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Chapter 5

Chitosan, Chitosan Derivatives and their Biomedical Applications

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Abstract
Chitosan is one of the most studied polysaccharides nowadays. Because of its biocompatibility, biodegradability and abundance in nature, it has had a wide number of applications. In this chapter, an overview of chitosan including its physicochemical properties and characterization methods is presented. Subsequently, the main chitosan chemical modifications via the hydroxyl and amino groups are discussed. These chemical modifications improve chitosan physical properties and expand its range of applications especially in the biomedical field which will also be studied.

Keywords: chitin, chitosan, hydrogels, chitosan derivatives, biomedical applications

1. Introduction
Chitin is the second most abundant polysaccharide in nature after cellulose and can be found in the exoskeletons of crustaceans and mollusks, insect cuticles and fungi [1]. Due to its low solubility in water as well as most organic solvents, chitin is usually converted to chitosan by deacetylation process, obtaining a soluble material in aqueous acid medium. Considering its nontoxicity, biocompatibility, biodegradability and indirect abundance in nature, chitosan has attracted much research interest and has found potential applications in pharmaceutical, textile, paper and food industries, as well as in agriculture and medicine [2, 3]. On the other hand, chitosan structure can be modified through its amino group and the hydroxyl groups. These chemical modifications improve chitosan mechanical properties and its solubility or bring new functional properties and promising applications. In this chapter, the generalities about chitosan will be discuss, including both chemical and enzymatic isolation processes, the characterization techniques and its physicochemical properties. Subsequently, we will focus
particularly on the current state of knowledge of chitosan functionalization methods, including carboxymethylation, cross-linking, copolymer grafting, among others. Finally, the most recent advances in the biomedical applications of chitosan and its derivatives, especially tissue engineering, drug delivery systems, gene therapy systems and wound healing will be discussed.

2. Chitin

Chitin is a linear polysaccharide composed by units of 2-acetamide-2-deoxy-β-D-glucopyranose linked through β(1, 4) bonds. This polysaccharide has a structure similar to cellulose but instead of having a hydroxyl group at carbon number 2, chitin has the N-acetyl group as can be seen in Figure 1.

Chitin was isolated in 1811 by French chemist and botanist Henri Braconnot, who found in some fungi an insoluble material in alkaline conditions [4, 5]; chitin was the first polysaccharide identified by man, even before cellulose which was discovered in 1838 by French chemist Anselme Payen [6]. In 1823, Antoine Odier found a similar material described by Braconnot while studying the cuticle of some insects and called it chitin, from the Greek “chiton” which refers to a robe or wrap. In 1824, Children extracted chitin from May bug elytra and found nitrogen by elemental analysis. Payen, Fischer and Leuchs also noted the presence of nitrogen in chitin in 1843. Later, Karrer and Zechmeister carried out experiments with chitin and found the N-acetylglucosamine as its main component. Finally, Meyer and Pankow confirmed the structure of chitin through X-ray diffraction studies in the first half of the twentieth century [7].

Chitin has average molecular weight (MW) between $1.03 \times 10^6$ and $2.5 \times 10^6$ Da and is a polysaccharide insoluble in water as well as in most organic solvents. Chitin can be dissolved in solutions with high ionic strength as hexafluoroacetone or dichloroethane with mineral

Figure 1. Chemical structure of: (a) chitin and (b) cellulose.
acids, and 5% lithium chloride in dimethylacetamide [2]. In 2006, Tamura and coworkers solubilized chitin in saturated methanol with dihydrate calcium chloride [8]. The insolubility of chitin is associated with a high degree of crystallinity due to the formation of different hydrogen bonds between the N-acetyl groups (N-H…O=C) at C-2 and hydroxyl group at C-3 and C-6 with N-acetyl group (O-H…O=C), keeping chitin chains tightly bound. There are three crystalline forms for this polysaccharide, depending on the source of chitin. α-Chitin is the most abundant form and is present in the shell of shellfish and fungi cell walls. In this crystalline form, chitin chains are organized in an antiparallel configuration, allowing an orthorhombic crystal to form and giving rigidity to the polymer. In β-chitin form, the chains are aligned in parallel, as do cellulose chains, forming monoclinic crystals. In this case, there are more intramolecular hydrogen bonds rather than intermolecular interactions. β-Chitin is commonly associated with proteins in squid and diatomaceae and has weak packing. Finally, there is a γ-chitin form, which is a mixture of the α and β forms. γ-Chitin combines the properties of both polymorphisms and swells when in contact with water [9].

3. Chitosan

Chitosan is obtained via partial or total chitin deacetylation (Figure 2) and can be classified as a copolymer of 2-amino-2-deoxy-β-D-glucopyranose (glucosamine) and 2-acetamide-2-deoxy-β-D-glucopyranose (N-acetylglucosamine). In general, when the content of N-acetyl groups is >50% is considered chitin, while for lower values is considered chitosan. It has nitrogen content of 6.80% or higher and is characterized by molecular weights between 1 × 10^5 and 5 × 10^5 Da. The discovery of chitosan is attributed to Rouget, who in 1859 found that when chitin was heated in alkaline medium, a material that was soluble in organic acids was obtained [4]. In 1894, Hoppe-Seyler called this material chitosan; however, only until 1950, its chemical structure was elucidated [5].

As a biopolymer, chitosan has potential biomedical applications, since it is biocompatible and biodegradable. Due to its solubility in acidic aqueous medium, many applications at industrial level can be found for chitosan; its solubility is related to the degree of acetylation, molecular weight, and distribution of the acetyl and amino groups along the chain. Additionally, antimicrobial activity is attributed to chitosan when the amino groups are in cationic form, which means that antimicrobial activity of chitosan is higher at low pH [10]. Chitosan has a
broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria [11]. Due to this property, chitosan is a natural antimicrobial agent with potential application in agriculture, food, biomedical and biotechnology fields [12].

4. Global distribution of chitin and chitosan in nature

Chitin is the second most abundant polysaccharide in nature after cellulose [13], it is widely distributed in fungi, diatomaceae, arthropods and other large number of animals and plants; today, a worldwide production of $10^{11}$ tons of chitin per year is estimated [14, 15]. The commercial exploitation of chitin has been mainly focused on products derived from the marine food industry, such as shrimp, crabs, lobsters and squid [16]. Each year 6–8 million tons of waste crab, shrimp and lobster shells are produced globally [17]. The major useful components and its percentage on a dry weight basis in commercial crustacean wastes are as follows: chitin 15–40%, protein 20–40% and minerals like calcium carbonate 20–50%. The production of chitin from seafood is reported in countries such as India, Poland, Japan, China, United States, Norway and Australia [3]. On the other hand, the production of chitosan from fungal mycelia has increased in recent decades; the study of different types of fungi as a source of chitosan have been reported, with yields ranging from 1 to 8%, average molecular weights between $5.6 \times 10^4$ and $1.6 \times 10^5$ Da and degree of deacetylation (DD) between 41 and 70% [18–20].

5. Isolation of chitosan

The production of chitosan is based on a hydrolysis of the acetamide group as shown in Figure 2. When fungi are used to produce chitosan, the alkaline treatment removes the protein and deacetylates chitin simultaneously. When the shells of crustaceans are used as source of chitosan, two pretreatment are required, one to remove traces of organic material and another to remove calcium carbonate. Nowadays, there are chemicals and enzymatic methods to produce chitosan. In this section, both methods will be discussed showing the advantage and disadvantage of each of them.

5.1. Chemical method

During chemical isolation of chitosan, after obtaining the shells from different sources they are washed, dried and size reduced by grounding into a powder [21]. Then demineralization is carried out using acidic treatment with HCl, $\text{H}_2\text{SO}_4$, $\text{HNO}_3$, among others. Subsequently, the deproteination of shells requests an alkaline treatment, which usually is carried out with NaOH. Finally, a deacetylation process of chitin is performed using NaOH again but in a higher concentration, with heating, to produce chitosan. This product is dissolved in acidic medium such as diluted acetic acid, formic acid or lactic acid and finally precipitated with dilute sodium hydroxide [2, 22].

The advantages of chemical production of chitosan include the short processing time and the applicability of this methodology at the industrial scale. However, some disadvantages are found:
1. This method is environmentally unfriendly, due to the large amount of alkaline waste and organic material.

