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 as platform for developing drug
16 delivery systems with improved biodistribution, increased specificity and sensitivity, and reduced
17 pharmacological toxicity. In particular, chitosan nanoparticles have been found appropriate for non-
18 invasive routes of drug administration: oral, nasal, pulmonary and ocular routes. These applications
19 are facilitated by the absorption-enhancing effect of chitosan. Many different procedures have been
20 proposed for obtaining chitosan nanoparticles. Particularly, the introduction of hydrophobic
21 moieties into chitosan molecules by grafting to generate a hydrophobic-hydrophilic balance
22 promoting self-assembling is a current and appealing approach. The grafting agent can be a
23 hydrophobic moiety to form 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 developed for
26 preparing chitosan nanoparticles by self-assembling by both procedures and illustrates the state of
27 the art of their application in drug delivery.

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

30 1. