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## **Chitosan based-nanoparticles and nanocapsules: Overview, physicochemical features, applications of a nanofibrous scaffold, and bioprinting**

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1 **Chitosan based-nanoparticles and nanocapsules: Overview, physicochemical**  
2 **features, applications of a nanofibrous scaffold, and bioprinting**

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14 **Abstract**

15 Recent advancements in the synthesis, properties, and applications of chitosan as the second after  
16 cellulose available biopolymer in nature were discussed in this review. A general overview of  
17 processing and production procedures from A to Z was highlighted. Chitosan exists in three  
18 polymorphic forms which differ in degree of crystallinity ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). Thus, the degree of  
19 deacetylation, crystallinity, surface area, and molecular mass significantly affect most  
20 applications. Otherwise, the synthesis of chitosan nanofibers is suffering from many drawbacks  
21 that were recently treated by co-electrospun with other polymers such as polyvinyl alcohol  
22 (PVA), polyethylene oxide (PEO), and polycaprolactone (PCL). Ultimately, this review focuses  
23 on the area of new trend utilization of chitosan nanoparticles as nanospheres and nanocapsules, in  
24 cartilage and bone regenerative medicine. Owing to its biocompatibility, bioavailability,  
25 biodegradability, and costless synthesis, chitosan is a promising biopolymeric structure for water  
26 remediation, drug delivery, antimicrobials, and tissue engineering.

27 **Keywords:** Chitosan; chitosan production; the degree of deacetylation; chitosan physicochemical  
28 properties; chitosan nanofibers; bioprinting.

## 29 **1. Chitosan source**

30 Chitosan consists of residues of 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose and 2-amino-2-  
31 deoxy- $\beta$ -D-glucopyranose (Fig. 1) and it is the second prevalent biopolymer in nature after  
32 cellulose. The -NH<sub>2</sub> and -OH in the chitosan structure are the main functional groups responsible  
33 for chitosan properties and its unlimited application. In 1859, Rouget discovered chitosan when  
34 he studied the deacetylated forms of chitin from crustacean marine [1]. Since the first  
35 international conference of chitin and chitosan (Boston, USA, 1977), scientific and industrial  
36 communities have shown exponential interest in chitin and chitosan as a costless biopolymeric  
37 structural form [2].

38 Crustaceans, insects, mollusks, and fungi are the main sources of chitosan. In industry,  
39 chitosan is made from the external chitinous exoskeleton of marine crustaceans such as crabs,  
40 shrimps, lobsters, crayfish, krill, and woodlice which are waste products that originated from the  
41 fishing industry [3]. First, the crude material is subjected to demineralization, deproteinization,  
42 deodorization, and drying to extract chitin. Then, the chitin is being deacetylated, cleaned, and  
43 left to dry to gather chitosan. This method is economically efficient since raw materials are not  
44 associated with any costs and could include the extraction of Astaxanthin. Currently, chitosan is  
45 commercially produced in the entire world and its price in powder form depends on the  
46 application, properties, and purity. The main characteristics that ascertain the biopolymer of  
47 chitosan are its molecular weight and the degree of deacetylation. Other properties are important,  
48 such as crystallinity, surface area, particle size, air humidity, and ash content [4, 5]. These  
49 characteristics are important in controlling the properties of chitosan, i.e. solubility, polycation  
50 nature, antimicrobial activity, biocompatibility, and bioadhesion. Consequently, different shapes  
51 and physical forms of chitosan such as films, nanofibers, nanoparticles, nanocapsules,  
52 microparticles, membranes, sponges, scaffolds, and hydrogels determine their possible utilization  
53 in water treatment, food processing, cosmetics, agriculture, and biomedicine [6-8].

### 54 **1.1. Processing and production of chitosan**

55 In general, two main processes are used for chitosan production: the ordinary process and  
56 the fermentation process [9]. In the ordinary method, the sources are crustaceans, insects, or  
57 mollusks. First, chitin is isolated from the raw materials using various chemical treatments that  
58 remove ash, proteins, color, and flavor. Chitin is converted to chitosan by alkaline deacetylation.  
59 Several phases are performed to purify chitosan such as dissolution, precipitation, centrifugation,  
60 and finally, the drying phase. In the fermentation process, chitosan is obtained from fungi, i.e.  
61 fungal biomass containing glucan complexes with chitin or chitosan is obtained by fermentation,  
62 [10]. Then the biomass is separated from the fermentation medium by filtration and drying and  
63 the next successive steps are performed to extract the chitosan [11]. Fig. 2 shows the main steps  
64 of the ordinary method to produce chitosan. This process usually begins with the  
65 demineralization phase. The main goal of this step is to reduce the ash and mineral content in the  
66 crustacean waste. Ash mainly consists of inorganic traces such as carbonates, phosphates, and  
67 other mineral salts [12]. Demineralization occurs mainly in dilute HCl solutions (1-10%) assisted  
68 by stirring at room temperature for short periods of nearly 1-3 hours [13]. This treatment  
69 guarantees complete removal of the mineral content without destroying the polymer chains of the  
70 chitin. Other acids like nitric acid, sulfuric acid, formic, acetic, and EDTA can be also used [14].  
71 Unfortunately, these nominated acids are effective, but they can damage the structure of chitin in  
72 the depolymerization process. The second step is deproteinization that includes the removal of  
73 proteins residues adhered to the demineralized powder. It proceeds with alkalis including sodium  
74 hydroxide, sodium bicarbonate, sodium carbonate, potassium carbonate, potassium hydroxide, or  
75 calcium hydroxide. The most common option is the use of sodium hydroxide (1-10%) with a  
76 gentle stirring at a temperature that reaches 100 °C and the period between 2 and 72 hours. A  
77 deproteinization requires effective control of time and temperature, as high temperatures and  
78 longer periods can lead to depolymerization and deacetylation. On the other hand, enzymatic  
79 treatment with proteases enzymes such as pepsin and trypsin is an alternative form of  
80 deproteinization but less efficient than alkaline treatment [15].

81 The third step in the isolation of chitin is deodorization/depigmentation responsible for  
82 removing pigments and odors. For this purpose, ethanol, acetone, potassium permanganate,  
83 sodium hypochlorite, or hydrogen peroxide are generally used [16]. It is important to notify that  
84 the above-mentioned phases are followed by several successive washing processes up to  
85 obtaining a controlled neutral pH value. The final product of demineralization, deproteinization,

86 and deodorization/depigmentation is chitin. It can then be dried for later use or immediately  
87 deacetylated.

88 The deacetylation reaction is the most important step in this processing, at this stage, chitin  
89 turns into chitosan. During the deacetylation of the chitin C-2 position, the acetamide group is  
90 converted into amino groups (-NH<sub>2</sub>) [17]. When the percentage of amino groups attains around  
91 50% or more, the polymer becomes soluble in aqueous acidic media, and in this case, chitosan  
92 can be obtained [18]. Alkaline hydrolysis with sodium hydroxide (40-50%) at high temperatures  
93 (80-120 °C) is the most common method for extracting chitosan from chitin [19]. The main  
94 factors influencing the deacetylation reaction are the alkali concentration, reaction time,  
95 solid/liquid ratio, temperature, and the size of the chitin particles. Several alternative methods  
96 have been studied to improve or replace conventional alkaline hydrolysis, including enzymatic  
97 agents, vapor explosion, microwave deacetylation, and ultrasonic deacetylation [20].

98 The purification step can be performed to produce high purity chitosan. In this case, the  
99 deacetylation product is dissolved in an acidic medium, obtaining a viscous solution with an  
100 insoluble fraction consisting of impurities, mainly ash, which has not been completely removed  
101 in the demineralization step. This viscous solution is centrifuged or filtered, and the insoluble  
102 fraction is discarded. The alkali is then added to the viscous solution, and consequently, a  
103 suspension with precipitated chitosan is formed. The suspension is centrifuged again to obtain  
104 pure chitosan (90-95%) in the form of a paste. The previous steps (dissolution / centrifugation /  
105 precipitation / centrifugation) can be repeated several times to obtain chitosan with high purity  
106 (purity greater than 99.9%) [21].

107 Drying is the last step and is necessary to obtain pure chitosan. This process is important to  
108 produce chitosan and to provide the moisture content which is required to conserve the product  
109 without changing the material. Typically, after drying, the desired product should have a moisture  
110 content of less than 10% (in the wet state) to provide good physicochemical and microbiological  
111 aspects after long-term storage. Polymerization reactions and Maillard reactions are the most  
112 important changes that should be avoided during the drying operation. Some methods, such as  
113 convection, floor drying, drying in the nozzle layer, spray drying, sun drying, oven drying,  
114 infrared drying, freeze-drying, and low-pressure overheating have been used to dry chitosan. All

115 these methods have their advantages and disadvantages, while air, temperature, and residence  
116 time are the key factors that must be controlled to obtain a good quality of chitosan product [22].

## 117 **1.2. Chitosan characteristics**

118 The main characteristics that determine the quality and flow rate of chitosan are the molecular  
119 weight, degree of deacetylation, crystallinity, surface area, and size of the particles as mentioned  
120 before [23]. Herein, the molecular mass of chitosan is a characteristic associated with the number  
121 of monomer units of a biopolymer. The evaluation and modification of this property are very  
122 important since molecular weight influences the properties like viscosity and solubility [24]. In  
123 general, the chitosan molecular weight ranges from 20 to 1200 kDa. Based on the molecular  
124 weight, chitosan can be classified as low, medium, and high molecular weight. Its molecular  
125 weight can be determined by HPLC and light diffusion but the viscometry method is the most  
126 common and simplest one [25]. In general, the molecular weight can be modified starting from a  
127 high molecular weight. Therefore, depolymerization methods are used to obtain low molecular  
128 weight edible chitosan films which more effective than high molecular weight in preserving  
129 papaya fruit and fish during their storage. The effect of the medium molecular weight of the  
130 polymer on the chitosan protein interaction was studied [26]. This study stated that increasing  
131 chitosan molecular weight from 15 to 200 kDa increases the binding constant between bovine  
132 serum albumin and chitosan in complexes.

