1 *Review*

2 Chitosan Based Self-assembled Nanoparticles in
3 Drug Delivery

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13 **Abstract:** Chitosan is a cationic polysaccharide usually obtained by alkaline deacetylation of chitin
14 poly(N-acetylglucosamine). It is biocompatible, biodegradable, mucoadhesive and non-toxic. These
15 excellent biological properties make chitosan a good candidate for a platform in developing drug
16 delivery systems having improved biodistribution, increased specificity and sensitivity, and
17 reduced pharmacological toxicity. In particular, chitosan nanoparticles are found to be appropriate
18 for non-invasive routes of drug administration: oral, nasal, pulmonary and ocular routes. These
19 applications are facilitated by the absorption-enhancing effect of chitosan. Many procedures for
20 obtaining chitosan nanoparticles have been proposed. Particularly, the introduction of hydrophobic
21 moieties into chitosan molecules by grafting to generate a hydrophobic-hydrophilic balance
22 promoting self-assembly is a current and appealing approach. The grafting agent can be a
23 hydrophobic moiety forming micelles that can entrap lipophilic drugs or it can be the drug itself.
24 Another suitable way to generate self-assembled chitosan nanoparticles is through the formation of
25 polyelectrolyte complexes with polyanions. This paper reviews the main approaches for preparing
26 chitosan nanoparticles by self-assembly through both procedures and illustrates the state of the art
27 of their application in drug delivery.

28 **Keywords:** chitosan; self-assembled; polyelectrolyte complex; nanoparticle; drug delivery

29

30 1. Introduction

31   Chitosan (CS) is a family of linear polysaccharides composed of glucosamine and N-
32 acetylglucosamine units linked together by β (1→4) glycosidic links (Figure 1). CS is obtained by the
33 partial deacetylation of the naturally occurring polysaccharide, chitin, which is essentially poly(N-
34 acetylglucosamine). Depending on the natural source and the conditions used to isolate and
35 deacetylate chitin, the resulting degree of acetylation (DA) and molecular weight of chitosan will
36 depend on the reaction parameters involved [1]. Molecular weight, the DA and even the pattern of
37 acetylation (the distribution of glucosamine and N-acetylglucosamine units along the chitosan chain)
38 will affect its chemical and biological properties [2, 3].
39   The degree of deacetylation (DD = 100 − DA) of chitosan is about 50% or higher. In dilute aqueous
40 acid solutions, the amino groups of chitosan become protonated, allowing its dissolution. In fact, the
41 solubility of chitosan in 1% or 0.1M acetic acid is a simple and practical criterion used to differentiate
42 it from chitin. However, chitosan solubility depends on its DD, the ionic concentration, the pH, and
43 the distribution of acetyl groups along the chain, as well as the conditions of isolation and drying. If
44 deacetylation of chitin is performed under homogeneous conditions chitosans with a DD about 50%
45 might dissolve, but if deacetylation is carried out under heterogeneous conditions, DD of 65% or
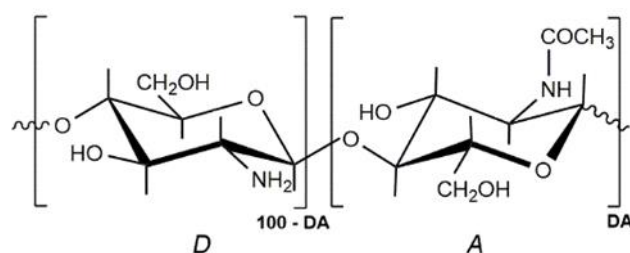46 higher is usually needed to achieve dissolution [4].

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Figure 1. Structural units of chitin and chitosan. (A) N-acetylglucosamine unit; (D) Glucosamine unit. In chitosan DA< 50.

Chitosan is a biocompatible, biodegradable and non-toxic material. It exhibits other significant biological properties, such as wound healing capacity, antimicrobial and hemostatic activities. It is an excellent film former and can be processed into fibers, gels, microspheres-microcapsules, and micro/nanoparticles [5]. Also, because it has free –OH and –NH$_2$ groups in its structure, it is amenable to chemical modifications that can potentiate some of its properties for certain applications. All these remarkable physical, chemical and biological properties have made CS an excellent candidate for applications in cosmetics, food industry, medicine and pharmacy [4]. The preference of chitosan in comparison with other cationic polymers such as polylysine, polyarginin or polyethyleneimine for many of these applications relies on its comparivelty lower toxicity [6].

Mucoadhesive and absorption-enhancing properties are also found in CS. It opens the tight junctions between cells so that the drug of interest can traverse the mucosal cells. [7,8]. These properties also make CS an ideal candidate for the delivery of drugs and bioactive molecules in general. Numerous reports show the applications of CS in drug delivery, with several reviews on the subject [6, 8-10]. Applications include CS as an excipient in tablets, chitosan hydrogels, films, fibers, micro/nanocapsules and micro/nanoparticles.

Nanoparticles of CS are applied in drug delivery, not only by the traditional administration routes (eg. oral and parenteral routes) but also via mucosal (nasal, pulmonary, vaginal) and ocular routes [11]. Chitosan nanoparticles are as well used in designing non-viral vectors for gene delivery and the delivery of vaccines [12].

Different approaches have been used to produce CS nanoparticles. These include ionotropic gelation [13, 14], spray drying [15], water-in-oil emulsion cross-linking [16], reverse micelle formation [17, 18], emulsion-droplet coalescence [19, 20], nanoprecipitation [21] and by a self-assembling mechanism [22, 23].

The self-assembling has been described as the association of certain molecules, macromolecules or composite materials with themselves to form tridimensional networks or other structures with new distinguishing properties. The self-assembling process can take place at the molecular or supramolecular level [24, 25]. It can occur by self-association or by an association with other structures through interactions such as hydrogen bond, van der Waals forces, and ionic or hydrophobic interactions. It can also be caused by an inclusion/complexation mechanism, like the iodine inclusion complex with starch [25].

CS self-assembled (also referred to as self-aggregated) nanoparticles (NPs) are particularly useful for encapsulating hydrophilic as well as lipophilic drugs [26]. Self-assembly can be provoked by the introduction of hydrophobic moieties into the CS molecules by grafting, in order to modify its hydrophobic-hydrophilic balance. The grafting agent can be a hydrophobic moiety such as cholesterol [27], cholic [28] and deoxycholic acid [29] or 5β-cholanic acid [30], to form micelles that can entrap lipophilic drugs or it can be the drug itself. Frequently, a water soluble CS derivative, such as glycol chitosan [31] or succinyl chitosan [32] is used instead of CS. Another suitable way to generate self-assembled chitosan nanoparticles is through the formation of polyelectrolyte complexes with polyanions [33]. The aim of the present article is to review the main approaches used for preparing chitosan nanoparticles by self-assembly through both procedures, and to illustrate the state of the art in drug delivery.

2. Polyelectrolyte complexes

Polyelectrolyte complexes (PECs) are formed when the solutions of two polyelectrolytes carrying complementary charges (i.e. a polycation and a polyanion or their corresponding salts) are mixed together. PEC formation is mainly caused by the strong Coulomb interaction between the oppositely charged polyelectrolytes. The formation of complexes brings about at least a partial charge neutralization of polymers [10]. The complexes obtained (also called polysalts) generally precipitate or separate from the solution forming a complex rich liquid phase (coacervate). However, under certain conditions the polyelectrolytes, with weak ionic groups and significantly different molecular weights at non-stoichiometric mixing ratios, can generate water-soluble PECs on a molecular level [34, 35].

The formation of polyelectrolyte complexes is accompanied by the release of small counter-ions into the medium. The increase in entropy produced by the release of these low molecular weight counter-ions to the medium is the main driving force for PEC formation. Although the electrostatic interaction between the complementary ionic groups of polyelectrolytes is responsible for PEC formation, hydrogen bonds and hydrophobic interactions also contribute to complexing. The arrangement of chains in a PEC can be envisaged as a combination of a disordered scrambled egg-like structure and a highly ordered ladder-like organization (Figure 2). Therefore, the actual structure having hydrophobic and hydrophilic regions makes PECs a particular class of physically cross-linked hydrogels that are sensitive to pH and to other environmental factors such as temperature and ionic strength.
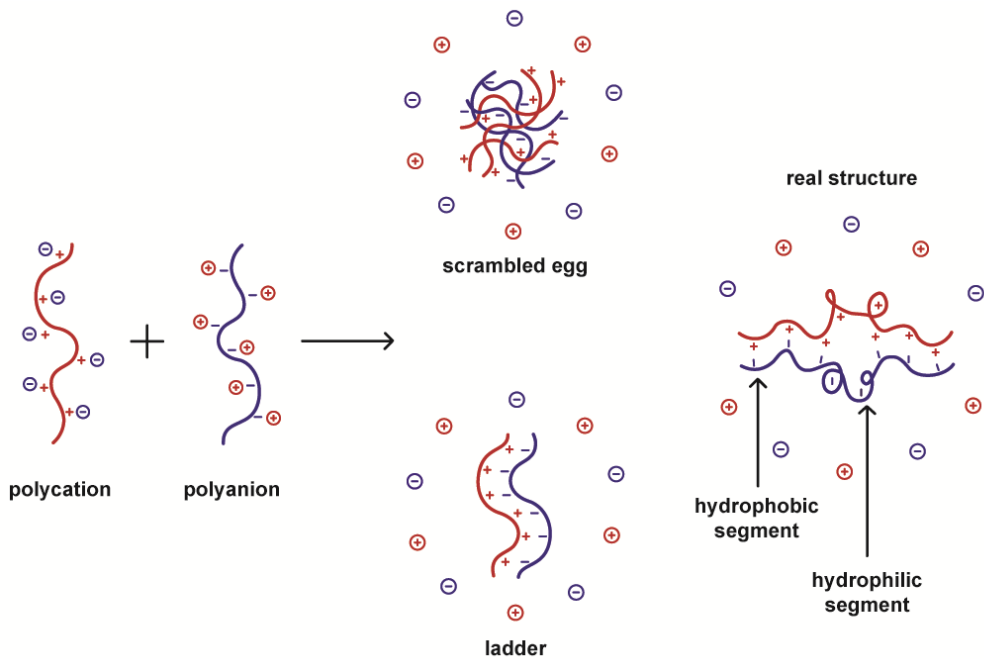

Figure 2. The structure of polyelectrolyte complexes. Scrambled egg and ladder arrangements illustrate extreme situations. The actual structure can be represented as an intermediate one combining hydrophobic ladder-like segments coexisting with disordered hydrophilic regions.

Many factors affect the structure and stability of PECs; -these include: the degree of ionization of each one of the polyelectrolytes and their charge density and charge distribution on the polymer chains, polyelectrolytes concentration, mixing ratio (Z), mixing order, the nature of the ionic groups on the polymer chains, molecular weights of the polyelectrolytes, flexibility of the polymer chains, interaction time and temperature and ionic strength, as well as the pH of the medium [36].

As a cationic biopolymer, CS may react with negatively charged polyelectrolytes, giving rise to the formation of PECs [37, 38]. Many reports show PECs produced with chitosan and carboxymethyl cellulose (CMC) [39, 40], alginate [41-45], poly(acrylic acid) [46, 47], pectin [48-51], carrageenans [52, 53], heparin [54] and other polyions [55-61].