2. There is a high cost because of the effluent treatment generated after acid and alkaline reagents.

3. The continuous hydrolysis of the polymer during the alkaline treatment causes a decrease in the molecular weight and therefore its mechanical properties.

5.2. Enzymatic method

Enzymatic treatments offer an alternative way to extract chitosan from crustacean shells. Lactic acid-producing bacteria have been used for demineralization of crustacean shells instead of acidic treatment. The obtained lactic acid reacts with calcium carbonate yielding calcium lactate, which can be precipitated and removed [23]. For the deproteinization of crustacean shells, proteases from bacteria are used. The treatment consists of a fermentation of the crustacean by different species of bacteria such as *Pseudomonas aeruginosa* K-187, *Serratia marcescens* FS-3, and *Bacillus subtilis* [24]. Commercial proteases have previously been used to produce chitosan [25]. Chitin acetyl groups are removed by chitin deacetylase [25]. This enzyme was first found in *Mucor rouxii*. However, *Serratia* sp. and *Bacillus* sp. are bacteria that also produce chitin deacetylase and can be used to generate chitosan [23].

The main advantages of using enzymatic methods are the following:

1. Acidic and alkali treatments, which could be a source of environmental problems, are avoided.

2. Decreasing chitosan molecular weight and mechanical properties is avoided, because selective enzymes are used in each step.

The disadvantage of this biological method is its high cost, which limits its use only to laboratory scale.

6. Physicochemical properties of chitosan

Polysaccharides were considered a source of metabolic energy at first; however, throughout their existence, they have found many applications, as they are nontoxic, biodegradable and abundant in nature. Polysaccharides can be classified according to their acid-base character; there are neutral polysaccharides such as glycogen, cellulose and dextran. Pectin, alginic acid and agar are examples of acidic polysaccharides. Finally, there are some highly basic polysaccharides such as chitin and chitosan [2].

As mentioned previously, chitosan is a linear polysaccharide that contains copolymers of β-glucosamine and N-acetyl-β-glucosamine linked by β(1, 4) glycosidic bonds. The degree of deacetylation (DD) is defined as the glucosamine/N-acetylg glucosamine ratio; in other words, DD is the percentage of glucosamine units present in the copolymer chain. Under acidic pH, the amino groups in the chitosan chain become protonated and the polymer dissolves in aqueous media. The protonation constants pKa of chitosan change depending on the molecular weight (MW) and DD; pKa of 6.51 and 6.39 were found when MW was 1370 and
60 kDa, respectively. On the other hand, pKa value was increased from 6.17 to 6.51 when DD decreased from 94.6 to 73.3% [26].

7. Characterization of chitosan

The molecular weight and deacetylation degree are the most important properties of chitosan and dictate the quality and applications of these biomaterials. Table 1 summarizes the most common methods used to characterize chitosan.

<table>
<thead>
<tr>
<th>Chitosan property</th>
<th>Characterization methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deacetylation degree (DD)</td>
<td>Potentiometric titration [27, 28]</td>
</tr>
<tr>
<td></td>
<td>Elemental analysis [29]</td>
</tr>
<tr>
<td></td>
<td>Fourier transform infrared (FTIR) [30–33]</td>
</tr>
<tr>
<td></td>
<td>Nuclear magnetic resonance (NMR) [34–36]</td>
</tr>
<tr>
<td>Molecular weight (MW)</td>
<td>Viscometry [37, 38]</td>
</tr>
<tr>
<td></td>
<td>Gel permeation chromatography (GPC) [37, 39, 40]</td>
</tr>
</tbody>
</table>

Table 1. Chitosan characterization methods.

8. Functionalization of chitosan

Chitosan is a biomaterial that after chemical modifications can find specific biomedical applications. The presence of amino and hydroxyl groups is considered an interesting functionality to modify or improve desired properties. The chemical reactions like cross-linking, carboxymethylation, etherification, graft copolymerization and esterification are the most common modification carried out with chitosan [41].

8.1. Cross-linking

A hydrogel is a natural or synthetic polymeric system, where the chains are cross-linked through covalent and noncovalent bonds, resulting in three-dimensional networks (Figure 3). These systems are able to swell rapidly, retain large volumes of water, while maintaining their original shape [42].