### 133 **1.2.1. Degree of deacetylation**

134 The degree of deacetylation (DD) is the ratio between 2-acetamido-2-deoxy- $\beta$ -D-  
135 glucopyranose and 2-amino-2-deoxy- $\beta$ -D-glucopyranose. When all the monomer units are 2-  
136 amino-2-deoxy- $\beta$ -D-glucopyranose, the biopolymer is completely deacetylated and DD is 100%.  
137 When the percentage of 2-amino-2-deoxy- $\beta$ -D-glucopyranose units reaches 50% or more, the  
138 polymer being normally soluble in aqueous acid media is called chitosan [27]. In the same way,  
139 like MW, DD influences the properties and applications of chitosan. For example, an increase in  
140 DD contributes to an increase in free amino groups in the chitosan chain. Therefore, the solubility  
141 and the polycation quality of chitosan also increase. The DD can be tested using spectroscopic  
142 methods such as Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and  
143 nuclear magnetic resonance (NMR) [28]. Chitosan with different DD can be obtained by varying

144 the deacetylation reaction conditions. The adsorption of anionic methyl orange dye by using  
145 chitosan with different degrees of deacetylation was reported. The results showed an increase in  
146 DD to 84% directly proportional to the adsorption capacity from 266 to 373 mg / g [29]. Similar  
147 findings were also obtained elsewhere for the adsorption of food dyes by chitosan in the binary  
148 system [30]. The DD has been shown in the literature to influence the mechanical properties  
149 (elongation and tensile strength) of chitosan films [31].

### 150 **1.2.2. Crystal structure**

151 The crystallinity of chitosan is the ratio between the crystalline and amorphous fractions of a  
152 biopolymer. This property is quantified as a crystallinity index (CI) which is determined by the  
153 relation between the characteristic peaks of X-ray diffraction (XRD). In the solid-state, chitosan  
154 is a semi-crystalline and polymorphic biopolymer, its unit cell is orthorhombic and contains two  
155 antiparallel chains free from water molecules [32]. Additionally, chitosan is found in three  
156 polymorphic phases that differ in the degree of crystallinity ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), depending on the source  
157 and arrangement of the chains. The commercial chitosan is a semi-crystalline polymer and CI is a  
158 function of DD. Also, the quantitative assessment of CI is important because this property affects  
159 the swelling, porosity, hydration, and absorption properties. Thakhiew's et al. [33] works showed  
160 that different methods of drying affect the physical and mechanical properties of chitosan films.  
161 They used hot air drying (HD) and low-pressure steam drying (LPSSD). They found that LPSSD  
162 provides films with a lower CI value (3-5%) than films made from HD (10-16%). As a result, the  
163 swelling degree was extended to 48-100% (LSSPD) and 75-110% (HD) [26, 34].

### 164 **1.2.3. Surface area and particle size features**

165 The surface area and size of the particles depend on the source and the extraction process  
166 associated with the porosity, pore-volume, and pore size distribution of the chitosan particles.  
167 Chitosan powder or flakes are known to have a reduced surface area (less than 10 m<sup>2</sup>/g) being  
168 non-porous materials. Particles smaller than 1 mm is commonly used in most applications. The  
169 surface area and particle size are crucial for applications such as adsorption and immobilization  
170 of enzymes since many accessible sites and porous structures are required in these vital fields  
171 [35, 36]. Some modifications have been made on chitosan to increase surface area because  
172 naturally, it is a non-porous material. The surface area is usually determined by N<sub>2</sub> adsorption-

173 desorption isotherms by the Brunauer-Emmett-Teller (BET) method. Particle size can be  
174 obtained using scanning electron microscopy (SEM), sieving test, or using a particle size  
175 analyzer. The effect of the particle size, surface area, and pore volume of chitosan on the  
176 adsorption of some food azo dyes FD&C red 2 and FD&C yellow 5 was studied before [37]. The  
177 particle sizes used were 0.10, 0.18, and 0.26 mm with surfaces of 4.2, 3.4, or 1.6 m<sup>2</sup>/g.  
178 Accordingly, the results indicated that the adsorption potential is increased by an increase in  
179 surface area and a decrease in particle size.

### 180 **1.3. Properties of chitosan**

181 The properties of chitosan depend on its characteristics which are therefore responsible for  
182 the quality and extent of use of chitosan. Among the most important properties of chitosan are  
183 solubility, polycationic nature, film formation, antimicrobial activity, and biocompatibility [38,  
184 39].

#### 185 **1.3.1. Solubility and polycationic nature**

186 The solubility and polycationic nature of chitosan are fundamental properties to produce more  
187 derivatives and it is usually a strong base with primary amino groups that have a pKa of 6.3.  
188 Therefore, this biopolymer is soluble in acid solutions diluted to pH 6.0. At low pH values, the  
189 H<sup>+</sup> ions in solution protonate the -NH<sub>2</sub> groups, which leads to the formation of (NH<sub>3</sub>)<sup>+</sup>. Therefore,  
190 chitosan is converted into a polycationic biopolymer structure. On the other hand, when the pH is  
191 above 6.0, deprotonation occurs and chitosan becomes insoluble [40]. An insoluble-soluble  
192 transition occurs when pKa with a pH is between 6.0 and 6.5. Formic acid is the most suitable  
193 solvent, while acetic acid is used more often. Other alternatives are hydrochloric acid and nitric  
194 acid. Chitosan is insoluble in sulfuric and phosphoric acids as well as concentrated acids are not  
195 indicated since they can lead to depolymerization and deformation of the polymeric chains of  
196 chitosan [41]. The solubility of chitosan and chitosan-based materials depends on several  
197 important factors, including time, temperature, DD, MW, particle size, type, the concentration of  
198 acid, and reduction methods.

#### 199 **1.3.2. Antimicrobial properties**



200 Chitosan provides antimicrobial activity observed in fungi, viruses, and bacteria (Gram-  
201 negative and gram-positive). This is a key property that allows it to be used in areas such as food  
202 storage, pharmacy, and biomedicine more than other materials [42-45]. Previous studies showed  
203 that the antibacterial property of chitosan is due to its polycationic nature. The polycationic  
204 chitosan molecule strongly interacts with the surface of the microbial cells, leading to something  
205 like a progressive shrinkage in the cell membrane and eventually to cell death. At the same  
206 concern, chitosan bactericidal activity is attributed to the electrostatic interaction between the  
207  $(\text{NH}_3)^+$  chitosan groups and phosphoryl groups of the cell membrane component, phospholipid  
208 [46]. In general, the polycationic nature of the chitosan molecule communicates with the  
209 components of the microorganism's anionic surface antigens (lipopolysaccharides and proteins),  
210 which contributes to the release of intracellular response: to fluctuations in the permeability  
211 barrier. Also, it prevents bacterial growth by stopping the entry of nutrients into the cell; once in  
212 the cell, binds to DNA and thus inhibits the synthesis of proteins and RNA [44, 47, 48].

213 In some cases, a modification of chitosan is beneficial for developing a nanoantibiotic  
214 system able to antagonize both opportunistic and obligatory pathogenic bacteria (Fig. 3). In this  
215 study, the authors developed functionalized chitosan with 3,5-dinitro salicylic acid and hooked in  
216 antibiotic linezolid. This combination is tough on Methicillin-Resistant Staphylococcus Aureus  
217 (MRSA) and other microbes [49].

### 218 **1.3.3. Biocompatibility**

219 Biocompatibility can even be categorized as "compatibility with living tissues or a living  
220 system because it is non-toxic, physiologically reactive and does not cause immune rejection".  
221 One of the principal characteristics of chitosan is its biocompatibility which allows for the use of  
222 chitosan in tissue engineering, wound dressing, bone regeneration, and other related areas [50-  
223 53]. *In vitro* and *in vivo* assays have been used to evaluate the cytotoxicity, safety, and  
224 biocompatibility of chitosan inside the body [54].

### 225 **1.4. Chitosan hydrogel**

226 Hydrogels can be represented as a 3-dimensional polymeric matrix composed of natural or  
227 synthetic materials that would have a high degree of flexibility depending on their high water  
228 content [55, 56]. They can hold significant amounts of water and are distinguished by a

229 consistency such as soft rubber-like living tissue, making them a suitable material for different  
230 utilization. For different applications, chitosan can be used as a hydrogel but the compressive  
231 strength, in this case, is fragile. Therefore, the combination of chitosan with other materials is  
232 usually used to produce hydrogels. Some methods of producing such hydrogel include radiation  
233 crosslinking technology, freeze-thawing, dissolution/lyophilization, and others [57]. The quality  
234 of the hydrogels is checked using SEM, FTIR, XRD, DSC / TGA in the same way as other  
235 chitosan-based composites. Also, depending on the application, specific features like swelling,  
236 elastic modulus, gel resistance, mechanical properties were previously studied. Cheng et al. [58],  
237 developed the heat-sensitive chitosan-based hydrogel as a local eye drug delivery system of  
238 latanoprost for the treatment of glaucoma. Hydrogel has been characterized based on its  
239 rheological properties, the release of drugs *in vitro*, its biocompatibility *in vivo*, and *in vitro*  
240 study. The release of latanoprost from the developed hydrogel was achieved and showed a  
241 prolonged release profile. In the case of the rabbit glaucoma model, the Triamcinolone-Induced  
242 Intraocular Pressure Elevation was significantly reduced over one week. In addition, a weekly  
243 topical dose of 250 µg / ml containing latanoprost hydrogel remained within normal limits until  
244 the end of the study period. The hydrogel has desirable characteristics which correlate to its  
245 delayed latanoprost release. This new composition of chitosan in the future could be a non-  
246 invasive alternative to traditional eye drops for long-term intraocular pressure control. Fan et al.  
247 [59] produced a chitosan /gelatin/ polyvinyl alcohol (PVA) hydrogel for wound dressings utility.  
248 The gelatin was blended in a combination of PVA and chitosan using radical initiator  $\gamma$ -  
249 radiation. Various experimental conditions were modified, and hydrogels were widely  
250 characterized. The Maximum tensile force was 2.2 MPa. All the hydrogels had a strong clotting  
251 effect which further indicated that the hydrogels' hemostatic effect was optimal. Hydrogels have  
252 demonstrated strong resilience to water pH, swelling and evaporation rates.

## 253 **2. Chitosan nanoparticles and nanocapsules form**

254 Chitosan and its derivatives are widely used to develop nanoparticles that increase the  
255 bioavailability of the drug when taken orally. They have a good mucoadhesive capacity,  
256 enhanced bioavailability, and dissolution rate of hydrophobic drugs. Besides, chitosan improves  
257 the stability of labile drugs, the bioavailability, and the controlled release of the drug, as the  
258 particles are beyond the nanoscale range to effectively overcome barriers and enhance

259 permeability. However, this polysaccharide as a drug release system has some disadvantages Its  
260 low solubility at physiological pH affects the intake and causes a pre-systemic metabolism with  
261 oral administration of drugs. The ability of chitosan alone to encapsulate water-insoluble drugs is  
262 also harmful because the loading efficiency is generally quite low. Various chitosan derivatives  
263 have been created and have been proven to be effective, especially when they are in nanoparticle  
264 form to remedy these deficiencies [60].