125   *2.1. Chitosan based PEC nanoparticles and their application in drug delivery*

126   Because of the above mentioned biological properties of CS, many applications of these PECs
127   have been proposed for biomedical purposes, particularly for drug delivery [62]. Hence, researchers
128   have shown special interest in the preparation of chitosan PEC nanoparticles for the delivery of drugs,
129   proteins, genes and vaccines [36, 63, 64].
130   When chitosan PEC particles are formed, they tend to aggregate because of charge
131   neutralization, therefore, at least two conditions are mandatory to avoid aggregation and to obtain
132   nanoparticles: the polyelectrolyte solutions must be diluted, and one of the polyions must be in the
133   appropriate excess required so that the charge ratio $(n_+/n_-) \neq 1$. (Figure 3).
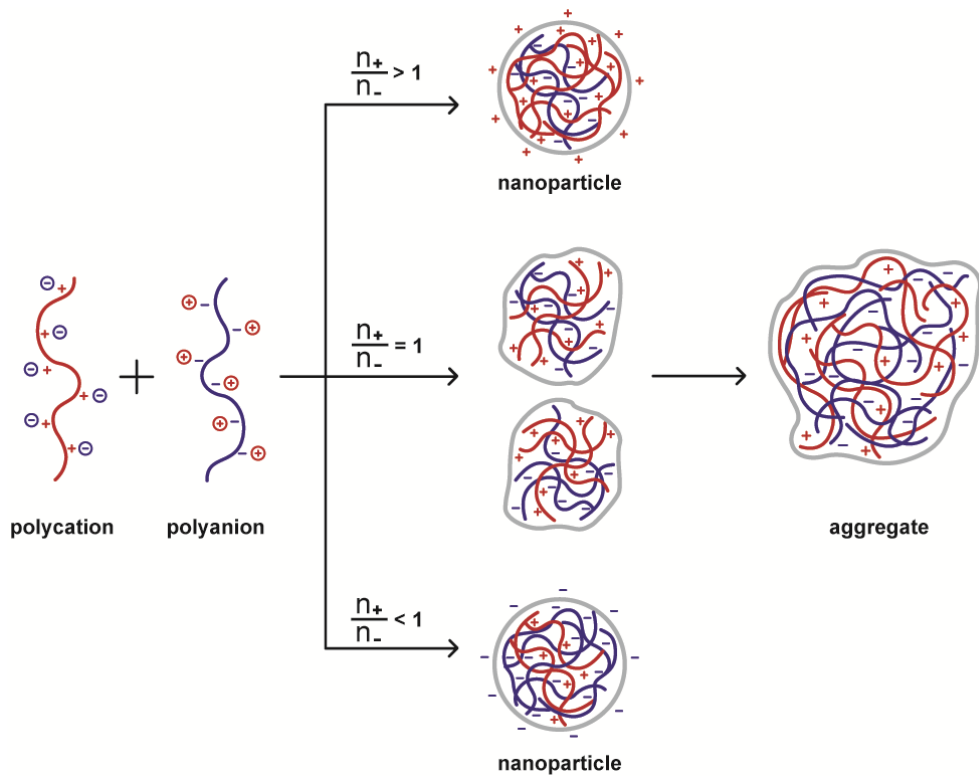

134
135   Figure 3. Effect of the polyelectrolytes charge ratio on the size and charge of the PEC formed.
136   When the charge ratio is different from one, the nanoparticles formed are charged with the same
137   charge as the polyion in excess. If the charge ratio equals one, uncharged particles are formed,
138   thereby producing large aggregates.

139   Other conditions such as pH (particularly important in weak polyelectrolyes), ionic strength and
140   the mixing rate should be adjusted to the particular chitosan-polyanion pair system selected, since
141   these variables will also influence the size and charge of nanoparticles.
142   Different preparation methods will result in diverse kinds of nanoparticles, which can be
143   classified as nanoaggregates, nanocapsules or nanospheres. The particular procedure selected can be
144   largely determined by the water solubility of the active agent that will be encapsulated and the
145   polyanion used.
146
147   2.1.1. Chitosan-alginate PEC nanoparticles
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149   Alginates are a family of anionic polysaccharides extracted from brown algae. They are
150   composed of α-L-guluronic acid (G) and β-D-mannuronic (M) acid units linearly linked by 1,4-
151   glycosidic bonds (Figure 4). The M/G ratio and their distribution along the chains (chain
152   microstructure) are strongly dependent on the particular species of algae from which it was extracted
153   [65]. Alginate (ALG) is non-toxic, biocompatible and biodegradable, mucoadhesive and non-
154   inmunogenic. The gelling capacity of ALG in the presence of calcium ions in the so-called "egg-box"

155 model has been extensively employed to prepare gels, capsules, and micro- and nanoparticles for
156 drug delivery [66]. The guluronic units are responsible for the crosslinking reaction; and the
157 properties of the beads formed, such as strength and porosity, will therefore depend on the alginate
158 source. Other parameters affecting the characteristics of beads are ALG molecular weight, and the
159 concentration of $CaCl_2$ and alginate solutions [65].

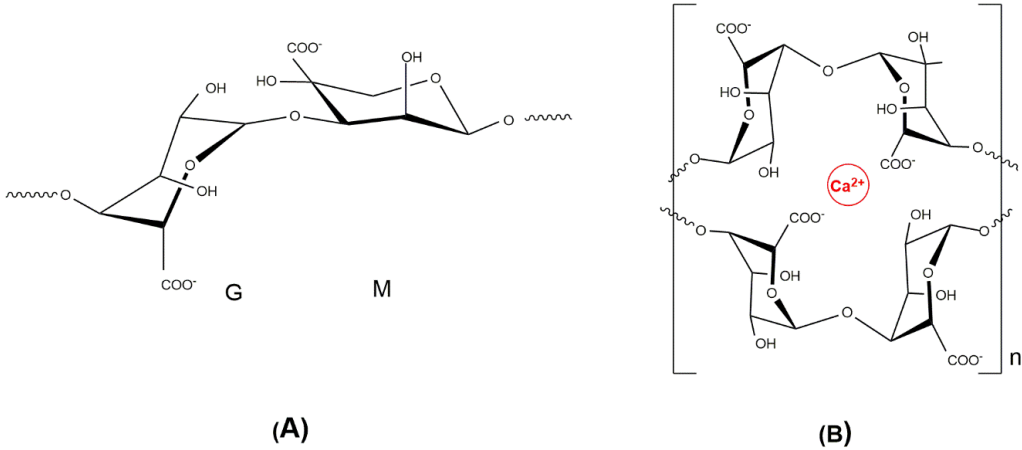(A)   (B)

160

Figure 4. (A) Structural units in alginate. (G) Guluronic acid; (M) Mannuronic acid. (B) Representation of two G-blocks forming an 'egg box' sequence with a calcium ion.

Chitosan-alginate PEC nanoparticles are usually prepared by one of the following three procedures.

2.1.1.1. Plain complex coacervation by mixing dilute solutions of CS and ALG.

In this procedure, the order of addition of one polysaccharide into the other, the CS/ALG ratio, the molecular weight of both polyelectrolytes and the pH and the ionic strength of the solutions are important factors in determining the relevant parameters of nanoparticles (size, particle charge, stability, encapsulation efficiency).

This procedure was used by Liu and Zhao [66] to prepare negatively or positively charged CS/ALG nanoparticles by dropping a CS solution over the ALG solution. They found that particle sizes varied from 320 nm to 700 nm, depending on the pH and ionic strength of the solution. At pH 4.80 in deionized water the sample displayed a narrow unimodal size distribution with an average hydrodynamic diameter (Dh) of 329 ± 9 nm. The Zeta-potential of NPs was also dependent on pH and ranged from +6.34 mV at pH 3.0 to –44.5 mV at pH 10.0. The loading capacities of NPs for ibuprofen and dipyridamole were 14.18% and 13.03%, respectively. Drug release was governed simultaneously by the solubility of the drug and the permeability of the CS/ALG nanoparticles [67].

In a modification of this procedure, a CS solution containing Tween 80 (stirring the chitosan solution with Tween 80 generated chitosan drops) was dropped into a previously prepared solution of an alginate complex with doxorubicin (DOX). The NP suspension was stirred overnight and the doxorubicin loaded CS/ALG NPs were separated by centrifugation. The size of the NPs was 100 ± 35 nm, with a polydispersity index (PDI) of 0.40 ± 0.07, the Zeta-potential was of +35 ± 4 mV and the encapsulation efficiency (EE) achieved was 95 ± 4% for the optimal formulation (CS/ALG = 2:1) [68].

The reverse procedure was used to encapsulate amoxicillin in CS/ALG nanoparticles. Essentially a mixture of chitosan, Pluronic F-127 (surfactant) and amoxicillin was prepared in various concentrations of all components. An aqueous solute on of ALG was sprayed into this mixture with stirring, to form NPs. Both solutions were at pH 5.0. The process was optimized for variables such as pH and the mixing ratio of polymers, concentrations of polymers, drug and surfactant, using the 33 Box-Behnken design. The resulting particle size, surface charge, drug entrapment percentage, in-vitro mucoadhesion and in-vivo mucopenetration of nanoparticles in rat models were analyzed. The optimized formulation with particle size, Zeta-potential and encapsulation efficiencies of 651 nm, +59.76 mV and 91.23%, respectively, showed comparatively low *in-vitro* mucoadhesion compared to plain chitosan nanoparticles, but excellent mucopenetration and localization [69].

194 A modified hybrid blending system was developed by Goycoolea *et al.*, which combined the
195 complex coacervation of CS and ALG with the ionotropic gelation of CS with trisodium
196 tripoliphosphate (TTP). The purpose of this combination was to increase stability in the biological
197 media and for better pharmacological performance than with conventional CS-TPP nanoparticles. In
198 this method, an ALG solution containing TPP was mixed under rapid stirring with the CS solution
199 forming the CS-TPP-ALG nanoparticles. Insulin loaded CS-TPP-ALG nanoparticles were obtained
200 by adding insulin into the ALG-TPP solution before mixing with the CS solution. The average particle
201 size of the insulin-loaded CS-TPP-ALG NPs was 297 ± 4 nm (PDI 0.25), similar to that of the unloaded
202 NPs, which was 307 ± 5 nm (PDI 0.22). High positive Zeta-potential values ~ +42 mV   were obtained
203 in both cases, providing good stability to the NPs. Insulin loading efficiencies (defined as insulin
204 loaded per weight of nanoparticles) as high as 50.7% were attained [70].

205 2.1.1.2. Ionotropic pregelation of alginate followed by complexation with chitosan.

206 This is a very common method in which pregelation is usually attained with $CaCl_2$, but other
207 divalent ions may also be used. The active agent can be dissolved or dispersed in the ALG solution
208 or it can be loaded into the resulting CS/ALG nanoparticles.   Azevedo *et al.* used this procedure by
209 setting the initial pH of the ALG and CS solutions to 4.9 and 4.6, respectively. In their formulation,
210 the average size of CS/ALG NPs was 120 ± 50 nm with a Zeta-potential of -30.9 ± 0.5 mV. Vitamin B2
211 loaded NPs were obtained by dissolving the compound in the ALG solution before the pregelation
212 step. The average size of nanoparticles with vitamin B2 was 104 ± 67 nm (PDI 0.32 ± 0.07) with a Zeta-
213 potential of -29.6 ± 0.1 mV. The nanoparticles showed EE and loading capacity (LC) values of $56 \pm$
214 6% and $2.2 \pm 0.6\%$, respectively [71].