Physical or chemical chitosan hydrogels can be formed, sensitive to external stimuli such as pH or ionic strength. In an acidic medium, free chitosan amino groups are protonated generating electrostatic repulsion that promotes the swelling of the polymeric material [43]. To carry out the formation of chemical hydrogels, chitosan can use both hydroxyl groups at C-3 and C-6, as well as the amino group at C-2. Due to the nucleophilic character of amino groups, they are used widely to react with the cross-linking agent. Therefore, chitosan chains are typically cross-linked with dialdehydes: glyoxal and glutaraldehyde [29]. Figure 4 shows the scanning electron microscopy (SEM) for a chitosan hydrogels cross-linked with glyoxal and
glutaric acid. The cross-linking of chitosan with glutaraldehyde and sulfosuccinic acid has been used to generate membranes with a good proton conductivity, which makes them useful in fuel cells [44]. Genipin, a compound extracted from the fruit of Gardenia jasminoides and American Genipa has also been used as cross-linker agent [45]. Bodnar and coworkers reported the preparation of chitosan nano-hydrogels, using dicarboxylic and tricarboxylic acids as nontoxic cross-linking agents, which contribute to the biocompatibility of the material [46]. Succinic, glutaric and adipic acid were used recently to prepare chitosan hydrogels [47]. The degree of cross-linking affects hydrogels features such as pore size, mechanical strength and percent swelling. It is clear that small amounts of cross-linking agent increase the absorption capacity. Increasing the cross-link density reduces the swelling due to decreased mobility.

![Diagram](image)

**Figure 3.** Three-dimensional representation of the polymeric network in a hydrogel.

![SEM images](image)

**Figure 4.** SEM of chitosan hydrogels cross-linked with: (a) glyoxal and (b) glutaric acid. From Laboratorio de Polímeros, Universidad del Valle.
of the polymer chains. Chitosan hydrogels are also used as controlled release systems, both as epidermal and intracorporeal implants, because they are able to maintain a constant drug concentration in a particular environment for a prolonged time [48, 49]. Due to the biocompatibility of chitosan, hydrogels are not only used for controlled release of drugs such as insulin [50], but also used to produce hemodialysis membranes, biodegradable sutures, artificial substituents skin burns healing agents, immobilizing enzymes and cells, among others.

8.2. Graft copolymerization

There are various techniques being used to tailor the surface characteristics of chitosan to introduce or improve desired properties. However, graft copolymerization of synthetic polymers with chitosan is of the most importance. Chitosan and carboxymethyl chitosan have been grafted with metacrylic acid by using ammonium persulfate (APS) as initiator in aqueous media. After grafting, the chitosan derivatives had much improved water solubility (Figure 5a) [51, 52]. The copolymerization of aniline with chitosan in the presence of APS has been carried out, producing films and fibers, which have shown protonic conductivity (Figure 5b) [53]. Different initiators such as, potassium persulfate (PPS), ceric ammonium nitrate (CAN), thiocarbonationpotassium bromate (TCPB), potassium diperiodatocuprate (III) (PDC), 2,2-azobisisobutyronitrile (AIBN) and ferrous ammonium sulfate (FAS) have been used to initiate grafting copolymerization [54].

Due to its solubility in both water and organic solvents, low toxicity, good biocompatibility and biodegradability, poly(ethylene glycol) (PEG) is a suitable graft-forming polymers. PEG grafted with chitosan have been synthesized using the hydroxyl groups of chitosan (Figure 5c). The new material was soluble in water and aqueous solutions of wide pH range [55].

8.3. Carboxymethylation

The low solubility of chitosan is a disadvantage in many of its potential applications. Thus, the preparation of chitosan derivatives has been carried out to improve chitosan solubility in aqueous media. One modification widely used is the carboxymethylation. The procedure consists in dispersing chitosan in isopropanol and NaOH aqueous solution under magnetic stirring and room temperature. Then, a monochloroacetic acid/isopropanol mixture is added to the suspension [56]. Carboxymethyl-chitosan is an amphoteric polymer, and the solubility depends on pH. Although during the reaction O- and N-carboxymethylation may occur simultaneously (Figure 6), under controlled reaction conditions (like temperature and stoichiometry) is possible to yield only one of them [37].