265         Polymeric nanoparticles are commonly defined as solid colloidal particles of between 10 nm  
266 and 1  $\mu\text{m}$  in size [61]. Nanoparticles are used as a method for the integration and storage of active  
267 substances in different fields and consist of macromolecular and/or molecular structures in which  
268 active substances such as drugs are dissolved, stored, encapsulated, or even adsorbed, or  
269 maintained on the external interface. Nanoparticles can be classified into two major groups based  
270 on their morphology: nanospheres, as a rule, are solid structures with a homogeneous matrix  
271 structure in which resources are distributed uniformly; and nanocapsules with a traditional hollow  
272 shell structure consisting of a polymer membrane and an internal core of the included drug [62].

273         Among the various types of innovations in nanotechnology that can be found in the  
274 nanoparticles and nanocapsules field, it is important to emphasize that both materials are the main  
275 target of the inclusion of active substances, have a high carrying capacity, and provide controlled  
276 release. The fundamental significance in the use of nanoparticles/nanocapsules in  
277 biotechnological applications instead of microparticle systems lies in their ability to increase the  
278 absorption rate, boost bioavailability and ensure delivery of targeted drugs for cancer treatment  
279 and IV distribution systems, and so on [63]. These systems, as a means of drug release, ensure  
280 the targeted release of drugs to cells instead of nonspecific diffusion and improve the  
281 solubilization and release of poorly water-soluble drugs, ensure the delivery of two or more drugs  
282 together and protect the drugs from undesirable degradations [64].

## 283         **2.1. Chitosan nanoparticles and nanocapsules preparation methods**

284         A production method in which the chitosan particle size was reduced to a nanometer size was  
285 first described in 1994 by Ohya et al [65]. The processes used to produce nanoparticles can be  
286 divided into different categories: (i) physicochemical processes in which the nanoparticles are  
287 precipitated by using preformed polymers, with yield induced by emulsification-solvent

288 evaporation, diffusion, or reverses salting-out, [25]; (ii) by in-situ chemical synthesis of  
289 macromolecules which lead to polymerizations or interfacial polycondensation reactions; and (iii)  
290 mechanical processes using high energy devices such as high-pressure homogenizers, ultrasonic  
291 devices, or wet high energy milling. The main methods for producing chitosan nanoparticles and  
292 nanocapsules are ionic gelation, emulsification and crosslinking, complexation with  
293 polyelectrolytes, self-assembly and drying processes. Some features of each method are listed  
294 below with emphasis on the main aspects of the processes.

### 295 **2.1.1. Ionic gelation**

296 Ionic gelatin is probably the most common method of producing chitosan-based  
297 nanoparticles. Chitosan is a cationic polysaccharide able to gel on contact with polyvalent  
298 polyanions as tripolyphosphate (TPP). This interaction led to the formation of electrostatic  
299 interactions between opposite charges of the components [63, 66]. At acidic pH, it can  
300 spontaneously transform into particles in the submicron size. This process seemed to be very  
301 interesting because reproducible nanoparticles can be obtained in a soft medium (aqueous  
302 medium) and avoid the use of organic solvents and high temperatures. Using chitosan TPP the  
303 nanoparticles were prepared by dissolving it in a diluted solution of glacial  $\text{-CH}_3\text{COOH}$ . The  
304 resulting solution was magnetically stirred at room temperature until completely dissolved.  
305 Therefore, the pH increased to the expected value by adding NaOH. A TPP solution can be  
306 prepared in various concentrations by dissolving the TPP in high purity water. Both solutions are  
307 filtered through a small pore filter. Then, the TPP solution is added dropwise to the chitosan  
308 solution with constant stirring until it forms an opaque suspension, which indicates the formation  
309 of particles with average diameter controlled by the molar ratio of chitosan/TPP [67]. The  
310 mechanism of chitosan nanoparticles formation by ionic gelation using TPP as well as particle  
311 internal structure and surface topology are determined by the following parameters the degree of  
312 acetylation and MW of chitosan, intrinsic viscosity, concentration, and the molar ratio of -  
313  $\text{NH}_3^+$ /TPP [68]. Other factors may also influence the properties of the nanoparticles, such as  
314 operating temperature, stirring speed, and flow rate of TPP addition. This can affect particle size  
315 and polydispersity, as they appear to significantly reduce the amount of nanoparticle aggregation.  
316 Smaller particles can be isolated from larger particles by filtration and ultracentrifugation [69].

317 This method effectively captures small hydrophobic and hydrophilic molecules, as well as  
318 peptides, proteins, and genes. The formation of electrostatic interactions within the components  
319 of the matrix is considered to be the main retention mechanism of these biologically active  
320 compounds. In addition to TPP, other polyanions can be used for crosslinking, for example, the  
321 active molecule adenosine triphosphate (ATP), fucoidan, and sodium metabisulphite ( $\text{Na}_2\text{S}_2\text{O}_5$ )  
322 [70]. Catechol-containing molecules such as hydrocaffeic acid have demonstrated the ability to  
323 increase the mucoadhesion of various polymers, including chitosan. Chitosan/TPP hydrocaffeic  
324 conjugated nanoparticles obtained by ionic gelation showed 6 times greater mucoadhesion in the  
325 rabbit's small intestine than the unmodified nanoparticles [71]. Besides, by the same technique,  
326 chitosan/calcium pyrophosphate hybrid microflowers shape was prepared and efficiently applied  
327 in dye removal and enzymatic catalysis (Fig. 4) [72].

### 328 **2.1.2. Polyelectrolyte complex (PEC)**

329 PEC's forming of chitosan nanoparticles is based on the ionic interaction of chitosan with  
330 negatively charged polymers. The process occurs when polymers are mixed with opposite  
331 charges and lead to self-assembly or the formation of strong reversible electrostatic bonds in self-  
332 assembly systems. These direct interactions between the polymer chains result in the formation of  
333 complex polyelectrolyte networks with proportional structures, thus avoiding the use of covalent  
334 crosslinking agents. In particular, these polymer network systems are commonly used as  
335 biocompatible hydrogels and are sensitive to changes in surrounded environmental conditions  
336 [73]. The emergence and stability of the PEC are based on several factors including the degree of  
337 ionization within each polyelectrolyte with an opposite charge, density of the charges present in  
338 the structure of each polyelectrolyte, concentration, and proportion of polyelectrolytes in the  
339 mixture, a sequence of the mixture and the type and location of ionic groups. In polymer chains,  
340 the stability is also correlated with the MW of the polyelectrolytes, the flexibility of the polymer  
341 chain as well as the temperature, ionic resistance, and pH of the reaction medium [74]. In the  
342 presence of a common solvent, the excess polyelectrolytes charges (positive or negative) are  
343 consolidated, and a non-stoichiometric complex is formed. In contrast, the stoichiometric  
344 polyelectrolytic complex contains equal quantities of each opposite charge, and the total charge  
345 in the resulting complex is zero. In the case of chitosan, various polyelectrolyte complexes can

346 form when the positively charged surface of chitosan interacts with anionic biopolymers such as  
347 sodium alginate, pectin, carboxymethylcellulose, chondroitin sulfate, dextran sulfate [75], etc.

### 348 **2.1.3. Drying processes**

349 This process is frequently used in drugs, chemicals, cosmetics, materials, and food science  
350 technology. The first patent for this technology originated in the early 1870s. Even during the  
351 drying process, the water/solvents are separated through evaporation from liquids, solids, or  
352 semi-solids and the resulting vapor is collected by vacuum. Hot air, a microwave oven, spray  
353 drying, freezing, supercritical drying, and natural drying with air are common methods for  
354 drying. The techniques of spray drying and supercritical drying are often used in the production  
355 of chitosan particle systems because they are fast, light, continuous, reproducible, and adapt well  
356 to the active material. They are scaled-down in one phase without modification. These techniques  
357 are important and can achieve particles of various sizes and high stability [76]. Spray drying has  
358 been reported to be used to produce dry granules and powders from mixtures of drugs and  
359 excipients in the form of a solution or suspension. The versatility of this approach has been  
360 developed to include the synthesis of microparticles from various protein-filled polymeric  
361 materials, vaccine antigens, and therapies. Spray drying provides a comprehensive, convenient,  
362 one-step, and protein-friendly method for protein-loaded chitosan micro/nanoparticles [77].  
363 Besides, it is widely used in compounds including heat-sensitive substances such as extracts,  
364 pigments, drugs, aromatic oils, and flavors in various types of conveyors, which lead to the  
365 formation of polymeric nanoparticles or microparticles as well as nanocomposites [78]. The  
366 fundamental theory of spray drying is dependent on the use of a flux of hot air to dry spray drops.  
367 This method requires the preparation of an aqueous chitosan solution that is sprayed into a drying  
368 chamber through a nozzle to achieve the desired particles. The resulting particles have a smooth  
369 surface, a spherical shape, and a reduced size distribution, and they also exhibit good drug  
370 stability with high efficiency when the drug is turned on [79]. Other benefits of this process  
371 include the ability to attach the surface and cover the balls in the stages of development which  
372 can increase stabilization and control the release of the drug. The size of the particles produced is  
373 affected by the velocity of the sprayed flow, the degree of crosslinking, and the flow rate of the  
374 airflow [80].

### 375 **2.1.4. Emulsion system (diffusion process of oil/water emulsion)**

376 Nanoparticles can be obtained using nanoemulsion in various ways. Emulsification processes  
377 usually require high mechanical energy to obtain small droplet sizes. The low power method  
378 should be considered according to the physical behavior of the system based on the mixing of oil  
379 and water in the presence of surfactants [81]. Firstly, a dispersion of chitosan in a glacial acetic  
380 acid solution is prepared as an aqueous component. Then, a drop of a hydrophobic solvent, such  
381 as cyclohexane, cyclohexanol, soybean oil, or another solvent with similar properties is added to  
382 the chitosan solution, all mixing continuously. The resulting mixture is treated with a surfactant,  
383 for example, Triton X-100 or Tween with constant stirring until the mixture becomes translucent,  
384 which indicates the formation of emulsion containing nanoparticles [82]. Another option is to  
385 pre-mix the surfactant in one of the solutions, saving a step in the process. In general, the  
386 particles formed in the emulsion system have nanocapsules structures, high drug loading  
387 efficiency, and better bioavailability [83].