215 2.1.1.3. Oil-in-water (o/w) microemulsion of alginate followed by ionotropic gelation and further
216 complexation with chitosan.

217 This method is usually preferred for encapsulating hydrophobic drugs. Preparation of
218 nanocapsules is carried out by emulsifying a solution of the drug (oil phase) into the aqueous sodium
219 alginate solution containing a surfactant, followed by gelation with calcium chloride and CS.
220 Bhunchu *et al.* used this method to prepare CS/ALG NPs containing curcumin diethyl
221 disuccinate (CDD). CDD dissolved in acetone (1 ml) was dropped into 20 ml of a dilute ALG
222 solution (0.6 mg/ml) containing a non-ionic surfactant (Pluronic F127, Cremophor RH40™ and
223 Tween 80®). Four ml of a $CaCl_2$ solution (0.67 mg/ml) was added while stirring, followed by
224 sonication. Four ml of the CS solution at various concentrations (0.15 - 0.45 mg/ml in 1% (v/v) acetic
225 acid) were added with continuous stirring at 1000 rpm for 30 min. After standing overnight for
226 equilibration the CDD loaded CS/ALG NPs were obtained as a dispersion in the aqueous solution.
227 Pluronic F127 gave the smallest particle size, 414 ± 16 nm (PDI 0.63 ± 0.05) with the highest Zeta-
228 potential, -22 ± 1 mV. The EE and LC of these NPs were $55 \pm 1\%$ and $3.33 \pm 0.08\%$, respectively. These
229 NPs improved cellular uptake of CDD in Caco-2 cells, compared to free CDD [72].

230 A list of some selected examples of CS/ALG PEC nanoparticles based on the different
231 `procedures mentioned above is given in Table 1.
232
233 Table 1. Chitosan-Alginate PEC nanoparticles. The intervals shown generally indicate extreme values
234 obtained under different preparation conditions.

Procedure	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
Complex coacervation				
CS added into ALG	Ibuprophen Dipyridamole	320 to 700[b]	+6.34[b] to − 44.5[b*]	[67]
	Gatifloxacin[a]	347[c]	+38.6[c]	[73]
	Doxorubicin	100 ± 28[b]	$+36 \pm 3$[b]	[68]

CS into ALG-DOX		100 ± 35^c	$+35 \pm 4^c$	
ALG added into CS	Amoxicillin[a]	264 to > 601	+ 35 to + 61.9	[69]
	Fluorescein isothiocyanate	338 ± 16^b 266 ± 7^c	$+34 \pm 8^b$ $+30 \pm 4^c$	[74]
ALG into Thiolated CS	Fluorescein isothiocyanate	338 ± 16^b 266 ± 7^c	$+34 \pm 8^b$ $+30 \pm 4.^c$	
ALG+TPP added into CS	Insulin	260 - 525	+41 to +50	[70]

Ionotropic pregelation of alginate plus PEC coating with CS

CS into Ca/(ALG+drug)	Insulin	781 ± 61^b 748 ± 217	-15 ± 2^b -6 ± 2^c	[75]
	Vitamin-B2	120 ± 50^b 104 ± 67^c	-30.9 ± 0.5^b -29.6 ± 0.1^c	[71]
	Acetamiprid	201.5	-32.1	[76]
CS+EGF into Ca/ALG	EGF-antisense[a]	194 - 1435	~ +30	[77]
CS+plasmid into Ca/ALG	pEGFP plasmid	161	+29.3	[78]

o/w ALG microemulsion followed by ionotropic gelation and further complexation with CS

	Turmeric oil	522 - 667	-21.8 to -22.2	[79]
	A.A.	400		[80]
	CDD	410 ± 20	22 ± 1	[72]
LMWAlg + OligoCS	BSA	134 - 229		[81]

235   [a]Optimization performed; [b]unloaded particle; [c]loaded particle; A.A., aminoacid derivatives; CDD,
236   curcumin diethyl disuccinate; *pH 3.0

237   Inspection of Table 1 reveals that a wide variation in particle size and Zeta-potential is reported
238   for all of the three general procedures devised for preparing CS-ALG PEC nanoparticles. The same
239   happens with the EE and the LC. This is a consequence of the already mentioned dependence of these
240   parameters on multiple variables.
241   In plain complex coacervation a surfactant is sometimes added to improve the entrapment
242   efficiency and the solubility of the drug [69, 73], but it might increase the size of particles and decrease
243   the Zeta-potential [69]. EE values reported in these methods vary from around 50% [69,70] to 95%
244   [67]. The LC is not always declared, but values of 14.18% and 13.03%, depending on the drug [66]
245   have been reported.
246   In the method based on the pregelation of alginate encapsulation efficiencies reported are in general
247   higher than 50%. For instance, Azevedo *et al.* [71] reported an EE of $56 \pm 6\%$ for vitamin B2, while
248   other authors declared $73 \pm 2\%$ for insulin [75], 62% for acetamipirid [76] and 95.6% for EGF-antisense
249   [77]. However, loading capacities reported were only $2.2 \pm 0.6\%$ [71] and $10 \pm 2\%$ [75]. For the NPs
250   loaded with vitamin B2 the PDI was 0.32 ± 0.07. The other reports did not declare the PDI obtained.
251   The method based o/w microemulsion of ALG followed by ionotropic gelation and
252   complexation with CS produced in general nanocapsules with sizes of about 400 to 660 nm [72, 79-
253   80]. The PDI was reported only in reference [74] and was 0.63 ± 0.05. When using low molecular
254   weight polysaccharides particles sizes ranging from 134 to 229 nm were reported [81]. Encapsulation

255 efficiencies informed were 55 ± 1% for curcumin dietil disuccinate (LC, 3.33 ± 0.08%) [72] and 88.4%
256 for BSA [81].
257
258 2.1.2. Chitosan-Pectin PEC nanoparticles
259
260   Pectin is an anionic hetero-polysaccharide derived from plant cell walls, consisting primarily of
261 1,4 linked α-D-galactopyranosyl uronic acid residues with 1,2-linked α-L-rhamnopyranose residues
262 interspersed with varying frequencies (Figure 5). Pectin structure also presents a certain amount of
263 neutral sugars (arabinose, galactose, rhamnose, xylose and glucose). A number of galacturonic acid
264 residues in the pectin are methyl or acetyl esterified. The percentage of galacturonic acid residues
265 that are esterified is known as the degree of esterification (DE).
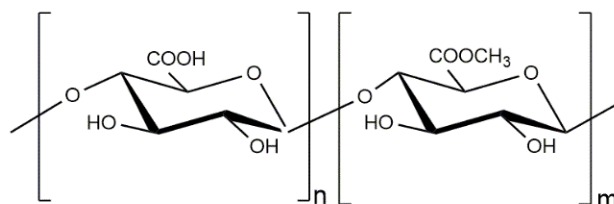

266
267   Figure 5. Chemical structure of partially acetylated polygalacturonic acid in pectin.

268   Pectin is hydrophilic, biocompatible and biodegradable and has low toxicity. As in ALG, pectin
269 with a low methoxyl content (DE <50%), has the ability to gel in the presence of Ca^{2+} ions generating
270 junction zones between chains with an egg-box structure. Pectins with higher DE can also form gels,
271 provided there are a sufficient number of blocks of non-esterified uronic acid residues per molecule
272 to allow the formation of a sufficient number of junction zones to form a network. These properties
273 of pectin have been employed to prepare diverse formulations for drug delivery applications.
274   Galacturonic acid provides pectin with a negative charge in solutions with pH higher than 3.5
275 permitting the formation of polyelectrolyte complexes with chitosan. The strength of the interaction
276 dependes on the degree of esterification of the pectin, with pectins of a relatively low DE (36%) readily
277 forming PECs with CS [82]. PEC formation is also affected by the ratio of pectin to CS and the pH of
278 the solutions [83].
279   CS-pectin PEC nanoparticles can be prepared by the same methods previously described for CS-
280 ALG PEC nanoparticles. Birch and Schiffman prepared nanoparticles by the complex coacervation
281 technique adding pectin at the appropriate CS-to-pectin ratio to the CS solution. They thereby
282 obtained particle sizes ranging from 560 ± 10 nm to 1000 ± 40 nm. The Zeta-potential varied from +20
283 ± 1 mV to +26 ± 1 mV.   When the addition order was reversed the particle size increased from 460 ±
284 20 to 1110 ± 30 nm and the Zeta-potential changed from +19 ± 1 to +28 ± 1 mV [84].
285   Rampino *et al*. prepared CS-pectin PEC nanoparticles by two different procedures: a) coating, by
286 adding a dispersion of low molecular weight CS NPs previously prepared by the ionotropic gelation
287 of CS with TPP to a pectin (from apple and citrus fruit) solution; and b) blending, by adding a CS
288 solution to a solution of pectin and TPP. Nanoparticles were charged with ovalbumin (OVA) and
289 bovine serum albumin (BSA) as the model proteins. They pointed out that the blending technique
290 can be advantageous because, by being a one-step preparation, it is highly desirable for a scale-up
291 process. Additionally, it gives the possibility of tuning the size and Zeta-potential by properly
292 selecting the ratios of CS, pectin and TPP. However, they found that there was a decrease in the
293 loading of BSA and OVA in the case of the blending technique (loading efficiency, ranging between
294 16% and 27%) due to the electrostatic interactions of CS with the protein and pectin, both negatively
295 charged. Therefore, they concluded that the selected technique would depend on the
296 physicochemical characteristics of the polymer and protein involved [85]. Some of the parameters
297 reported in their work are listed in Table 2, together with some selected examples of CS-pectin
298 preparation procedures reported by other authors.
299

300   Table 2. Chitosan-Pectin PEC nanoparticles. The intervals shown generally indicate extreme values
301   obtained under different preparation conditions.