8.4. Etherification

Hydroxypropyl chitosan was prepared from chitosan and propylene epoxide under alkaline conditions. This functionalization allows to carry out graft copolymerization, for example, maleic acid was grafted onto hydroxypropyl chitosan to improve the antimicrobial applications, showing good inhibition effect against *S. aureus* and *E. coli* [51].
8.5. Esterification

Other chemical modification that is widely documented in the literature is the esterification of chitosan. Special attention has been paid to the preparation of N,O-acyl chitosan using acetyl chloride in MeSO\(_3\)H as solvent (Figure 7). Under these conditions, O-acetylated chitosan is the
major product compared to the N-acetylated chitosan [57]. It has been shown that acetylation of chitosan substantially improve its antifungal activity [58].

8.6. Phosphorylation

There are some methods to obtain phosphorylated derivatives of chitosan. These derivatives are important due to their interesting biological and chemical properties. They could exhibit bactericidal and osteoinductive properties. Phosphorylated chitosan can be prepared by heating chitosan with orthophosphoric acid in N,N-dimethylformamide (DMF). Another way to prepare phosphorylated chitosan is by the reaction of chitosan with phosphorous pentoxide in methanesulphonic acid, this method is considered very efficient (Figure 8) [59].

The methods described here promote phosphorylation of the hydroxyl groups in carbons 3 and 6 of chitosan.
9. Biomedical applications of chitosan

Biomedical applications of chitosan are related with its most important properties, biocompatibility and biodegradability. Biocompatibility is defined as the ability of a material to be biologically inert in the human body or any other guest while performing certain function (ASTM F2475-11). In other words, biocompatibility is directly related to cytotoxicity that may present the material. The cell culture method is the most simple and widely used way to study both the toxicity and the chitosan-cell interaction. L929 fibroblasts studies supported on chitosan have shown biocompatibility of this biopolymer trough no interference with cell growth, on the contrary, favoring its proliferation [60, 61].

On the other hand, biodegradability is defined as the ability of a material to decompose into low molecular weight molecules like carbon dioxide, methane and water by an enzymatic process (ASTM D-5488-944). Biodegradation of chitosan is related to its depolymerization, oligomers produced are subsequently decomposed to the corresponding monomers glucosamine and N-acetylglucosamine. To carry out this process of depolymerization, nature uses the bacterial enzyme known as chitinase, which is present in the digestive tract of vertebrates and many invertebrates such as fish, lizards, birds and mammals such as pigs. For humans, biodegradation of chitosan is mainly associated with the enzyme lysozyme, widely available in macrophages [62].

9.1. Tissue engineering

Tissue engineering involves the use of scaffolds for the formation of new viable tissue for a medical purpose. To generate tissue, it is necessary to use a supporting material including natural and synthetic polymers. Polylactic acid (PLA) and polyglycolic acid (PGA) are commonly used for fibroblasts growth at laboratory scale [63, 64]. However, chitosan is now one of the leading cell culture media due to its biocompatibility and because it accelerates the growth process. In many of these studies, chitosan, PLA and PGA are mixed to form films, porous structures or beads [65].

Electrospinning is the production of fiber using electric field to draw charged threads of polymer solutions. This method allows to produce scaffolds with tissue engineering applications from different polymeric materials. Due to the repulsive forces between ionic groups within polymer backbone in chitosan, it is restricted the formation of continuous fibers during the electrospinning process. However, some fiber of chitosan have been prepared successfully using trifluoroacetic acid, which can destroy the rigid interactions between the chitosan molecules, improving the electrospinning process and the diameter of the chitosan fibers [66, 67]. Figure 9 shows PLA nanofibers with a chitosan coating produced by coaxial electrospinning. These nanofibers nets are used to grow osteoblasts and promote the regeneration of bone tissue.