388 Changes in the methodology can contribute to the formation of nanocapsules, the use of  
389 ultrasound and an increase in the reaction temperature can collaborate with the process. There are  
390 various modifications in the preparation of an emulsion using water/ oil or oil in the water  
391 system, for example, the addition of gelling agents such as chlorides, TPP, or polycations is  
392 performed [84]. Although the emulsification method facilitates particle size control, strong  
393 crosslinking agents are used in this process and complete removal of unreacted crosslinking  
394 agents can be an issue [85].

#### 395 **2.1.5. Self-organized nanoparticles**

396 Self-assembled nanoparticles can be formed in aqueous media of amphiphilic polymers.  
397 Hydrophobic interactions between amphiphilic polymer components tend to minimize interfacial  
398 energy and allow nanoparticle formation. Although chitosan is not an amphiphilic biopolymer,  
399 the self-organizing nanoparticles can be obtained by structural modification of chitosan to bind a  
400 compound such as a palmitoyl, which causes self-aggregation of chitosan molecules [86]. The  
401 hydrophobicity of chitosan is increased with the introduction of phthaloyl, polyester, fatty acids,  
402 cholesterol [87].

403 Structural changes in chitosan derivatives can make it amphiphilic, resulting in nanoparticles  
404 self-assembling and be used as drug delivery vehicles. Besides, the quaternary chitosan

405 derivatives were synthesized using (5-pentyl) trimethylammonium and dodecyl aldehyde. Auto-  
406 aggregates formed in an aqueous solution have a dimensional distribution of 250 to 500 nm and  
407 have a hydrophobic core, which can be used as a carrier for hydrophobic substances [88].

## 408 **2.2. Chitosan nanoparticles and nanocapsules characterization techniques**

409 Dimensions and size distribution are important tools because of their ability to recognize the  
410 physicochemical and pharmaceutical behavior of the compounds present in the systems.  
411 Depending on the form of preparation, laser diffraction and a coulter counter can be used to  
412 determine particle size. However, the most common method is dynamic light scattering (DLS),  
413 which is used for measuring particle size distribution. The uniformity of the particle size is  
414 obtained by using polydispersity (PDI), with PDI values between 0.10 and 0.25 indicating a  
415 reduced dimensional distribution and higher values indicating a wide dimensional distribution  
416 [89]. Particles containing chitosan or other charged molecules show a zeta potential which  
417 corresponds to the electrical potential represented by the surface of the material in the solvent and  
418 is highly dependent on the particle's composition. The zeta potential uses the electrophoresis  
419 principle that reflects the electrical charges on the surface of the particles. Electrical fields are  
420 induced from scattered particles, whereby the particles move to the oppositely - charged electrode  
421 at a rate comparable to the zeta potential value. For a dispersion system, a high level of negative  
422 or positive zeta potential indicates the stability of the particles towards aggregation, which means  
423 a stable system. This parameter can be associated with particle stability and the zeta potential  
424 values less than  $\pm 30$  mV contribute to low stability leading to a possible aggregation of the  
425 particles [90]. Various microscopy methods can be used to confirm particle size, to observe and  
426 characterize the morphology and structure of these nanoparticles. Atomic force microscopy  
427 (AFM) and SEM are some examples of devices used for this purpose, although transmission  
428 electron microscopy (TEM) is the most used method since provides information on the structure,  
429 shape, and actual particle size as well as an estimation of the wall thickness in capsules [91]. The  
430 structural modifications of chitosan were mainly characterized by FTIR,  $^1\text{H-NMR}$ , and  $^{13}\text{C-NMR}$ .  
431 These modifications are generally used to illustrate the physicochemical properties of the  
432 resulting nanoparticles, to detect interaction with active substances, and study how chitosan binds  
433 with specific cellular receptors [91].



434 The specific surface characteristics of the resulting particle system can be integrated by X-ray  
435 photoelectron spectroscopy (XPS). This technique provides details on the elements through  
436 which particles, chemical bonds, and extensive high sensitivity molecular information derive.  
437 Besides, XPS extends a detailed composition of the surface and the proximal surface chemistry  
438 analysis of the particles [92].

### 439 **2.3. Applications of chitosan nanoparticles and nanocapsules**

440 Nanosystems based on chitosan can be used in various fields of applications, depending on  
441 the system's preferred structure and chemical aspects of biopolymers, such as pKa, degree of  
442 deacetylation. Chitosan has two types of hydroxyl groups in its structure: the primary hydroxyl  
443 group in the Carbon-6 position and the secondary hydroxyl group in the Carbon-3 position in the  
444 glucosamine unit. Additionally, the Carbon-2 position works for a group  $-NH_2$ . Therefore, the  
445 structure of chitosan can be manipulated and modified by inserting new functional groups or  
446 through cross-linking reactions, whereby the obtained derivatives are appropriated for the desired  
447 application. Chitosan nanoparticles modified with acetophenone by Schiff-based condensation  
448 were fabricated by ionic gelation in the presence of TPP as a gelling agent. The excellent  
449 adsorption capacity was observed towards various divalent metals:  $Cu^{2+}$ ,  $Cd^{2+}$ ,  $Hg^{2+}$ , and  $Pb^{2+}$  at  
450 pH 7.0. It was being used to eliminate these metal ions contaminating tap water, seawater, and  
451 industrial wastewater, as a result of which the adsorption balance is established within 30 minutes  
452 after the contact time between the absorbent and metal ions [93]. Also, the 100 nm chitosan  
453 nanoparticles can remove the hexavalent chromium ( $Cr^{6+}$ ) from water, which requires the  
454 presence of TPP for the efficient adsorption of the metals.  $Cr^{6+}$  is adsorbed on the surface of  
455 chitosan/ TPP/nanoparticles and reduces  $Cr^{6+}$  to  $Cr^{3+}$ , the reduced form of  $Cr^{3+}$  is less or non-  
456 toxic. Consequently, this system can be effectively used for detoxification and purification of  
457 water from  $Cr^{6+}$  [94].  $Co^{2+}$ ,  $Ni^{2+}$ , and  $Cu^{2+}$  can also be removed from water using modified  
458 chitosan nanoparticles by preparing chitosan -poly(methacrylate) using PEC technique with  
459 particle size from 100 to 120 nm [95]. Recently, modified chitosan nanoparticles were used to  
460 eliminate  $Cr^{6+}$  and/or Rhodamine B (RHB) dye from a complex water system. About 98.4% of  
461  $Cr^{6+}$  and 96.2% of RHB was adsorbed on the surface of modified chitosan nanoparticles. The  
462 mechanism was disused based on hydrogen bonding interaction, electrostatic attraction, and  $\pi$ - $\pi$   
463 stacking force (Fig. 5). Accordingly, this nanoadsorbent system is preferential adsorbent

464 compared to others reported materials dealing with  $\text{Cr}^{6+}$  and RHB pollutants owing to the  
465 functionality of the unsubstituted amino group in the chitosan structure. Besides, green  
466 microwave method was adopted in this paper which save cost, time, and required low energy for  
467 complete the functionality process [96].

468 In another study, chitosan or starch were added to poly (vinyl alcohol) NPs ( $\text{PVA}_n$ ) as  
469 nano adsorbent for oil spills from wastewater [61]. The optimal adsorption amount ( $q_{\text{g/g}}$ ) of  
470 PVAn blended chitosan was 33.6, 73.96, 93.1 g/g for hydraulic oil, kerosene, and toluene oils,  
471 respectively. This study reveals the correlation between different types of oil and their adsorption  
472 efficiency, and the mechanism was illustrated based on the diffusion mechanism. The  
473 incorporation of chitosan enhanced the reusability performance of the binary blend even after six  
474 cycles. This property produces adequate recoveries between 71.8 to 96.3% correlating to the high  
475 mechanical strength of the nanoblends which can be used efficiently in the practical application  
476 of oil spill recovery (Fig. 6).

477 In agriculture, chitosan nanoparticles have been used as herbicidal and pesticidal  
478 formulations of such compounds as paraquat, clomazone, and acetamiprid. Paraquat is a non-  
479 selective herbicide that is often used to control weeds. However, its solubility in water and soil  
480 absorption can cause toxicity in humans. This herbicide has been encapsulated in chitosan  
481 /alginate and chitosan/TPP nanoparticles produced by Ca(II) complexation [97] and by the ionic  
482 gelation technique [98], respectively, with an obtained diameter of about 635 and 300 nm. These  
483 systems have shown less toxicity than a pure compound, which can reduce adverse effects on  
484 human health and the surrounded environment. Clomazone is a main component in rice fields  
485 and has been encapsulated in chitosan/alginate nanoparticles with a particle size of about 200 nm  
486 [99].

487 Polyphenols (PPH) are common in vegetables, fruit, tea, and coffee. PPH is of great  
488 importance for human nutrition because of their nutritional value and their impact on the  
489 prevention of chronic diseases. Furthermore, they have been examined for their antiallergic,  
490 antibacterial, anti-inflammatory, antitumor, antiviral action, and above all, for their strong  
491 antioxidant activity [100]. Nanoparticles based on chitosan have been used to encapsulate  
492 polyphenols extracted from various sources. The polyphenols in tea extract have been  
493 successfully encapsulated with chitosan nanoparticles with an average size of 150-350 nm [101].

494 Chitosan is used in the nutrition and food industry to protect food or certain nutrients for  
495 human consumption via packaging and control of foodborne pathogens. The nisin-containing  
496 chitosan -carrageenan nanocapsules were obtained using the PEC method with the smallest  
497 particle size of approximately 500 nm. This method was tested *in vitro* to assess the antimicrobial  
498 effects against *Micrococcus luteus*, *Pseudomonas aeruginosa* *Salmonella enterica*, and  
499 *Enterobacter aerogenes*, followed by an antibacterial activity test in tomato juice [102].