Procedure	Active agent	Particle size, nm	Z-potential, mV	Ref.
Complex coacervation				
Pectin added into CS	Insulin	441 ± 32[a]		[86]
		580 – 896[b]	+62 ± 3[b]	
		*650 ± 86[b]	+33 ± 4[b]	
	Curcumin	10 - 59 (dry NPs)		[87]
	Insulin	1175 – 2618[a]	-22.5 to +35.0[a]	[23]
		964 – 2510[b]	-22.4 to +33.2[b]	
	Nisin	301 - 712[b]		[88]
	None	560 - 1000	+20 to +26	[84]
CS added into Pectin	None	460 - 1110	+19 to +28	[84]
Combined ionotropic gelation and complex coacervation				
Pectin+TPP added into CS	Insulin	375 - 7239	+10.6 to +32.7	[86]
CS added into Pectin+TPP	OVA	250 - 750[a]	−20 to −29[a]	[85]
CS +TPP added into Pectin	BSA	200 – 400[a]	-15 to -45[a]	[85]
		700 - 1250[b]	-38[b]	
Ionotropic pregelation of pectin plus PEC coating with CS				
CS added into Pectin+CaCl2	OVA	419[a]	-30.4[a]	[84]
		302 – 409[b]	−21.9 to −26.0[b]	

302   [a]Unloaded particle; [b]loaded particle *The CS solution contained Ca^{2+}ions

303   Not all the references in Table 2 report parameters such as EE, LC and PDI. In plain complex
304   coacervation, Maciel, *et al.* [23] reported microparticles with size less than ~2500 nm using charge
305   ratios (n+/n− given by the chitosan/pectin mass ratio) of 0.25 and 5.00, with PDIs of 0.25 ± 0.06 and
306   0.40 ± 0.06, respectively. The highest EE (~ 62.0%) of the system was observed at a charge ratio (n+/n−)
307   5.00. Andriani *et al.* [87] added glutaraldehyde to the chitosan-pectin mixed solution. This way they
308   obtained encapsulation efficiencies varying from 24.0% (LC 6.30%) to 94.7% (LC 21.05%). Combining
309   ionotropic gelation and complex coacervation, Al-Azi, *et al.* [86] reported a PDI of 0.67-0.71. Insulin
310   association efficiency varied from 2.40 ± 0.33% (LC 0.31 ± 0.04%) at pH 3, to 4.06 ± 0.12% (LC 0.52 ±
311   0.01%) at pH 5. Using Ca^{2+} ions caused a marked improvement in insulin association efficiency of
312   nanoparticles.
313
314   2.1.3. Chitosan-Dextran sulfate PEC nanoparticles
315
316   Dextran sulfate (DS) is a biodegradable and biocompatible negatively charged branched
317   polyanion that is able to interact with positively charged polymers. It is a high-molecular weight,
318   branched-chain polysaccharide polymer of D-glucose containing 17-20% sulfur. The straight chain
319   consists of approximately 95% α-(1,6) glycosidic linkages. The remaining α- (1,3) linkages account for
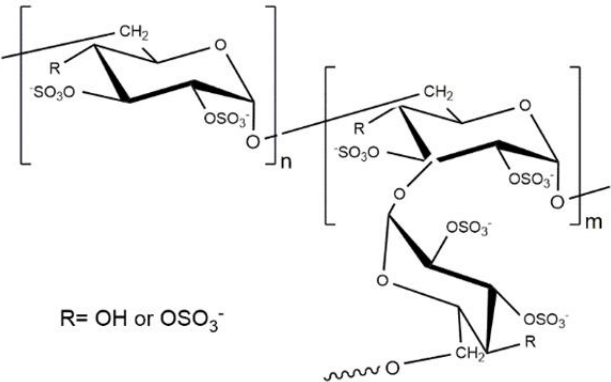320   the branching of dextran (Figure 6).

321

Figure 6. The chemical structure of dextran sulfate.

323   DS has been used as an anticoagulant and with applications in drug delivery. For instance, it
324 was used to mask the positive charge of doxorubicin (DOX) before its addition to a CS solution for
325 nanoparticle formation by ionotropic gelation with TPP. This modification doubled DOX EE relative
326 to the controls and made it possible to reach loadings of up to 4.0 wt-% DOX [89].
327   CS-DS PEC nanoparticles are almost invariably prepared by simple coacervation. The factors
328 affecting the mechanism for the formation of these nanoparticles: the mode of addition, charge
329 mixing ratio, pH and ionic strength of the media and the molar mass of both components have been
330 thoroughly reviewed by Schatz *et al.* [90, 91].
331   There are numerous reports on the preparation of CS-DS PEC nanoparticles with a potential
332 application for the delivery of proteins (insulin, BSA), growth factors [92-94], immunoglobulin-A [95]
333 and vaccines [96, 97]. Recently fluorescein isothiocyanate loaded CS-DS nanoparticles (FCS-DS NPs;
334 mean size, 400 nm (PDI 0.25 ± 0.01) and surface charge, +48 mV) were topically applied to the porcine
335 ocular surface where it was retained for more than 4 h. After 6 h under the topical FCS-DS NPs
336 treatment, particles were accumulated in the corneal epithelium but were not found in the corneal
337 stroma. However, when the epithelium was removed, the FCS-DS NPs penetrated the stroma. These
338 results indicate that FCS-DS NPs are potentially useful for drug/gene delivery to the ocular surface
339 and to the stroma when the epithelium is damaged [98].
340   Most of nanoparticles formulations reported describe processing factors affecting the
341 characteristics of CS–DS nanoparticles, including their physicochemical properties as well as the
342 optimal conditions for their preparation. Some examples are listed in Table 3.

343 Table 3. Chitosan-Dextran sulfate PEC nanoparticles. The intervals shown generally indicate extreme
344 values obtained under different preparation conditions.

Procedure	Active agent	Particle size, nm	Z-potential, mV	Ref.
Complex coacervation				
DS added into CS		>244[a]	-47.1 to -60[a]	[99]
	BSA	478 - 1138[b]	-28.0 to +56.4[b]	
	Rhodamine 6G	245 - 3521[b]	-31.0 to +34.0[b]	
CS added into DS	Insulin	489 - 665[b]	-0.4 to -21.5[b]	[100]
		527 - 1577[b]	-20.6 to +11.5[b]	[101]
	Amphotericin B	616 - 891[a]		[93]
		644 – 1040[b]	-27 to -37	
	REPIFERMIN®	239	-18.4	[94]
		306	-15.5	
Mixing with agitation	Hydralazine	290 ± 60[a]	-7 ± 4[a]	[102]
		340 ± 50[b]	-5 ± 1[b]	

345   aUnloaded particle; bloaded particle

346   In Table 3, reference [99] illustrates that different results are obtained for the same system when
347   used to encapsulate two different substances, BSA and Rhodamine 6G, by complex coacervation. In
348   this work, the size of the BSA loaded CS-DS NPs varied from 478 nm (PDI 0.64; EE 96.8%; LC 81.6%)
349   to 1138 nm (PDI 0.97; EE 53.2%; LC 29.3%). However, the Rhodamine 6G loaded nanoparticle sizes
350   were higher, varying from 545 nm (PDI 0.60; EE 98%; LC 31%) to 3521 nm (PDI 0.68; EE 42%; LC
351   18%). In both cases, the bigger NPs were more polydisperse and had lower LC and EE [99].
352   Sarmento et al. prepared CS-DS PEC nanoparticles containing insulin with association
353   efficiencies varying from 85.4 ± 0.5% to 72± 3% depending on CS/DS mass ratio [100]. In a later article
354   [101] these authors evaluated the pharmacological activity of insulin-loaded CS-DS PEC
355   nanoparticles following oral dosage in diabetic rats. On this occasion, they introduced small changes
356   in the preparation parameters and obtained somewhat lower association efficiencies, ranging from
357   69 ± 1% (LC, 2.3 ± 0.6%) to 24 ± 2% (LC, 2.0%) [101]. This influence of the preparation parameters on
358   the characteristics of the PEC nanoparticles can be used to modify them to meet the specific
359   requirements of a determined application.
360   PECs of soluble chitosan derivatives with DS have also been formulated to overcome the
361   insolubility of chitosan in neutral and basic media. Glycol chitosan (GC) and DS solutions were mixed
362   together to prepare GC-DS PEC nanoparticles loaded with the antifolic agent methotrexate (MTX)
363   aiming to increase its efficacy for the treatment of brain tumors.   EE was as high as 87% for a particle
364   size of 149 ± 41 nm (PDI 0.7 ± 0.1). *In vitro* experiments indicated their potential for the controlled
365   delivery of the drug to the brain [103].
366   PEC nanoparticles of water soluble N,N,N-Trimethyl chitosan (TMC) and DS were prepared by
367   adding DS solutions to TMC solutions at the desired pH values (5, 8, 10, and 12). The release efficiency
368   and ex-vivo nasal toxicity evaluation were assessed after loading a model drug, ropinirole
369   hydrochloride into an optimized PEC formulation at pH 10 (particle size, 255 ± 10 nm; Zeta-potential,
370   −4 ± 1 mV; LC = 82 ± 2 %; EE = 87.9 ± 0.6 %). Data indicated that the PECs produced at alkaline pH
371   have a reliable formulation for nasal administration. They are biologically compatible with the
372   mucosal surface, thereby being potentially applicable as carriers for nose to brain drug delivery [104].
373
374   2.1.4. Chitosan-Carboxymethyl chitosan PEC nanoparticles
375
376   O-Carboxymethyl chitosan (CMCS) is a water soluble amphiphilic derivative of chitosan that
377   conserves the biological properties of native chitosan with increased antibacterial activity [105]. The
378   structural unit of CMCS is shown in Figure 7. CMCS has been applied in biomedicine, especially in
379   drug delivery where CMCS nanoparticles prepared by ionotropic gelation have demonstrated
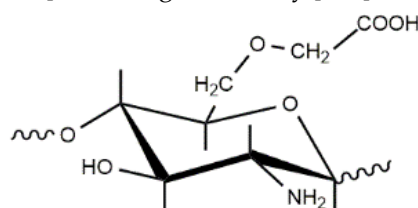380   promising results for drug [106, 107] and antigen delivery [108].

381
382   Figure 7. The structural unit of carboxymethyl chitosan.

383   The pKa of CMCS is 2.0-4.0, so that at pH above 4 it is negatively charged and forms
384   polyelectrolytes complexes with chitosan [109]. CS-CMCS PEC nanoparticles were produced by

385 complex coacervation. Wang *et al.* developed insulin-loaded nanogels with opposite Zeta-potential
386 by adding a previously prepared insulin-CMCS solution into a CS solution (particle size, 260 ± 5 nm;
387 PDI 0.08 ± 0.02; Zeta-potential, +17.2 ± 0.5 mV for insulin:CMCS/CS-NGs(+)) or inversing the order of
388 addition (particle size, 243 ± 4 nm; PDI 0.03 ± 0.01; Zeta-potential, −15.9 ± 0.5 mV for insulin:CMCS/CS-
389 NGs(-)), respectively. Encapsulation efficiencies of about 75% and loading capacities near 30% were
390 attained in both cases. They observed that negatively charged particles exhibited enhanced
391 mucoadhesion in the small intestine and had better intestinal permeability in the jejunum, indicating
392 there was a better performance in insulin:CMCS/CS-NGs(-) for blood glucose management than in
393 those positively charged [110, 111].

394 CS-CMCS nanoparticles have also been prepared by combining ionotropic gelation and complex
395 coacervation. CMCS and TPP at varying concentrations were blended with a previously prepared
396 mixture of DOX and CS solutions. Nanoparticles sizes between 249 ± 10 nm (Zeta potential, −27.6 ±
397 0.8 mV) and 362.7 ± 8.4 nm (Zeta potential, −42 ± 1. mV) with encapsulation efficiencies and loading
398 capacities of around 70.5% and 20% respectively were obtained depending on the preparation
399 conditions. Results from *in vivo* experiments indicated that CS/CMCS-NPs were efficient and safe for
400 the oral delivery of DOX [112]. After certain modifications of the preparation procedure, positively
401 charged CS/CMCS-NPs were obtained. This time, the DOX aqueous solution was premixed with
402 CMCS and the CS solution and TPP were subsequently blended with the mixture under agitation.
403 Nanoparticles sizes were of between 197 ± 11 nm (PDI 0.235; Zeta-potential, +37.6 ± 0.8 mV) and 442
404 ± 7 nm (PDI 0.635; Zeta-potential, +12.2 ± 0.6 mV), depending on the pH of the medium. *In vivo* studies
405 revealed that CS/CMCS-NGs had a high transport capacity by paracellular and transcellular
406 pathways, which guaranteed excellent absorption of encapsulated DOX throughout the entire small
407 intestine [113].
408
409 2.1.5. Chitosan- Chondroitin sulfate PEC nanoparticles
410
411 Chitosan-chondroitin sulfate PEC NPs have been prepared by complex coacervation and the
412 influence of the preparation conditions on the properties of nanoparticles was reported [114, 115].
413 Chondroitin sulphate is a linear glycosaminoglycan (GAG) composed of alternating D-glucuronate
414 and β(1,3) linked N-acetyl-d-galactosamine-4- or 6-sulfate (Figure 8). It is found in cartilage, bone and
415 connective mammalian tissue. Chondroitin sulphate (CHOS) has shown *in vivo* anti-inflammatory
416 properties in animal models and *in vitro* regulation of chondrocyte metabolism, such as the
417 stimulation of proteoglycan and collagen synthesis, and the inhibition of the production of cytokines
418 involved in cartilage degradation [116]. Its biological properties have stimulated the preparation and
419 evaluation of CS-CHOS nanoparticles for drug/gene delivery [117, 118] and delivery of platelet
420 lysates [119]. CS-CHOS nanoparticles have been suggested as a novel delivery system for the
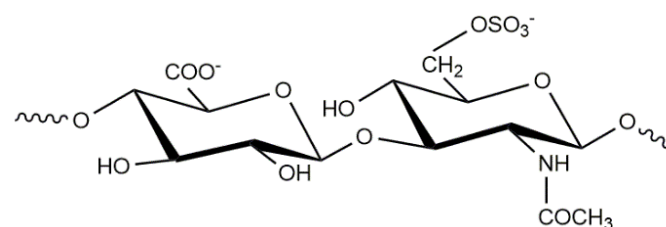421 transport of hydrophilic macromolecules [120].

422

423   Figure 8. Chemical structure of chondroitin sulfate.

424 2.1.6. Chitosan-Heparin and Chitosan-Hyaluronan PEC nanoparticles
425
426 CS PECs with other two glycosaminoglycans, hyaluronic acid (hyaluronan, HA) and heparin
427 (HEP) have also been used to prepare nanoparticles. HA is a high molecular weight linear
428 polysaccharide composed of β(1, 3) linked D-glucuronate and N-acetyl-D-glucosamine units. It is

429 present in all soft tissues of higher organisms and in particularly high concentrations in the synovial
430 fluid and vitreous humor of the eye. It plays a vital role in many biological processes such as tissue
431 hydration, proteoglycan organization, cell differentiation, and angiogenesis, and acts as a protective
432 coating around the cell membrane. On the other hand, HEP has a more heterogeneous composition,
433 but its main disaccharide unit is composed of D-glucuronate-2-sulfate (or iduronate-2-sulfate) and
434 $\alpha(1,3)$ linked N-sulfo-D-glucosamine-6-sulfate, which provides it with the highest negative charge
435 density of any known biological macromolecule (Figure 9). HEP can be found primarily on the cell
436 surface or in the extracellular matrix, attached to a protein core. Heparin is a well-known
437 anticoagulant drug and is extensively used in medical practice [121]. The important bioactivity of
438 both GAGs has stimulated the preparation of CS-HA and CS-HEP PEC nanoparticlesdue to their high
439 potential in applications as delivery systems for these macromolecules, particularly in tissue
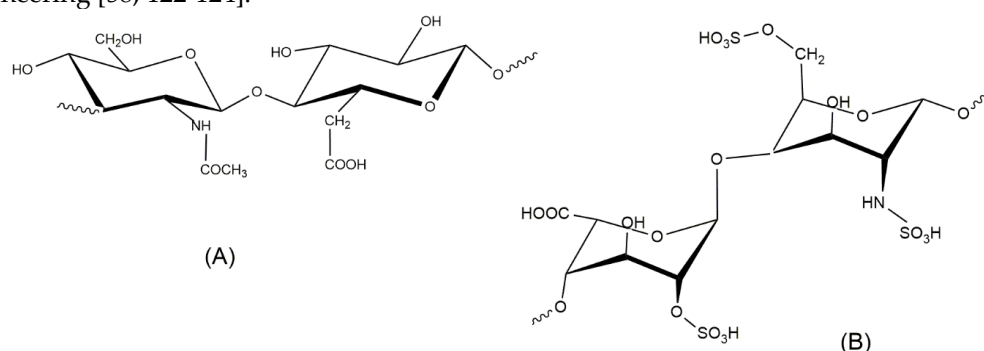440 engineering [58, 122-124].

441

442   Figure 9. Chemical structures of (A) Hyaluronic acid and (B) Heparin

443 2.1.7. Chitosan and Poly(γ-glutamic acid) PEC nanoparticles

444

445   Poly(γ-glutamic acid) (γ-PGA) is an anionic, natural polypeptide made of D- and L-glutamic
446 acid units, joined together by amide linkages between the α-amino and γ-carboxylic acid groups
447 (Figure 10). PEC formation between CS and γ-PGA has been evaluated in terms of physical and
448 chemical properties. In experimental trials, it has shown wound-healing efficacy with a potential
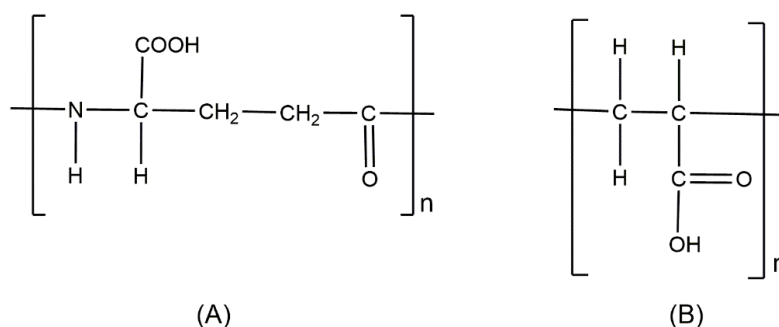449 application as a wound dressing material [125].

450

451   Figure 10. Chemical structures of (A) Poly(γ-glutamic acid) and (B) Poly(acrylic acid)

452   PEC nanoparticles of γ–PGA and low molecular weight CS were obtained by complex
453 coacervation by Lin *et al.* by adding an aqueous γ–PGA solution at pH 7.4 to a low molecular weight
454 CS solution at different pH values. The NPs prepared at pH 6.0 and a CS/γ -PGA ratio of 4.5:1.0 (w/w)
455 had a Zeta-potential of +32 ± 2 mV with a particle size of 146 ± 2 nm (PDI 0.21 ± 0.02). Insulin loaded
456 NPs were obtained by including insulin in the γ–PGA solution before its addition to the CS solution.
457 Nanoparticles with a mean size of 198 ± 6 nm (PDI 0.30 ± 0.09) and a Zeta-potential of 28 ± 1 mV were
458 obtained when the amount of insulin added was 84 µg/ml (EE 55 ± 3, LC 14.1 ± 0.9. Animal studies
459 indicated that the insulin loaded NPs enhanced insulin adsorption and reduced the blood glucose
460 level in diabetic rats [126]. Hajdu *et al.* [127] reported the effect of pH, polymer ratios, concentrations,
461 and orders of addition on the physicochemical properties of NPs

462   The same procedure was used to prepare exendin-4 loaded NPs, only that in this case the CS
463   solution contained distinct metal ions (Cu^{2+}, Fe^{2+}, Zn^{2+} or Fe^{3+}) to enhance the drug loading efficiency.
464   Loading efficiency of 61 ± 2% (LC 15 ± 2%) was achieved for exendin-4 loaded NPs formed with Fe^{3+}.
465   Their particle size was 261 ± 26 nm [128].
466   Nanoparticles of γ–PGA and CS have also been prepared by the combination of ionotropic
467   gelation and complex coacervation. To this end, the insulin and γ-PGA solutions were premixed.
468   Afterwards, TPP and $MgSO_4$ solutions were mixed together and added to the insulin and γ-PGA
469   mixture. The resulting solution was then added by flush mixing with a pipette tip into the aqueous
470   CS solution and the nanoparticles were formed. These NPs also resulted in a promising carrier for
471   the improved trans mucosal delivery of insulin in the small intestine [129, 130].
472   More recently Pereira *et al.* used the pregelation method to prepare CS/γ-PGA PEC nanoparticles
473   to be used as a nanocarrier system for the plant growth regulator gibberellic acid (GA3). To this end,
474   a $CaCl_2$ solution was added to a solution of γ-PGA at pH 4.9. Then, a CS solution at pH 4.5 was added
475   to the γ-PGA/$CaCl_2$ solution while stirring, using a peristaltic pump. To prepare GA3 loaded NPs,
476   the plant hormone was added to the γ-PGA/CaCl2 before the addition of the CS solution. The
477   unloaded γ-PGA/CS nanoparticles presented an average size of 117 ± 9 nm (PDI 0.43 ± 0.07) and Zeta-
478   potential of −29.0 ± 0.5 mV at pH 4.4. The corresponding values for the GA3 loaded γ-PGA/CS
479   nanoparticles were 134 ± 9 nm (PDI 0.35 ± 0.05) and −27.8 ± 0.5 mV at pH 4.4, respectively. The
480   encapsulation efficiency of the GA3 particles was 61%. In laboratory experiments using *Phaseolus*
481   *vulgaris* seeds, the γ-PGA/CS-GA3 NPs showed high biological activity, with an enhanced rate of
482   germination when compared with the free hormone. The encapsulated GA3 was also more efficient
483   than the free GA3 in the increase of leaf area and the induction of root development, demonstrating
484   the considerable potential of this system for its use in the field [131].
485
486   2.1.8. Chitosan-Poly(acrylic acid) PEC nanoparticles
487
488   Poly(acrylic acid) (PAA) is a biocompatible linear anionic polyelectrolyte that readily reacts with
489   CS generating polyelectrolyte complexes by the electrostatic interaction between its COO^- groups and
490   the NH_3^+ groups of chitosan [33, 38].
491   Hu *et al.* prepared CS-PAA PEC nanoparticles by template polymerization of acrylic acid in
492   chitosan solution using chitosan as the template. Positively charged NPs with the mean size and Zeta-
493   potential of 206 ± 22 nm and +25.3 ± 3.2 mV, respectively were obtained with 70 % yield.   These NPs
494   were loaded with silk peptide powder (SP) with an encapsulation efficiency of 82 %. Release
495   experiments showed a marked pH dependence of the peptide release profile.   They also obtained
496   CS-PAA PEC NPs by complex coacervation by dropping the CS solution into the solution of PAA
497   and vice versa, to study the effect of reversing the order of addition on the resulting nanoparticles.
498   When CS was added to PAA, negatively charged particles were obtained with a mean size of 436 ±
499   78 nm and a Zeta-potential of -22.2 ± 3.6 mV. On the other hand, adding PAA solution into the CS
500   solution produced positively charged NPs with a mean size and Zeta-potential of 358 ± 46 nm and
501   +47 ± 3 mV, respectively. The order of addition also influenced the microstructure of NPs.
502   Transmission electron micrographs of dry nanoparticles showed that NPs obtained by adding the CS
503   solution over the solution of PAA had a hollow core, in contrast with nanoparticles obtained with the
504   reverse addition method, which presented a compact core [132]. In a further study, it was found that
505   nanoparticle size was affected by the molecular weight of CS and PAA, the ratio of the amino group
506   to the carboxyl group (n_a/n_c) and incubation temperature [133].
507   Davidenko *et al.* examined the influence of some experimental parameters such as the pH of the
508   polyelectrolyte solutions, their concentrations and the purification procedure on the dimensions of
509   nanoparticles and their size distribution. NPs were formed by the dropwise addition of an aqueous
510   solution of PAA into the corresponding volume of an aqueous solution of CS of a determined
511   concentration with high-speed magnetic stirring (ca. 1300 rpm). The ratio of primary amino groups
512   in CS to carboxylic groups in PAA was fixed at 1.25. They showed that it was possible to obtain
513   nanometric particle suspensions at concentrations below 0.1%. The most convenient pH values for

514 obtaining CHI–PAA NPs with an optimum yield (nearly 90 %) are 4.5–5.5 for CS and 3.2 for PAA.
515 Under these conditions, the size of NPs was 0.477 ± 0.008 nm. Particle sizes of approximately 130-140
516 nm were obtained at other pH values, but yields were lower than 45 %. It was found that purification
517 by dialysis can provoke a drastic change both in the distribution profile and in the particle size of the
518 complex. To avoid this the pH of the NPs dispersion should be as near as possible to the pH of the
519 outer dialysis solution [134]. CS-PAA PEC nanoparticles obtained by this procedure were loaded
520 with 5-fluoruracil (5-Fu) and the release profiles at pH 2 and 7.4 were obtained. At pH 2 almost 100%
521 release was achieved after 2 hours, whereas at pH 7,4 only 65% of the loaded drug was released after
522 9 hours. At this pH constant release was observed after the first 90 minutes [135].
523 The complex coacervation procedure has also been used for preparing CS-PAA PECs nanofiber
524 structures with average fibre diameters of 210 nm to 910 nm and Zeta-potentials of $+39 \pm 1$ mV to -22
525 ± 3 mV, respectively. These parameters vary according tothe preparation conditions (volume ratio of
526 CS to PAA, final suspension pH, concentration and molecular weight of CS, incubation time and
527 reaction temperature). Nanofibers can bind plasmid DNA very well and show a potential to enhance
528 gene transfer in tissue engineering applications [136, 137].
529
530 2.1.8. Chitosan PEC nanoparticles with other polyanions
531
532 The preparation of CS PEC nanoparticles for the delivery of drug and therapeutic proteins is
533 continuously increasing. They include other polyanions of natural origin, like carrageenan [138, 139],
534 carboxymethyl gum kondagogu [140], and gum arabic [141], as well as synthetic ones. Examples of
535 the latter are poly(malic acid) [142], poly(2-acrylamido-2-methylpropanesulfonic acid) [143], and
536 polystyrene-block-poly(acrylic acid) [144]. The methods used for the preparation of these
537 nanoparticles are based on the general techniques already described and will therefore, not be
538 discussed here.

539 **3. Hydrophobic modification of Chitosan and derivatives for self-assembly**

540 The hydrophobic modification of chitosan and chitosan derivatives enables an appropriate
541 hydrophilic/hydrophobic balance to promote self-assembly in an aqueous or polar medium. This
542 modification is usually achieved by grafting hydrophobic moieties to the polysaccharide chains. The
543 hydrophobically modified chitosan chains self-aggregate in the hydrophilic media as illustrated in
544 Figure 11. The following sections are shown to illustrate the state of the art of this method of chitosan
545 and chitosan derivatives NPs preparation.

legend: chitosan chain — hydrophilic solvent — hydrophobic moiety — hydrophobic drug
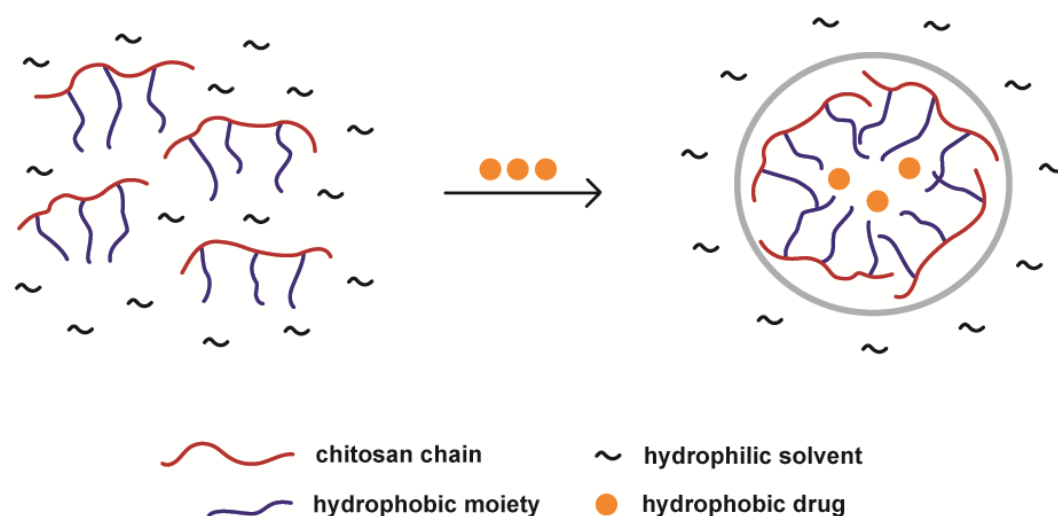
546
547 Figure 11. Schematic representation of hydrophobically modified chitosan self-assembly.
548 Aggregates can entrap hydrophobic drugs in their hydrophobic core.

549 *3.1 Hydrophobically modified Chitosan and Chitosan Oligosaccharides.*

550

551   Deoxycholic acid-modified CS self-aggregates have been proposed as a gene delivery system for
552 DNA transfection in cells [145, 146]. This system is based on the complex formation between plasmid
553 DNA and positively charged chitosan self-aggregates, which produces micelle-like nanoparticles
554 having controlled dimensions for the effective gene delivery to cells. The hydrophobic modification
555 of chitosan was accomplished with deoxycholic acid mediated by carbodiimide coupling (1-ethyl-3-
556 (3-dimethylaminopropyl) carbodiimide, EDC) for amide bond formation. Self-aggregates were
557 obtained by varying the chitosan/deoxycholic acid ratio (degree of substitution of chitosan, DS from
558 0.02 to 0.1) and the molecular weight of the reacting CS (molecular weight, MW from 5 to 200 kDa).
559 They exhibited hydrodynamic sizes ranging from 132 to 300 nm. For CS molecular weights higher
560 than 40 kDa, a transition from a bamboo-like cylindrical structure to a poorly organized bird nest-
561 like structure of self-aggregates was proposed. The DNA-CS complex formation had strong
562 dependency on the size and structure of CS self-aggregates and significantly influenced gene
563 transfection efficiency (up to a factor of 10) [146].

564   Similarly, Wang *et al*. prepared cholesterol-modified chitosan self-aggregates with succinyl
565 linkages mediated by EDC coupling amidation of CS, attaining a DS of 0.073 and hydrodynamic
566 diameters of 417.2 nm. Epirubicin was used as a model anticancer drug. It was physically entrapped
567 into the cholesterol-CS self-aggregates forming almost spherical nanoparticles of 338.2 to 472.9 nm
568 with the epirubicin loading content increasing from ca. 8 to 14%. The controlled release of epirubicin
569 from the loaded nanoparticles was slow, reaching a total release of 24.9% in 48 h [147].

570   CS-cholesterol self-aggregates were also synthesized with another approach. Prior
571 phthaloylation of CS enabled the esterification of the primary –OH group at C6 with EDC/N-
572 Hydroxysuccinimide pre-activated cholesterol succinate. Later, CS deprotection afforded 6-O-
573 cholesterol-modified chitosans (DS of 0.017, 0.04 and 0.059) that self-assembled, forming
574 nanoparticles of 100-240 nm size. These NPs were capable of physically entrapping the all-trans
575 retinoic acid with different drug loading contents, encapsulation efficiencies and particle sizes. The
576 sustained release of the all-trans retinoic acid extended over 72 h [148].

577   Chitosan oligosaccharides (low molecular weight CS produced by depolymerization) are
578 usually preferred over high molecular weight CS for pharmaceutical applications [149]. Chitosans
579 with molecular weights ranging from few hundreds daltons (c.a. trimers, tetramers) to 20kDa have
580 been referred to as chitosan oligomers [3]. Thus, Hu *et al*. prepared a CS oligosaccharide (ca. 19 kDa
581 weight average molecular weight) hydrophobically modified with stearic acid and encapsulated
582 paclitaxel or doxorubicin for their controlled delivery [149-152]. CS oligosaccharide (COS)
583 modification was conducted with stearic acid by an EDC mediated amide linkage reaction achieving
584 COS substitution degrees of 0.035, 0.05, 0.12, 0.255 and 0.42 [150-153]. Further glutaraldehyde cross-
585 linking of COS micelle shells before and after paclitaxel physical entrapping enabled drug loading
586 contents of up to 94% and to control the micelle size and paclitaxel release rate [150]. A reduction of
587 micelle diameters from 322.2 nm to 272.0 nm was observed after glutaraldehyde cross-linking for the
588 blank COS-stearic acid particles and from 355.0 to 305.3 nm for the doxorubicin-loaded COS-stearic
589 acid particles. The Zeta-potential of particles was reduced from +57.1 to +34.2 mV and from +69.1 to
590 +51.8 mV, respectively [150]. Shell cross-linking of doxorubicin-loaded COS-stearic acid micelles also
591 showed enhanced cytotoxicity to A549, LLC and SKOV3 cancer cell lines [150].

592   To reduce the observed initial burst release during the dilution of doxorubicin-loaded COS-
593 stearic acid micelles by body fluid, stearic acid was also physically encapsulated into the micelle core
594 [152]. The hydrodynamic diameter of stearic acid-loaded COS-stearic acid micelles increased
595 significantly from 27.4 nm to ca. 60 nm for a 10 wt-% of stearic acid/COS-g-stearic acid micelles, while
596 Zeta-potential decreased from +51.7 mV to ca. +35 mV [151]. The incorporation of stearic acid
597 physically entrapped in the core of doxorubicin-loaded COS-g-stearic acid micelle significantly
598 reduced the drug release rate.

