myocardial infarct model and suggested that CS might have a recovery effect on ROS laden microenvironment. ROS is one of the major consequences in normal tissues when exposed to radiation. Therefore, ROS scavengers, mitigators are of great interest for tissue regeneration post-radiation damage. CS and other biomaterials with such properties need to be studied using radiation models to get better insight into their potential.

In addition to stem cells, stem cell-derived products such as exosomes, 3D organoids could be more tested for regenerative application, for instance, by incorporating into hydrogels or scaffolds. EV therapeutics is currently an accelerating field and seem to hold great future in regenerative medicine. Storage of EVs is more challenging and studies that could investigate freeze-drying or lyophilization of EV incorporated hydrogels might solve the problem of storage, delivery, and in vivo sustained release of EVs.

Though we could not cover all the studies within the scope of this review, there are many interesting studies with acellular composite CS scaffolds in various small and large animal models of diseases.

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