500 Small interfering RNA (siRNA) may be an effective candidate for disrupting genes in  
501 undesirable eukaryotic cells that are associated with diseases such as cancer, viral infections, and  
502 other genetic conditions. The siRNA consists of 21–23 base pairs that can induce a reduction in  
503 target genes [103]. Due to its positive natural properties, chitosan-based nanoparticles provide  
504 electrostatic interaction with negatively charged siRNA and thus improve their bioavailability.  
505 Therefore, siRNA is destructive for DNA and achieving a high level of safe gene silencing [104].  
506 Studies conducted on a mouse model of ovarian cancer have led to significant transfection and *in*  
507 *vivo* accumulation of cells, leading to effective inhibition of uncontrolled proliferation *in vivo*. In  
508 this study, the nanoparticles-lactate of polyethylene glycol / folic acid chitosan oligosaccharide  
509 were obtained by ion gelation and showed an average size of about 200 nm. Various other studies  
510 have shown that chitosan-based nanoparticles are useful for the encapsulation and release of  
511 siRNA [105]. The small interfering RNA was successfully incorporated by ionic gelation into the  
512 chitosan TPP with a particle size of 78-200 nm [106]. These systems are *in vitro* or *in vivo* tested  
513 against melanoma B16 cells [107]; Lung cancer [105]; Cardiomyoblast cells [108]; breast cancer  
514 [109]. According to the drug delivery line established by chitosan and modified chitosan  
515 nanoparticles, the treatment of brain diseases (tumor diseases, neurodegenerative,  
516 cerebrovascular) in which drugs must penetrate the brain has been improved for these systems.  
517 Indeed most of the compounds do not reach the cerebral blood flow barrier due to the protection  
518 of the brain guaranteed by the regulation of the blood-brain barrier (BBB) [110]. The drugs were  
519 administered via the nasal passage to overcome the limitation imposed by the BBB. They settled  
520 in the respiratory epithelium, entered the system circulation and therefore the BBB could be  
521 crossed [111].

522 Chitosan exhibits mucoadhesive properties, which have been carefully studied and improved  
523 by modifying the structure of chitosan. Various systems have been used to incorporate many

524 drugs, such as a chitosan-coated lipid matrix containing lipophilic dye Nile Red and near-infrared  
525 dye Dir [112]; Buspirone hydrochloride encapsulated in chitosan nanoparticles thiolate obtained  
526 by ionic gelation (200-600 nm) [113]. The intranasal pathways using chitosan nanoparticles have  
527 shown effective biocompatibility and biodistribution through the human bronchial epithelium  
528 (16HBE14p) [114]. Also, chitosan nanoparticles have been used in various diseases, for example,  
529 anxiety disorders [115]; and Neuroprotection in Alzheimer's disease [116]. Further, the  
530 mucoadhesive property of chitosan may also be useful for the administration of drugs for  
531 controlled oral, nasal, ocular [84], vaginal [117] local, and transdermal administration [81].

532 The mechanism responsible for the penetration effect is based on its positive charges.  
533 However, a more pronounced cationic property was obtained by trimethylation of the primary  
534 amino group without improved permeability properties indicating a more complex mechanism  
535 than expected [118]. High molecular weight and a high degree of deacetylation improve  
536 epithelial permeability. PEGylation, N-succinylation, and thiolation can also significantly improve  
537 their mucoadhesive properties. Thiolated chitosan forms disulfide bonds with glycoproteins in the  
538 mucosa and the effect can be increased using mercaptonicotinamide [119].

### 539 **3. Chitosan nanofibers**

540 Nanofibrous scaffolds have attracted an intensive interest in the last years due to their  
541 feasibility to be used for numerous applications including water filtration, wound healing,  
542 dressing, and medical clothing [120]. It might be noticed that the obtained accompanied  
543 properties of produced nanofibers are susceptible to the preparation conditions. These conditions  
544 include the type of polymer, viscosity, injection rate, voltage, orientation, and even the humidity  
545 [121]. The obtained properties might be changed upon variation of the former conditions  
546 involving fibers' diameters, porosity content, and surface roughness. Therefore, the selection of  
547 the polymeric phase with adequate conditions could lead to a formation of nanofibrous scaffolds  
548 with appropriate behaviors. For instance, both fibers' diameters and content of porosity are the  
549 main controlling factors of mechanical properties. However, using these scaffolds for tissue  
550 engineering applications is preferred to be conducted with a highly porous surface. This is  
551 because high interconnected porosity could introduce a simple way for vascularization besides  
552 nutrients and oxygen transformation. Furthermore, keeping the moisture of injured skin could

553 encourage fast healing; therefore, tailoring the properties of the fabricated electrospun  
554 nanofibrous scaffolds should meet the requirement of the suggested application.

555 It can be mentioned that chitosan has been used intensively for heavy metals removal from  
556 aqueous solutions and preferred rather than different nanocomposites in the removal efficiency  
557 [122-126]. The presence of amino and hydroxyl anions in chitosan structure might induce  
558 electrostatic forces towards the targeted ions, other physical interactions help for adsorption of  
559 ions through a porous surface. Otherwise, besides nanofibers, chitosan is produced in numerous  
560 shapes for various utilizations including balls, micro/nano-particles, or sponges[127].

### 561 **3.1. Production of chitosan nanofibers**

562 There are several methods for producing chitosan nanofibers, such as electrospinning forcing,  
563 blowing a solution, coaxial electrospinning, centrifugal electrospinning [128]. Herein, we  
564 introduce a comprehensive description of techniques used for the preparation of chitosan  
565 nanofibers and their applications in different fields of technology.

#### 566 **3.1.1. Electrospinning**

567 Nanofibers might be produced via different techniques, however, one of the most facile and  
568 low costed ones is the electrospinning technique [129, 130]. The technique is based on applying a  
569 high voltage on a polymeric solution to be elongated and sputtered on a moving/fixed substrate to  
570 produce nanofibers. The optimum behavior of electrospun nanofibers could be assigned to the  
571 high surface to mass ratio ( $\sim 40\text{-}100\text{ m}^2/\text{g}$ ), which can be controlled considerably [131].

572 The electrospun nanofibers configuration is established on a uniaxial tension for a  
573 viscoelastic gel. A typical electrical torsion formation contains three main components: a  
574 capillary tube gathered to a needle, a high voltage source, and a substrate for fibers' collector,  
575 which is illustrated in Fig. 7 [132].

576 It is obvious that in the electrospinning technique, a high electric field is required and applied  
577 over the distance between the syringe tip and the collection plate. The polymeric viscous gel is  
578 placed in a syringe and injected into a pump, which creates a steady flow. As soon as the electric  
579 field reaches a certain critical value that the repulsive electrostatic forces exceed the surface  
580 tension, the flow of charged fluid is expelled from the conical protrusion, which is known as the

581 Taylor cone. Finally, the jet lengthens and breaks constantly, as a result of the nanofibrous  
582 scaffolds are deposited on the collector with instantly fast solvent evaporation [133].

583 The important parameters in the electrospinning process are solution parameters and process  
584 parameters. The parameters of the solution are determined dependent on the characteristics of the  
585 solvent and the polymer, including type and concentration of the polymer, molecular weight,  
586 viscosity, surface tension, dielectric constant, and conductivity. The process parameters include  
587 the voltage, distance between the tip of the nozzle and collector, flow rate of the polymer  
588 solution, design and position of the nozzle, composition of the collector, geometry, and speed of  
589 rotation. Environmental parameters include temperature, humidity, and air velocity [134, 135].

590 Concerning solution parameters, the increase in polymer viscosity or concentration  
591 contributes to increased fiber diameter. However, if the viscosity of the polymer solution is too  
592 low, the ejected jet can decompose into droplets and lead to electrospray [136]. As a rule, low  
593 surface tension values lead to the formation of pearl less fibers, consequently, low stresses can be  
594 applied during electrospinning. Also, low conductivity polymer solutions cannot be welded, as  
595 there is no surface charge on a drop of liquid needed to form a Taylor cone. On the other hand,  
596 high electrical conductivity causes a decrease in the tangential electric field across the surface of  
597 a drop of liquid thus inhibits Taylor's cones from forming. Electrospinning is a successful  
598 machine for generating controlled electric voltage on a polymer solution dissolved in a solvent  
599 with high dielectricity constants that led to the formation of thinner nanofibers [137]. Applying  
600 high voltage on the charged solution boosts the electrostatic force, which increases the jet's  
601 elongation, resulting in a thinner diameter of the fiber. Voltages between 5 and 40 kV are  
602 generally applied during electrospinning according to most of the automatically established  
603 machines. A larger distance between the row and the collector can increase the elongation time  
604 due to the obtained thinner fiber length. Some high volatility solvents, high temperatures, and  
605 low humidity will support solvent evaporation and causing porous fiber surface [138]. However,  
606 in some cases some of non-polymer molecules can be electrospun without the use of polymer as a  
607 carrier [139].

608 Chitosan has proven to be a problem for the electrospun. Unfortunately, the polycationic  
609 nature of chitosan in acidic aqueous solution with a high number of amino groups in its backbone  
610 prevents chitosan to be electrospun. To date, nanofiber structures have been successfully obtained

611 by electroforming chitosan solutions in 90 wt. % aqueous acetic acid or by using a certain solvent  
612 such as trifluoroacetic acid (TFA), dichloromethane (DCM), and formic acid. However, traces of  
613 solvent or toxic organic acid in the products are harmful, especially if applied to human skin or  
614 injured tissues [140].

615 Recently, the nanofiber mats have been made of electrospun chitosan with biodegradable  
616 synthetic polymers such as PVA, polyethylene oxide (PEO), poly (vinylpyrrolidone) (PVP), poly  
617 (lactic acid) (PLA). Polycaprolactone (PCL) and polyethylene terephthalate (PET). The  
618 biocompatible, mechanical, antimicrobial activity, and other properties of chitosan nanofibers  
619 have been significantly improved with the addition of PVA, PLA, PCL, PEO, PET, and PVP  
620 [141]. To improve the biological efficiency of chitosan-derived nanofibers, researchers sought to  
621 squeeze chitosan using natural polymers such as collagen, gelatin, and elastin [142].

### 622 **3.1.2. Coaxial electrospinning**

623 Coaxial electrospinning is a modern and economical approach for producing coaxially  
624 structured nanofibers. The typical coaxial configuration consists of two concentric capillaries  
625 (two different solutions), the solutions of the core and the shell of which they come out and join  
626 at the end, forming a core-shell nanofiber (Fig. 8). The key benefit of this approach is to  
627 strengthen material that is less rotating using a highly rotating material [143].

628 As a result, various materials including inorganic and organic compounds have been  
629 successfully combined as functional nanocomposites for high added value. The technique has  
630 been widely used to control secondary fibrous structures, to encapsulate drugs or biological  
631 agents in fibers, to produce nanofibers from non-fibrous materials, and to incorporate functional  
632 fluids into the fiber matrix. Various biologically active drugs and substances, such as DNA,  
633 proteins, antibiotics, can be entrapped directly into the nucleus that is protected by the formation  
634 of the upper layer [144].