599   Hu *et al*. also studied the dual functionalization of COS with stearic acid and doxorubicin cis-
600 aconitate [152]. To this end, a previously prepared COS-stearic acid conjugate (DS in stearic acid of
601 ca. 0.06) was further reacted with doxorubicin cis-aconitate by EDC mediated amidation. This

602   produced COS conjugates with doxorubicin contents of 3, 6 and 10%. DOX-g-COS-g-stearic acid self-
603   aggregated in an aqueous medium giving micelle sizes of 40.1, 70.7 and 105.8 nm respectively, and
604   Zeta-potential values of +43.7, +40.2 and +32.0 mV, respectively [152].
605   Chitosan has also been hydrophobically modified with different acyl groups mediated by amide
606   linkage formation with different anhydrides and acyl chlorides such as DL-Lactide (PLA unit
607   modifying the CS), propionic and hexanoic anhydrides, nonaoyl chloride, lauroyl chloride,
608   pentadecanoyl chloride, stearoyl chloride [153, 154]. It was observed that the micelle size of blank CS-
609   PLA increased with an increase of the degree of substitution with PLA units or with the increase of
610   side chain length for the different acyl groups (propionate, hexanoate, nonanoate, etc.). Furthermore,
611   the Zeta-potential changed from +26.0 mV for propionyl chitosan to +10.2 mV for hexanoyl chitosan
612   and remained ca. +13 to +15 mV for the other acyl chitosans. Drug loading content and drug release
613   rate were also influenced by the CS substitution degree or the chain length of the acyl substituents of
614   CS. Rifampin loading content increased and drug release rate decreased with the increase of CS
615   substitution with PLA units [154]. Vitamin C loading content increased and drug release rate
616   decreased with the chain length of the acyl group modifying CS [154].
617   Water soluble chitosan N,O6-acetyl chitosan was prepared for future hydrophobic modification
618   with different steroids and DL-α-tocopherol for the sustained release of agrochemicals, testosterone
619   and vitamin E [155]. Drug content achieved values between 11.8 and 56.4 wt-%. The CS-steroid and
620   CS-tocopherol micelles formed showed hydrodynamic sizes of ca. 200 to 360 nm in phosphate
621   buffered saline solution (PBS) with Zeta-potential values varying from +7 to +22.7 mV in bi-distilled
622   water. Sustained releases were achieved for the steroids and tocopherol from the CS particles and the
623   biological activity of the released drug appeared unaffected [155].
624   Amphiphilic block or graft copolymers of phthaloyl chitosan with different materials as
625   poly(ethylene glycol), N-vinyl-2-pyrrolidone and ε-caprolactone have a wide range of
626   pharmaceutical applications [156-162]. For example, N-phthaloylchitosan-g-mPEG micelles have
627   been physically loaded with camptothecin and all-trans retinoic acid for their controlled release [156-
628   158]. These micelles exerted a protective effect from hydrolysis on the loaded drug (camptothecin,
629   which is sensitive to hydrolysis of the lactone group) or photodegradation (all-trans retinoic acid).
630   Furthermore, a continuous release without an initial burst of prednisone acetate from N-
631   phthaloylchitosan-g-polyvinylpyrrolidone micelles was achieved [159].
632   There are also several reports showing that chitosan-graft-polycaprolactone nanomicelles have
633   been physically loaded with 7-ethyl-10-hydroxy-camptothecin, BSA, paclitaxel and 5-fluorouracil
634   [160-163].
635   Another amphiphilic copolymer of CS was synthesized from N-acetyl histidine as the
636   hydrophobic segment and arginine-grafted chitosan by EDC carbodiimide-mediated coupling for the
637   controlled delivery of doxorubicin [164]. The key finding was the effectiveness of doxorubicin loaded
638   N-acetyl histidine and arginine-grafted CS for the suppression of both the sensitive and resistant
639   human breast tumor cell line (MCF-7) in a dose- and time-dependent pattern.
640   More details of prepared hydrophobically modified CS and CS oligosaccharide conjugates can
641   be found in Table 4.

642   Table 4. Hydrophobically modified chitosan and chitosan oligosaccharides.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
deoxycholic acid	DNA	162 ± 18[a]		[145]
		~ 300[b]		
		130 – 300[a]		[146]
cholesterol	Epirubicin	417 ± 18[a]		[147]
		338 – 473[b]		
6-O-cholesterol	All-trans retinoic acid	100 – 240[a]	+24.5 to +25.9[a]	[148]
		192 – 222[b]		

stearyl	Paclitaxel	28.1 – 74.6[a]	+39.0 to +53.2[a]	[149]
		35.8 – 175.1[b]	+44.0 to +58.7[b]	
	Doxorubicin	272 – 322[a]	+34.2 to +57.1[a]	[150]
		305 – 355[b]	+51.8 to +69.1[b]	
		27.4 ± 2.4[a]	+52 ± 3[a]	[151]
		20.4 ± 1.1[b]	+53.1 ± 14.4[b]	
stearyl+doxorubicin	Doxorubicin	40.1 – 105.8[b]	+32.0 to +43.7[b]	[152]
Acyl	Rifampin	154 – 181[a]		[153]
		163 – 210[b]		
	Vitamin C	444 – 487[a]	+10.2 to +28.9[a]	[154]
		216 – 288[b]	+5.9 to +18.4[b]	
N,O6-acetyl+steroid	Steroids	197 – 358[b]	+7 to +22.7[b]	[155]
N,O6-acetyl+tocopherol	Vitamin E	275 ± 5[b]	+14.9 ± 0.7[b]	
phthaloyl	Camptothecin	~ 170[a]		[156]
		~ 200 – 267[b]		
		~ 50 – 100[a]		[157]
		~ 100 – 250[b]		
	All-trans retinoic acid	~ 50 – 100[a]		[158]
		~ 80 – 160[b]		
	Prednisone acetate	89.8[a]		[159]
		143.3[b]		
polycaprolactone, (Chitosan-grafted)	7-Ethyl-10-hydroxy-camptothecin	47 – 113[a]	+26.7 to +50.8[a]	[160]
		63 – 152[b]	+25.6 to +48.8[b]	
	BSA	168.44[b]		[161]
		200.7[b]		
		435 ± 25[a]		
	Paclitaxel	408 – 529[b]	+27.5 ± 1.1[a]	[162]
		61.4 – 108.6[a]	+30.9 to +33.3[b]	
	5-Fluorouracil	67.9 – 96.7[b]	+18.9 to +43.1[b]	[163]
N-acetyl histidine	Doxorubicin	218[a]	+40.1 ± 2.8[a]	[164]
		185.3 – 218.3[b]	+36.3 to +40.1[b]	

643   [a]Unloaded particle; [b]loaded particle; * 5 mg/mL

644   *3.2 Hydrophobically modified glycol chitosan*

645

646   The limited water solubility of CS and the precipitation of some self-aggregated chitosan
647   conjugates, restricts its application in medical practice as a drug delivery system. In contrast, glycol
648   chitosan (GC) exhibits good water solubility at all pHs, good biocompatibility, and is widely applied
649   as a hydrophobic drug and gene carrier [167-172]. The structural units of GC are shown in Figure 12.
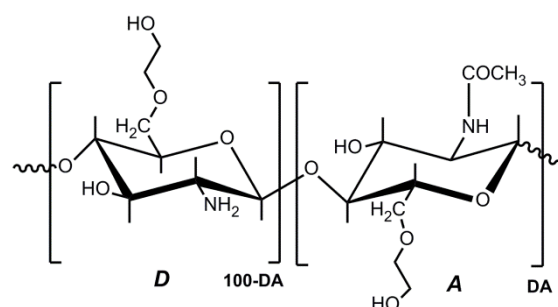
650
651   Figure 12. The chemical structure of glycol chitosan.

652   GC has been functionalized with cholanic acid, cholesterol, deoxycholic acid, vitamins,
653   testosterone, doxorubicin and other hydrophobic compounds using mostly an EDC-mediated
654   coupling reaction to achieve the amidation of CS amine groups with the desired carboxylic acid or
655   acyl chloride of the hydrophobic substituent. Further physical encapsulation of anticancer drugs or
656   bioactive compounds in the core of self-assembled GC hydrophobically-modified micelles is usually
657   performed.
658   Hwang *et al*. introduced cholanic acid in GC. The resulting GC-cholanic acid micelles can be
659   easily loaded with the anticancer drug docetaxel [165]. Docetaxel loaded GC-cholanic acid
660   synthesized spontaneously and was self-assembled as 350 nm aggregates in an aqueous medium.
661   During *in vivo* experiments in mice, these docetaxel loaded nanoaggregates showed higher anticancer
662   efficacy to A549 lung cancer cells and reduced toxicity when compared to the free drug.
663   The anticancer drug camptothecin has also been encapsulated into self-aggregates of GC-
664   cholanic acid, with a drug loading efficiency of above 80% [166]. GC-cholanic acid micelles protected
665   the lactone ring of camptothecin from hydrolysis and camptothecin loaded micelles showed
666   significant antitumor activity towards MDA-MB231 human breast cancer cells implanted in nude
667   mice. The 5β-cholanic hydrophobic functionalization of both GC and polyethylenimine and later
668   mixing of both modified polymers, made it possible to obtain self-assembled nanoparticles of ca. 350
669   nm with a Zeta-potential of +23.8 mV, for the delivery of siRNA in tumor-bearing mice [167]. The
670   siRNA-GC-polyethylenimine complex transfected the B16F10 tumor cells, efficiently inhibiting the
671   RFP gene expression of RFP/B16F10-bearing mice. Thus, GC-polyethylenimine self-aggregates were
672   revealed as promising gene carrier for cancer treatment [167]. GC-cholanic acid self-aggregates have
673   also been proposed for the delivery of RGD peptide and indomethacin [168, 169].
674   The hydrophobic modification of GC with deoxycholic acid and the later physical encapsulation
675   of palmityl-acylated exendin-4 peptide in formed self-assembled nanogels for a long-acting anti-
676   diabetic inhalation system was studied by Lee *et al*. [170]. Results were promising, with the ca. 72 h
677   residence of the administered anti-diabetic drug (palmityl-acylated exendin-4 peptide) in the lungs,
678   good hypoglycemic response and acceptable toxicity.
679   In another approach, the hydrophobic modification of GC with the drug to be delivered has been
680   explored. Quiñones *et al*. synthesized GC hydrophobically-modified with ergocalciferol
681   hemisuccinate, tocopherol hemiesters and testosterone 17β-hemisuccinate for the controlled release
682   of vitamin D2, vitamin E and testosterone [171-173]. The degrees of substitution of GC with the
683   vitamins and the testosterone reached values of 0.039 for vitamin D2, 0.21 to 0.36 for vitamin E and
684   0.015 for testosterone. The GC-vitamin and GC-testosterone conjugates formed self-assembled NPs
685   in an aqueous medium with hydrodynamic sizes from 280 to 500 nm andZeta-potential values of +7.7
686   to +36.5 mV. The sustained release of covalently linked vitamins and testosterone from the GC self-
687   aggregates was observed in an acidic medium for 3 to 4 days.
688   The hydrophobic modification of GC with an N,N-diethylnicotinamide-based oligomer enabled
689   a high paclitaxel loading content with an EE of up to 98% [174]. The hydrodynamic diameter of the
690   blank hydrophobically modified GC was 313 ± 20 nm in PBS. Paclitaxel loaded modified GC particles
691   with a drug loading content of 9.8, 18.9 and 23.9 wt-% exhibited hydrodynamic sizes of 331 ± 25 nm,
692   354 ± 23 nm and 363 ± 32 nm respectively. A sustained release of paclitaxel from the GC self-

693 aggregates was observed. Overall, the anticancer assessment of the paclitaxel loaded GC particles
694 appears promising in cancer therapy.
695   Doxorubicin encapsulation in GC-3-diethylaminopropyl self-aggregates and the hydrophobic
696 functionalization of GC with doxorubicin was also accomplished for the evaluation of doxorubicin
697 delivery systems for cancer therapy [175, 176]. The hydrodynamic parameters of GC-based self-
698 aggregates discussed are summarized in the Table 5.