9.2. Drug delivery systems

Like agar, glucomannan and pectin, chitosan with low molecular weight can be used as carrier for solid drug formulations because of their easy bioabsorption, with the advantage that it acts as an antacid, preventing stomach irritation [2]. Chitosan has been studied for colon-targeted delivery of a drug, due to its pH sensitivity and its complete digestion by the colonic bacteria [68].
9.3. Gene therapy systems

Delivery systems using DNA as a kind of therapeutic agent are considered gene therapy systems, which are a powerful tool for curing many hereditary diseases and treating acquired diseases such as multigenetic disorders [69]. Chitosan is a cationic polyelectrolyte whose positive charge provides a strong electrostatic interaction with negatively charged DNA and protects it from nuclease degradation [70, 71]. For that reason, chitosan is used for nonviral gene delivery. Nanoparticles of folic acid-chitosan-DNA have been used to improve the targeting and transfection rates in gene therapy. These nanoparticles have lower cytotoxicity and good DNA condensation. In this case, folic acid is attached to chitosan for targeting cell membrane with folate receptor overexpressed on human cancer cells [72]. Deoxycholic acid-modified chitosan were prepared and characterized to yield self-aggregates/DNA complexes in aqueous media. This chitosan self-aggregates were used as a delivery carrier for the transfection of genetic material in mammalian cells [69]. Reverse microemulsion was used as a template to fabricate chitosan-alginate coreshell nanoparticles encapsulated with enhanced green fluorescent protein (EGFP)-encoded plasmids. These chitosan-alginate coreshell nanoparticles endocytosed by NIH 3T3 cells causes swelling of transport vesicles which renders gene escaping before entering digestive endolysosomal compartment and promotes gene transfection rate [73, 74].

9.4. Wound healing

Wound healing is a biological process related with growth and tissue regeneration. The wound healing process has five important stages: homeostasis, inflammation, migration,
proliferation and maturation [75]. A number of studies have reported the use of chitosan membranes to produce wound healing with potential application in patients with deep burns, wounds, etc. PVA have been mixed with chitosan for the preparation of composites and blends films. PVA-chitosan membranes have shown an increasing of the mechanical properties [41]. Chitosan films with oleic acid and glycerol at 1% were prepared previously. These chitosan films exhibited suitable morphology, and in vivo studies with Wistar® rats showing that implanted chitosan films were biocompatible and bioabsorbable leaving the tissue healthy. For all this, chitosan films may allow their use for wound healing [76]. Due to the importance of antibacterial resistance in the field of wound care to avoid complications such as infection and delayed wound healing, nanoparticles of silver combine with chitosan has been used as an antibacterial agent [75, 77].

9.5. Antioxidant and antimicrobial properties

Chitosan is a polysaccharide with antimicrobial properties [78]. The antimicrobial mechanism of chitosan is currently unknown. However, it has been suggested that the polycationic nature below pH 6.5 is a decisive factor. The positive charge in the backbone chain interacts with negatively charged components in microbial cell membranes, altering their barrier properties, and thereby preventing the entry of nutrients or causing the leakage of intracellular contents [79]. Due to its ability to form films, chitosan is employed to produce food packaging as a potential food preservative. However, the limited solubility of chitosan restricts its applicability. At the same time, chitosan have been considered a natural antioxidant [80–82]. Thus, different functionalizations have been carried out to improve both the antimicrobial and antioxidant activities of chitosan.

Functionalization of chitosan with epigallocatechin gallate (EGCG) by a free radical-induced grafting procedure improves both its antimicrobial and its antioxidant activity [83]. Chitosan antioxidant activity can be enhanced by grafting on it protocatechuic acid which is a natural phenolic antioxidant [84]. Chitosan modified with monomethyl fumaric acid presented antibacterial activity against Gram-positive and Gram-negative bacteria [85]. Other chitosan derivatives with antimicrobial activity included the following: chitosan modified with thioglycolic acid [86] and chitosan with polyethylene glycol diacrylate (PEGDA) [87].

10. Summary

Chitin is the second most abundant polysaccharide in nature after cellulose. However, due to its solubility problems, scientific research has focused more on studying its main derivative, chitosan. Chitosan is a compound of great interest due to its biocompatibility and biodegradability. At the same time, antimicrobial and antioxidant properties have been found in chitosan. For all these properties, chitosan has potential application at industrial level as well as biomedical applications. The chemical modifications of chitosan through its functional groups have extended its range of applications, improving its mechanical properties, its solubility,
among others. Chitosan is a promising material because in addition to various applications, it is a product that is produced from waste seafood, offsetting the negative impact they may have. For all this, we are about to find chitosan commercially available in many everyday products.

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