635 It has been stated that some hard-to-process polymer solutions could be co-electrospun to  
636 create ultrafine cores in the shells of other polymer materials. Chitosan nanofibers have recently  
637 been obtained by coaxial electrospinning. Core and coating nanofibers can be made of synthetic  
638 polymers with high spinning properties. Recently, produced chitosan nanofibers by coaxial  
639 electrospinning using PEO as a coating model were obtained, optimum ones, in the condition of

640 merging chitosan with PEO at the ratio of 80:20 shell to the core, voltage 25kV and the flow rate  
641 3ml/hr. [145].

### 642 **3.1.3. Characteristics of chitosan nanofibers**

643 Nanofibers have a large surface area, small pore sizes, and the ability to create three-  
644 dimensional structures. Also, its chemical, physical, and biological properties attract attention in  
645 the field of materials science, mechanics, electronics, medicine, plastics, and energy. Therefore,  
646 characterization methods are needed to effectively track the size, morphology, and dimension of  
647 these nanomaterials to evaluate and develop their production process. Also, it is necessary to  
648 know the properties of nanofibers which determines whether the resulting material is suitable for  
649 a specific application, and some of these characterizations are shown in Table (1) [146].

## 650 **4. Some applications of chitosan nanofibers**

651 Chitosan nanofibers can be used in various fields mainly due to the presence of -NH<sub>2</sub> and  
652 -OH groups, along with their structure. Some of the possible uses of chitosan nanofibers are in  
653 the areas of drug delivery, biosensor, food packaging, water purification, tissue engineering, and  
654 wound dressing. This biopolymeric nanofibrous offers enormous structural possibilities for  
655 chemical modifications that create new properties, particularly in the biomedical field.

### 656 **4.1. Purification and treatment of contaminated water**

657 The demand for high-quality water has increased in recent decades [96, 125, 147, 148].  
658 Drinking contaminated water can cause millions of deaths every year, mainly due to pathogen  
659 contamination. Membrane filtration is a recognized technology for reducing the contamination of  
660 drinking water. Further, bacteria can be easily killed by the ultrafiltration membrane upon using  
661 antibacterial surfaces that were made up of silver ions and other metals [149]. Also, the viruses  
662 were removed through nanofiltration membranes according to the size exclusion principle. One  
663 of the materials used for filtration is chitosan nanofibers owing to their significant properties in  
664 wastewater treatment, agriculture, pharmaceuticals, food science, textiles, and biotechnology  
665 [150]. Recently, chitosan nanofibers have been extensively studied for water treatment. This  
666 nanofiber material is described as a natural polymer, which is suitable for the elimination of  
667 heavy metals and colored dyes from water.  $\beta$ -cyclodextrin/chitosan/PVA nanofiber webs were



668 designed by electrospinning and utilized in the dynamic adsorption of organic micropollutants  
669 and heavy metal ions from water [151].

670 Surgutskaia et al. manufactured aqueous PEO/chitosan nanofibers to adsorb nickel ( $\text{Ni}^{2+}$ ),  
671 cadmium ( $\text{Cd}^{2+}$ ), lead ( $\text{Pb}^{2+}$ ), and copper ( $\text{Cu}^{2+}$ ) from a solution. The authors observed that the  
672 maximum adsorption capacity of  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$  ions through a PEO/chitosan nanofiber  
673 membrane follow the order:  $\text{Ni}^{2+} > \text{Cu}^{2+} > \text{Cd}^{2+} > \text{Pb}^{2+}$ . The measured thermodynamic parameters,  
674  $\Delta G^\circ$ ,  $\Delta H^\circ$ , and  $\Delta S^\circ$  demonstrated that the adsorption of this system was feasible, spontaneous,  
675 and endothermic in nature. After five cycles of adsorption-desorption, the reusability was  
676 calculated and the results showed that the PEO/chitosan nanofibers can be widely used in the  
677 continuous adsorption process [152].

678 Recently, created chitosan nanofibers have been functionalized with  $\text{TiO}_2$  NPs to remove  
679 heavy metal ions without electrospinning [153]. The authors showed that  $\text{Au@TiO}_2$  was  
680 successfully prepared using the photo precipitation process and then loaded into a matrix of  
681 chitosan fibers, the resulting material showed plasmonic fibers effect. Photocatalytic and  
682 photoreduction efficiency were studied for many pollutants that were exposed to visible light  
683 irradiation. Plasmon fiber has a high decomposition efficiency compared to  $\text{Au@TiO}_2$ .

#### 684 **4.2. Wound dressing material**

685 The antibacterial resistance of microorganisms, as a rule, is one of the main problems in the  
686 field of wound care and their treatment, which leads to complications such as infections and  
687 delayed healing [154]. An appropriate dressing ought to be capable of absorbing exudates from  
688 the wound area and this allows water to evaporate at a certain point, preventing microbial  
689 transport [155]. Several studies are primarily developed innovative prophylactic antibiotics  
690 resistant microorganisms contaminated wounds. Titanium fibers of chitosan have been  
691 considered dressings because of their high efficiency against different bacterial strains [156].

692 Antimicrobial mats from colloidal nanofibers dispersions of AgNPs based on chitosan mixed  
693 with PVA for wound dressings were constructed by electrospinning [157]. This study showed  
694 that the PVA/ chitosan blend has a well antibactericidal activity against different bacterial strains.  
695 Also, the rotating webs made from the mixtures including AgNPs were better. Thus, the

696 involvement of AgNPs in combination with PVA/ chitosan solutions has enhanced not only  
697 electrospinning efficiency but also the antibacterial ability of electrically shaped fibers sheet.

698 In a recent study, honey was added to the PVA/chitosan polymer matrix before  
699 electrospinning to evaluate the ability of these nanofibers to heal a wound. The results showed  
700 that PVA/chitosan/honey has a positive effect on the healing of burn wounds. This study reported  
701 that topical use of this bio fiber displayed significant healing activity in the burning region [158].

702 The capabilities of 3D printed chitosan for chronic wound healing utilization were used. The  
703 results displayed that after 24 exposure to the human fibroblasts cell line, more than 90 % of cells  
704 still were viable, which demonstrated the high biocompatibility of these scaffolds and encourage  
705 their utilization for clinical applications [159]. Furthermore, N. M. Ergul et al. examined 3D  
706 printed polymeric blend containing chitosan/PVA encapsulated with hydroxyapatite (HAP) for  
707 tissue engineering. It was shown that grains size of HAP was around 1.21  $\mu\text{m}$ , while the elasticity  
708 modulus of the fabricated scaffold reached around 91.14 MPa for the ratios of  
709 chitosan/PVA/HAP (15 wt. %) based on the added contents of HAP [160]. In addition, Min Zhu  
710 et al. studied the photoactivated antibacterial behavior of chitosan films doped with Ag/MoS<sub>2</sub>.  
711 The results exhibited that antibacterial efficiency reached around 98.66% and 99.77% against *S.*  
712 *aureus* and *E. coli*, respectively under visible light after 20 minutes of continuous exposure [161].  
713 Moreover, A.A. Menazea et al. investigated the effect of AuNPs embedded through a polymeric  
714 blend of chitosan/PVA for wound healing applications. They found that photoactivated  
715 antibacterial blend illustrated great potency towards both *E. coli* and *S. aureus* with inhibition  
716 zones of 13.1 $\pm$ 1.3 mm and 14.8 $\pm$ 2.4 mm for the highest additional AuNPs [162].

### 717 **4.3. Tissue engineering**

718 Polymer nanofibers are still of significant concern in regenerative medicine as building  
719 materials for restoring, preserving, or enhancing the function of human tissues damaged due to  
720 aging, sickness, injury, or birth defects. Tissue engineering can also be segregated into several  
721 segments depending on the tissue/organ class such as bone, ligaments, cartilage, tendons, liver,  
722 nerves, and skin [163]. There are some widely accepted basic requirements for the construction  
723 of polymer forests. The structure must have high porosity with the adequate distribution of pore  
724 sizes, a large surface, biodegradability, adequate mechanical properties, and biocompatibility.

725 Various biocompatible polymers have been developed from natural materials such as collagen,  
726 elastin, laminin, etc. [164].

727 Poly( $\epsilon$ -caprolactone)/chitosan nanofibrous was fabricated using an electrospinning  
728 technique to create bone and skin tissue [165]. The presence of chitosan in the chitosan /PCL  
729 structure led to a significant improvement in hydrophilicity. Further, the cytocompatibility of the  
730 chitosan /PCL framework was studied and proved to be non-toxic. All these results showed that  
731 the chitosan /PCL nanofiber structure would be an excellent system for bones and skin tissue  
732 engineering.

733 Another example produced chitosan /PVA fibers with  $\text{CaCO}_3$  incorporation. The authors  
734 observed that a tensile test of fibers reinforced with  $\text{CaCO}_3$  at content 4% by weight gave an  
735 elastic modulus sixty times higher than the value of the fibers without  $\text{CaCO}_3$ . The  
736 biocompatibility of the  $\text{CaCO}_3$ -loaded fibers was also tested in a 14-day incubation with  
737 simulated biological fluid (SBF) to induce the formation of apatite minerals and in a 14-day  
738 analysis of cell proliferation with ATDC5 cells. Chitosan/PVA- $\text{CaCO}_3$  is perhaps the most  
739 effective medium for cell growth as compared to chitosan/PVA and chitosan/PVA-apatite.  
740  $\text{CaCO}_3$ -enhanced chitosan / PVA can be a viable substitute for cartilage [166].

#### 741 **4.4. Delivery of drugs**

742 Owing to large specific surface area nanofibers can deliver platforms for drugs, peptides,  
743 and vaccine antigens. The release of the drug may be immediate, delayed, or modified depending  
744 on the type of interaction between the polymer with the drug [167]. Usually, an immediate  
745 release is noticed when a composition of a water-soluble substance and a water-soluble polymer  
746 is used. However, the extended-release profile can be tailored by integrating the drug into other  
747 nanocarriers, such as nanoparticles, liposomes, dendrimers, then loaded into nanofibers or use  
748 hydrophobic polymers [168].