699 Table 5. Hydrophobically modified glycol chitosan.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
Cholanic acid	Docetaxel	350[b]		[165]
	Camptothecin	254[a]		[166]
		279 – 328[b]	+23.8 ± 0.9[a]	
	siRNA	350[a]	+10.0 ± 0.8[b]	[167]
		250[b]		
	RGD peptide	224[a]		[168]
		189 – 265[b]		
Cholesterol	Indomethacin	228[a]		[169]
		275 – 384[b]		
Deoxycholic acid	Palmityl-acylated exendin-4	~ 52 – 250[a]		[170]
Ergocalciferol	Vitamin D2	279 ± 7 (PBS)	+7.7 ± 0.1	[171]
DL-⊚-tocopherol	Vitamin E	284 – 496 (PBS)	+11.7 to +36.5	[172]
Testosterone	Testosterone	332 ± 4 (PBS)	+9.7 ± 0.6	[173]
N,N-diethylnicotinamide-based oligomer	Paclitaxel	313 ± 20[a]		[174]
		331 – 363[b]		
3-Diethylaminopropyl	Doxorubicin	102[a]	-0.9[a]	[175]
Doxorubicin	Doxorubicin	238[a]		[176]
		342[b]		

700 [a]Unloaded particle; [b]loaded particle

701 *3.3 Hydrophobically modified carboxymethyl chitosan*
702
703   O-Carboxymethyl chitosan, typically named carboxymethyl chitosan (CMCS), has been
704 hydrophobically modified with oleoyl chloride in pyridine/dichloromethane or with linoleic acid
705 using an EDC-mediated amide linkage reaction [177-179].
706   Oleoyl-modified CMCS formed self-aggregates in an aqueous medium with average
707 hydrodynamic diameters that depended on the molecular weight of the chitosan used to prepare the
708 CMCS [177, 178]. Hydrodynamic diameters of 157.4 nm (CS with molecular weight of 50 kDa), 161.8
709 nm (CS with molecular weight of 38 kDa), 274.1 nm (CS with molecular weight of 170 kDa) and 396.7
710 nm (CS with molecular weight of 820 kDa) have been reported for different oleoyl-modified CMCS.
711 The Zeta-potential values observed for blank oleoyl-modified CMCS particles were +16 ± 1 mV, +17.2
712 ± 0.9 mV and +20 ± 1 mV. Rifampicin and microbial antigens were physically entrapped in the oleoyl-
713 modified CMCS micelles with drug loading efficiency of 20% for rifampicin and ca. 52 to 62.5% for
714 microbial antigens. The sustained release of encapsulated drugs was extended until 40-48 h [177, 178].
715   Linoleic acid modified CMCS self-aggregated micelles were loaded with the anticancer drug,
716 adriamycin, for a sustained release [179]. The average hydrodynamic diameter of the blank linoleic-

717   modified CMCS was 418 ± 18 nm. Adriamycin was slowly released from the micelles for about 3 days.
718   Results are summarized in Table 6.

719   Table 6. Hydrophobically modified carboxymethyl chitosan.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
Oleoyl	Rifampicin	161.8[a]		[177]
	Microbial antigen	157.4 – 396.7[a]	+15.6 to +19.6[a]	[178]
		237.6 – 482.3[b]	+14.2 to +17.1[b]	
		331.6 – 573.9[b]	+12.8 to +16.3[b]	
Acyl	Adriamycin	418 ± 18[a]		[179]

720   [a]Unloaded particle; [b]loaded particle

721   *3.4 Hydrophobically modified succinyl chitosan*

722

723   Water soluble succinyl chitosans have been prepared by amidation (N-succinyl chitosan) and
724   esterification (O6-succinyl chitosan) of chitosan by its reaction with succinic anhydride (Figure 13).
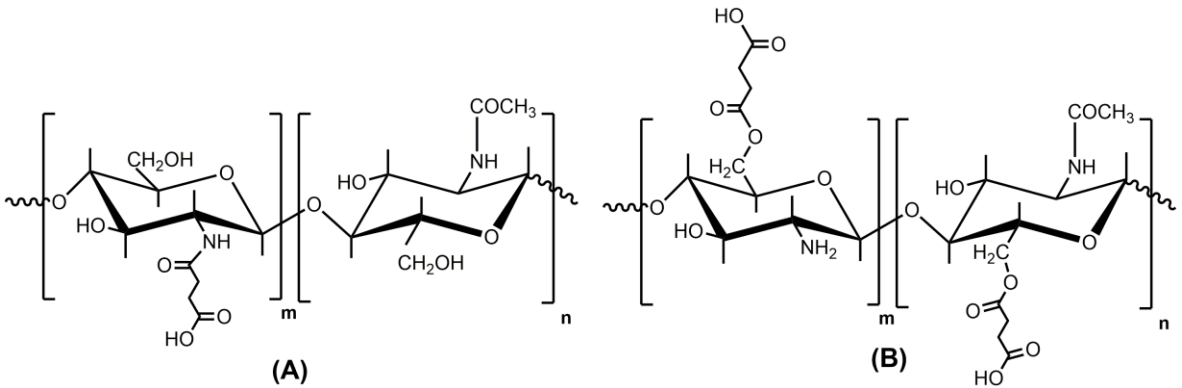
725

726
727   Figure 13. The chemical structure of N-succinyl chitosan (A) and O6-succinyl chitosan (B).

728   Xiangyang *et al*. reported the preparation of N-succinyl-N'-octyl chitosan micelles as
729   doxorubicin carriers for an effective anti-tumor activity [180]. Average hydrodynamic sizes of
730   doxorubicin loaded modified succinyl chitosan (SCS), which depended on the amount of octyl chains
731   and the drug loading content, was between 100 to 200 nm. Doxorubicin loaded SCS particles showed
732   a sustained release and more cytotoxic activity against HepG2, A549, BGC and K562 cancer cell lines
733   than parent doxorubicin.
734   In another study on SCS, the interactions between the polymer and BSA in the nanoaggregates
735   are inspected using different techniques [181]. The authors concluded that no significant change on
736   the conformation of BSA occurred during chain entanglements between the protein and the N-
737   succinyl chitosan. The hydrodynamic sizes of the micelles formed are reported in Table 7.
738   The synthesis of O6-succinyl chitosan involves phthaloyl protection of chitosan, the reaction
739   with succinic anhydride and deprotection (removal of the phthaloyl groups). Further hydrophobic
740   modification of free amine groups of O6-succinyl chitosan with tocopherol succinate mediated by an
741   EDC activated coupling reaction, made it possible to prepare cationic self-assembled SCS
742   nanoparticles with hydrodynamic diameters of 254 ± 4 nm and Zeta-potential values of +36.3 ± 0.9
743   mV [172]. The sustained release of covalently linked vitamin E (tocopherol) was extended up to 96 h.
744   The results are shown in Table 7.

745   Table 7. Hydrophobically modified succinyl chitosan.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.

Octyl	Doxorubicin	130.4 – 150.1[a]		[180]
		155.4 – 170.1[b]		
Acyl	BSA	~ 50 – 100[a]		[181]
		~ 100 – 200[b]		
DL-α-tocopherol	Vitamin E	254 ± 4	+36.3 ± 0.9	[172]

746   [a]Unloaded particle; [b]loaded particle

747   *3.5 Hydrophobically modified Trimethyl chitosan*

748

749   N,N,N-Trimethyl chitosan (TMC) is a water soluble derivative of chitosan prepared by
750   exhaustive N-methylation of some free amine groups of CS using iodomethane.
751   TMC has been hydrophobically modified with octyl, decanoyl, lauryl, lactose and palmitoyl
752   substituents for hydroxycamptothecin and harmine encapsulation in the hydrophobic core [183-85].
753   N-octyl-N-trimethyl chitosan and N-lauryl-N-trimethyl chitosan were self-assembled in an aqueous
754   medium as micelles of 23.5 nm and 20.8 nm, while N-decanyl-N-trimethyl chitosan formed micelles
755   with a hydrodynamic diameter of 277.2 nm.
756   Hydroxycamptothecin loaded N-alkyl-N-trimethyl chitosan micelles showed a sustained release
757   of the anticancer drug with improved pharmacokinetic properties and the stability of the
758   camptothecin lactone ring *in vivo* [182]. On the other hand, the harmine loaded hydrophobically
759   modified TMC released 65.3% of the encapsulated drug in 3 days at pH 7.4 [183].
760   Mi *et al*. investigated the preparation of self-assembled NPs by TMC and poly(γ-glutamic acid)
761   for the oral delivery of insulin [185]. The hydrodynamic diameters and Zeta-potential values of blank
762   and insulin loaded TMC/poly(γ-glutamic acid) NPs are presented in Table 8.

763   Table 8. Hydrophobically modified trimethyl chitosan.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
Alkyl	Hydroxy-camptothecin	20.8 – 277.2[a] 26.0 – 273.1[b]		[182]
Palmitoyl	Harmine	193.4 ± 3.1[b]	+26.67[b]	[183] [184]
Acyl	Peptide drugs	101.3 – 106.3[a] 522 ± 6[b]*	+30.6 to +36.2[a] +14.2 ± 0.6[b]*	[185]

764   [a]unloaded particle; [b]loaded particle; *pH 7.4

765   *3.6 Other hydrophobically modified chitosan derivatives*

766

767   N-octyl-O-sulfate chitosan (NOSC) micelles have been prepared from chitosan for the sustained
768   release of physically entrapped paclitaxel for cancer therapy [186-188]. Paclitaxel loaded N-octyl-O-
769   sulfate chitosan micelles showed hydrodynamic diameters of ca. 200 nm and Zeta-potential values
770   of ca. -30 mV [186, 187]. On the other hand, the additional modification of N-octyl-O-sulfate chitosan
771   with polyethylene glycol monomethyl ether, reduced the hydrodynamic sizes of the paclitaxel loaded
772   NOSC to ca. 100 nm [188]. The anticancer drug loaded NPs exhibited reduced toxicity and improved
773   the bioavailability of encapsulated paclitaxel [186-188].
774   Pedro *et al*. synthesized N-dodecyl-N'-glycidyl(chitosan) for the delivery of quercetin [189]. The
775   hydrodynamic parameters of quercetin loaded hydrophobically modified CS micelles were
776   measured by dynamic light scattering showing sizes from 140 to 260 nm and Zeta-potential values
777   from +18.7 to +30.4 mV at pH 7.4. At pH 5.0 the sizes ranged from 150 to 300 nm and the Zeta-potential
778   values varied from +14.1 to +29.9 mV, showing the dependence of both parameters on sample
779   concentration at both pHs. pH was also found to play a key role on quercetin release from the
780   micelles. The results are summarized in Table 9.

781   Table 9. Other chitosan derivatives.

Hydrophobic moiety	Active agent	Particle size, nm	Zeta-potential, mV	Ref.
Octyl	Paclitaxel	~ 200[b]		[186]
		200.8[b]	-31.1[a]	[187]
			-28.8[b]	
		104.3 – 133.4[b]		[188]
Acyl	Quercetin	140 – 300[a]	+14.1 to +30.4[a]	[189]

782   [a]Unloaded particle; [b]loaded particle

Conclusions

784   A considerable amount of research is going on for the self-assembling preparation of chitosan
785   nanoparticles in drug delivery applications. In particular, the nanoparticle preparations by
786   polyelectrolyte complexation and by the self-assembly of hydrophobically modified chitosans are
787   able to encapsulate the drug under mild conditions without losing their stability and
788   biocompatibility. Therefore, chitosan based self-assembled nanoparticles have great potential, as well
789   as multiple applications for the future, in the design of novel drug delivery systems.

790   **Author Contributions:** Javier Pérez, Hazel Peniche and Carlos Peniche equally contributed to the paper.

791   **Conflicts of Interest:** The authors declare no conflict of interest.

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