749 Chitosan nanofibers have recently exhibited controlled drug release affinity [169]. This  
750 material may be useful for the delivery of proteins/peptides, anti-inflammatory drugs, antibiotics,  
751 imaging agents. Fabrication of poly (d, l-lactide-glycolide) (PLGA)/ chitosan nanofiber structure  
752 by electrospinning was also assessed [170]. The results of this study showed that the drug release  
753 rate increased with increasing chitosan content, as the addition of chitosan improved

754 hydrophilicity. The release rate from an aligned PLGA nanofiber skeleton was also lower than  
755 that of a randomly oriented PLGA/ chitosan nanofiber skeleton.

756 Gold nanorods (AuNRs) containing double-fiber hybrid PVA/ chitosan nanofibers were  
757 prepared using a simple electrical conjugation process. The authors found how these nanofibers  
758 are used as carriers for the doxorubicin anticancer (DOX) drug that is inserted into the nucleus of  
759 the cells. The integrated AuNRs and DOX in fibers effectively inhibit the growth and  
760 proliferation of ovarian cancer cells and, thanks to their unique optical properties, can also be  
761 used as a cell imaging medium. The nanofiber matrix, which integrates two roles of cell imaging  
762 and the delivery of drugs may have great potential for use in biomedical fields [171].

## 763 **5. 3D chitosan printable scaffold**

### 764 **5.1. Chitosan printed in cartilage and bone regenerative medicine**

765 The architecture of chitosan and its stability in physiological environments are very  
766 analogous to that of glycosaminoglycan. Furthermore, chitosan degradation products are often  
767 elements of cartilage synthesis. These advantages of chitosan are widely used in the construction  
768 of bones and cartilages. Also, a coating of chitosan on the PCL scaffold contributed to better  
769 fixation and penetration of the cells. It is also recommended the chitosan to be mixed with other  
770 materials, such as biological glass and collagen, to improve the chemical, osteoconductive and  
771 mechanical properties of chitosan [172]. In a previous study, a new rapid prototyping system was  
772 developed for the delivery of robots to attain chitosan/HAP structures [173]. They added the  
773 HAP/ chitosan base to improve the osteoconductivity and mechanical strength of the scaffolds. In  
774 their analysis, they employed acetic acid to create chitosan hydrogel and HAP chitosan solution,  
775 supported by extrusion to obtain a gelatinous precipitate in a bath mixture of sodium hydroxide  
776 and ethanol. The increased use of sodium hydroxide led to the rapid deposition and poor mobility  
777 of the chitosan molecules at the edges of the contact structure. Conversely, a very low  
778 concentration of sodium hydroxide reduces the deposition rate and causes poor dimensional  
779 stability. Tests on cell culture showed acceptable results as well. Rapid prototype robotic delivery  
780 (RPBOD) may therefore be an effective method for producing chitosan-based structures.

781 Ascertain printing parameters such as outlet pressure, exit speed, and initial exit height  
782 was a problem in a study by Ang et al. [173]. These parameters depend on the solution's

783 viscosity; therefore, they differed for each composition and concentration. The macroscopic  
784 analysis, such as visual inspection, was used to solve this problem and establish coherent and  
785 uniform filaments. It is difficult to select the right binding to 3D print. It has been documented  
786 that different acids have been studied, such as citric acid, phosphoric acid, lactic acid, and  
787 sulphuric acid [174]. It was found that a shorter curing period was obtained with 20-40 wt.% of  
788 binding citric acid, and the 30-40 wt.% lactic acid [175].

789         Several chitosan binders were tested for 3D printing and obtained nearly identical results for  
790 lactic acid. They were double-checked using lactic acid, citric acid, and acetic acid as the binder  
791 by printing a similar composition (chitosan/HAP). They found the 40 wt. % lactic acid boosts  
792 medium viscosity, fast solidification time, and high wettability. Therefore, lactic acid has the  
793 correct structure relative to other binders.

794         There was an improvement in structural stability with a ratio of 20 wt. % of chitosan and  
795 40 wt. % of binder lactic acid and after processing 40 wt. % of lactic acid. The three-dimensional  
796 printing structures, which consist of 25 wt. % of chitosan and 75 wt. % of HAP is shown in Fig.  
797 9. The resulting printed samples showed a compressive strength of 16.32 MPa, an elastic  
798 modulus of 4.4 GPa, and a porosity of 37.1%. The low porosity is due to the destruction of the  
799 open pores due to the high concentration of lactic acid. They thought that the chitosan chemical  
800 structure allows attractive bio-stimulation. Their findings suggested that the elevated chitosan  
801 /HAP structure is a promising alternative to the bone regeneration process.

802         In another study, the mechanical property and cytocompatibility of a hybrid constitute  
803 synthetic polymer (polyethylene glycol diacrylate (PEGDA)) and chitosan was evaluated [176].  
804 This study showed that chitosan molecular weight and the feeding ratio are effective parameters  
805 in the shaping and precision of the structure. The ratio of PEGDA to nutrition with chitosan was  
806 set at 5:1 and 10:1, which was the optimal range. Low chitosan molecular weight requires an  
807 excessive concentration of sodium acetate due to its higher deacetylation to neutralize its  
808 protonated amino groups. They used Stereolithography (SLA) to create frames, while a 7.5:1  
809 ratio showed a boost printability effect. SLA can monitor the frame composition and porous  
810 structure, depending on the anatomical requirements of the patient. They designed bezels in an  
811 ear-shaped form with a laser beam of 405 nm. The presence of strengthened chitosan in the resin  
812 increases the coefficient of swelling. Adequate mechanical properties and enormous potential for

813 cell attachment contributed to the growth of an innovative design for the cartilage tissue  
814 engineering field with complex geometry.

815 Also, chitosan /alginate was chosen as the desired combination because of its similarity to  
816 natural cartilage glycosaminoglycans (GAGs) such as hyaluronic acid, keratin sulfate, and  
817 chondroitin sulfate. The compositional form can give a desirable property such as compressive  
818 strength or swelling potential. Besides, a neutral pH skeletal preparation provides a green  
819 environment that guards the combined growth factor for a drug against denaturation. They had to  
820 use a modern manufacturing process, integrating 3D printing with directional freezing to monitor  
821 building micro- and macro-channels. The macro channels were generated using 3D printing of  
822 positive and negative shapes in the methodology used, and the microchannels were developed by  
823 spatial freezing of the solution. These scaffolds with simultaneous lamellar capillaries and  
824 uprights made the cartilage quite normal for replication and showed better absorption of  
825 inflammation. Besides, better distribution of cells together with the height and appropriate  
826 diameter of the scaffolds also made a significant contribution to the process of regeneration  
827 [177]. On the other hand, chitosan was mixed with gelatin without the need for further  
828 treatments. This solution was combined with  $\beta$ -glycerophosphate forming hydrogel at the  
829 physiological temperature and enhanced the cellular interaction of chitosan. This solution was  
830 supplemented with hydrochloric acid and deionized water. Cells were introduced to the solution,  
831 and a method of bioprinting was carried out. Their findings showed that solid contamination,  
832 accumulation, and gelation during the 3D bioprinting can escalate to needle clogging, which can  
833 be prevented by cooling the prepared solution; centrifugation and degassing under vacuum  
834 prevent these problems during printing. They proved that even an increase in chitosan  
835 concentration results in a greater viscosity and an intensified gelation time which reduces fiber  
836 size. Furthermore, the distance between the needle and the impressed surface is an important  
837 factor to use agarose gel in this space. The diameter of the fiber decreased with the increasing  
838 speed of advance. The new bioprinter offers a simple and inexpensive way to print bi-substrates  
839 of chitosan /gelatin and at the same time maintains high cell viability [178].

840 It was observed that these new low stability chitosan hydrogels are easily dissolved, as  
841 mentioned in some cases in a previous study [179]. A new gelatin/sodium  
842 alginate/carboxymethyl hydroxycarbonate- chitosan composition filled with bone marrow stem

843 cells for three-dimensional bioprinting was reported. The addition of chitosan improves  
844 proliferation, cell adhesion, and mechanical force owing to the ionic bond between the  $\text{-NH}_2$   
845 group of chitosan and the  $\text{-COOH}$  group of the alginate. To enhance water solubility, chitosan  
846 was modified into carboxymethyl chitosan by dissolving gelatin in double distilled water, then  
847 alginate and carboxymethyl chitosan subsequently added to the solution and the ions were  
848 crosslinked with 2%  $\text{CaCl}_2$ . The stem cells of the bone marrow were loaded into a hydrogel and a  
849 three-dimensional bioprint was performed. The mechanical feed system has been set to a speed  
850 range of 1 to 10 mm and a pressure range of 20 to 60 psi. They showed that the decomposition  
851 rate of hydrogels with carboxymethyl chitosan has decreased compared with hydrogels without  
852 chitosan, which means that carboxymethyl chitosan has improved the stability of the hydrogel.  
853 Furthermore, the antibacterial activity with carboxymethyl chitosan has been improved compared  
854 to the hydrogels without chitosan.

855 Chitosan was modified for 3D bioprinting of artificial bone tissue in a previous study [180].  
856 In this study, chitosan was modified to N, O-carboxymethyl chitosan to react with the  
857 polyphosphate and improve the biologically active properties of the hydrogel. The N, O-  
858 carboxymethyl chitosan was dissolved in physiological solution and added polyphosphate to the  
859 solution, followed by centrifugation to remove a gas bubble. The solution was mixed with an  
860 alginate aqueous solution and then, bioprinted using a 3D bio plotter. Next, Ca was added for  
861 networking and the hardening of bio-printing structures. The proposed composition induced  
862 SaOS-2 bone cells for biomineralization. These scaffolds for bioprinting were *in vivo* studied and  
863 demonstrated significant activity of regeneration induction in lower leg defects of rats. This  
864 composition seems a very productive material for the custom design of frames in the bones.

## 865 **5.2. Printed chitosan in the formation of a vascular network structure**

866 Nowadays, chitosan has been used in multiple applications in tissue engineering and  
867 regenerative medicine, especially in the design of vascular networks. Because of its outstanding  
868 properties, chitosan is extensively used in vascular tissue engineering by the blending of chitosan  
869 with polycaprolactone. This combination shows desirable mechanical degradation and superior  
870 biological properties [165].

871 Recently, alginate and chitosan hydrogels with different content have been used to print  
872 channels with a wall thickness of less than 200 microns, in which a robotic printer directly  
873 printed microfluidic channels in the shape of a vessel tube. 1.0 M sodium hydroxide was used as  
874 the crosslinking agent of chitosan and acetic acid to dissolve chitosan. The high content of  
875 chitosan solution (4%) required higher pressure, which led to an increase in shear stress and a  
876 decrease in cell viability. On the contrary, the mechanical behavior is decreased at low chitosan  
877 content (2%). Therefore, chitosan with a concentration of 2.5 to 3% was chosen as the optimal  
878 concentration range. On the other hand, they have shown that the distribution of polymers or the  
879 rate of crosslinking affects the diameter of the channels.

880 Higher delivery speeds obtained by extrusion led to thicker walls and build wider channels.  
881 The printed alginate microfluidic channels were continual and more homogeneous based on their  
882 findings, but the printed chitosan samples were more fragile and easily breakable. Even though  
883 the concentration and delivery speed of selected materials are effective factors during  
884 microchannel printing. Hence, the viability of the cells is increased in the form of a single  
885 channel or mass incorporated in the hydrogel and this process is considered a promising  
886 production route for the development of the vascular network [181].

### 887 **5.3. Printed chitosan in skin regeneration**

888 Chitosan has attracted great attention in the areas of dressings and skin regeneration  
889 because of its antimicrobial and hemostatic properties. It may even be used as an adjuvant for the  
890 recruitment of polymorphonuclear leukocytes or neutrophils in the wound area, fibroblast  
891 proliferation, hyaluronic acid synthesis, angiogenesis, and deposition of collagen. It contributes  
892 to accelerating the process of wound healing and preventing scar formation. The fragile attitude  
893 of chitosan in skin tissue engineering can sometimes be also rectified by mixing with collagen,  
894 alginate, or other polymers [182].

### 895 **5.4. Cast chitosan for tissue engineering applications**

896 Hard tissues in mammals consist of two main constituents: hydroxyapatite (HAP) as a  
897 mineral part and collagen fibers as the biological contributor. It might be reported that HAP  
898 seems to be configured in nanorods with diameters in the range of 5-10 nm and length about 30-  
899 50 nm. Furthermore, these rods are embedded vertically through the nanofibers of collagen. It



900 could be concluded that while HAP represents the predominant component to resist compression  
901 stresses, collagen fibrous contribute mainly to deal with tensile stresses. In other words, the great  
902 mechanical properties of hard tissues could be assigned to their hierarchical structure. Therefore,  
903 a design of bone possesses the upper hand in mechanical properties than the types of constituents.  
904 Besides, HAP is a distinct inorganic agent with a hexagonal structure. Besides its high  
905 biocompatibility, bioactivity, low cytotoxicity, and osteoconductivity, it possesses numerous  
906 crystallographic sites that are able to be substituted partially whereas crystal structure is  
907 remaining. The biological HAP is usually substituted with versatile ions with different contents  
908 such as carbonate which comes from the ambient atmosphere. Therefore, mimicking of hard  
909 tissues in mammals might be reached via designing a multi-doped HAP to be embedded  
910 through a bio-polymeric agent, which could introduce adequate mechanical behaviors and avoid  
911 annealing procedures. In this issue, Mg/Ag co-doped HAP was encapsulated into chitosan using a  
912 casted technique. The morphological features of the obtained scaffolds were shown in Fig. 10 (a-  
913 f) [183].

## 914 **6. Advantages, drawbacks, and future trends of chitosan 3D printing**

915 3D bioprinting is a modern fabrication process to place biological materials, cells, and  
916 biomolecules accurately on scaffolds. The production control facilitates the approximation of  
917 natural textiles with customized biological and mechanical properties suitable for repairing  
918 tissues [184]. Though 3D printing was a breakthrough in the development of scaffolding, it still  
919 represents a long way to organ reproduction. One of 3D printing's most significant problems is  
920 the development of a functional organ filled with vascular lumens of different sizes. All cell types  
921 need a network of vessels to gain access to nutrients and oxygen, as well as to expel waste.  
922 Therefore, the creation of a complete and complex network of vascularization in the printed  
923 structure remains a mystery [185]. However, 3D printing technology has more possibilities than  
924 traditional scaffolding methods for developing the boundaries of the vascular system by checking  
925 the parameters. Interestingly, the impression of the native organ has many problems. Biological  
926 imitation of the whole organ, considering its various types of cells and microstructures, is a  
927 rigorous procedure that requires great effort.

928 Up to date, there is an important issue that should be taken into consideration which is the  
929 economic factor for the preparation of 3D printed chitosan, in addition to the preparation

930 drawbacks such as the huge time of the crosslinking process and poor mechanical features. Such  
931 issues limit the utilization of chitosan 3D printing for most medical purposes (tissue engineering).  
932 These issues can be avoided *via* adding new substances such as gelatin compounds which can act  
933 as mediators for the creation of new functional groups [186]. Based on that, such gelatin has been  
934 added to enhance the printability of chitosan. Additionally, gelation has been used to enhance the  
935 thermal stability of chitosan decomposition occurred around 220°C. The amazing features of  
936 modified chitosan facilitate its application in many biomedical purposes due to its proper  
937 biocompatibility and adhesion with the cells. Therefore, it is necessary to explore the most  
938 parameters and materials that can be used for changing the undesired features of chitosan [187].  
939 Furthermore, the added compounds represent an important factor that affects the shape and  
940 precision of the structure of interest. It has been reported that chitosan with low molecular weight  
941 is more favorable than the chitosan with high molecular weight. To get high properties, chitosan  
942 with low molecular weight should be then used. Moreover, chitosan with high molecular weight  
943 forms a rigid crystalline structure owing to the presence of huge inter and intramolecular  
944 hydrogen bonds along with its structure, which in turn, restricts its application in SLA  
945 bioprinting. Besides, acetic acid which is used for dissolving chitosan can be used to overcome  
946 partially the formation of free radicals. In a short, in SLA of chitosan, the photoinitiator  
947 concentration is a very important aspect. It has been reported that the utilization of photoinitiator  
948 has a threshold value. Above the threshold value, the photoinitiator can lead to poor cell adhesion  
949 and cell viability [180]. Based on the aforementioned findings, it was observed that the  
950 concentration of both solvent and materials is a very important factor affecting the mold structure  
951 in terms of mechanical features and stability. Moreover, different parameters such as physical or  
952 chemical agent impacts on the process and the crosslinking agent have been also checked, in turn,  
953 leading to an increment in the diameter of both core and channels. Thus, 3D printing produced  
954 chitosan with high viscosity and the presence of crosslinking agents with high concentration can  
955 create drawbacks such as limiting the feeding, transportation of oxygen, and cell migration. For  
956 that, viscosity and crosslinking concentration are highly appreciable for the formation of good  
957 chitosan 3D printing [188]. On contrary, it is clarified that 3D chitosan can swell after the  
958 printing process leading to decreasing the diameter and struts of the pores. Thus, for decreasing  
959 the swelling factor of 3D chitosan, a crosslinking agent should be added in a high concentration  
960 [189].

961 Previously, there is much-published work documented the development of a superior  
962 method (solvent-free) such as laser absorption of two photons and single-photon during shear  
963 deformation for the generating of chitosan with various molecular weights and allyl substituted  
964 derivatives scaffolds. The advantage of using such a method concerns the avoidance of organic  
965 and aqueous solvents with no problem associated with viscosities and distributors [190].

966 As previously mentioned, the evaluation of cytotoxicity for 3D chitosan illustrated the  
967 viability of cells and cell growth formation. However, there is a lack of studies about other  
968 factors that should be extensively examined. Some of these factors are biodegradability,  
969 absorption of water, and mechanical features of the formed 3D prints. All these categories will be  
970 discussed in detail in future studies. Moving to our discussion about the amazing properties of 3D  
971 printing structures; such morphological structure is a very important issue that should be  
972 accompanied by the prepared scaffolds that affect cellular behaviors. The 3D printing is more  
973 favorable to be prepared with a morphological structure containing huge porous structures with  
974 small sizes to be efficient during the penetration of cells. It has been documented in previous  
975 studies that 3D printed chitosan has been prepared with a pore size of less than 250  $\mu\text{m}$ . Indeed,  
976 chitosan with a positive charge is more desired for cartilage tissue engineering domains [191].

977 The rapid gelation of a negatively charged polyanion in an aqueous medium acted as an  
978 inhibitor of the diffusion of various parts of bioprinting. As in the study which reported that  
979 chitosan has a sufficient gelling time, acceptable for 3D bioprinting. Besides, it has maximum  
980 printing precision, long life, and is the cheapest gel compared to other approved materials used  
981 before in the materials for clinical purposes. Although bioprinting skin tissues developed with  
982 chitosan and chitosan /collagen have not received FDA approval. Given the similarity of  
983 composites with other approved materials, the regulatory approval may be relatively simple in the  
984 future [192].

985 It could be observed that upon variation of compositional contributions, the surface  
986 morphology has been changed considerably. HAP with dimensions of 4  $\mu\text{m}$  has varied to be 1.25-  
987 5  $\mu\text{m}$  upon the changing of dopant concentrations. Furthermore, HAP grain was scattered through  
988 chitosan fiber which has diameters in the range of 2.5-5  $\mu\text{m}$ . The compressive strength was  
989 conducted and displayed that additional Mg (II) might introduce high resistance to compressive  
990 stresses. The optimum compressive value was achieved at around 15.0 MPa [183].

## 991 **Conclusion**

992           The materials based on chitosan have several technological applications that have already  
993 been investigated and many others that are possible. For an effective investigation of chitosan-  
994 based materials, it must be considered that all areas of applications depend on the properties of  
995 the biopolymer. The properties, in turn, depend on the production process, which depends on the  
996 raw materials. Therefore, a series of clearly defined steps must be followed by the raw material to  
997 produce chitosan. Also, chitosan is easily modified in various ways to obtain special properties  
998 that are used in different trends. Traditional and advanced analytical methods should be used in  
999 all these phases to verify the main results. Besides, in this review, the chitosan nanofibers are  
1000 limited owing to the cationic property and solubility of chitosan in acids and this also was  
1001 discussed. To evaluate and develop the chitosan nanofibers production process, it is necessary to  
1002 analyze its morphology, crystallinity, chemical composition, structural changes, and mechanical  
1003 properties. Since chitosan is a natural polymer with useful biological properties, its nanofibers  
1004 hold promise for a variety of applications. The presence of -NH<sub>2</sub> and -OH groups in chitosan  
1005 nanofibers allows their use in fields such as biotechnology, wastewater treatment, medicine, and  
1006 pharmaceuticals.

## 1007 **Declaration of Competing Interest**

1008 All authors declare that there is no conflict of interest.

1009

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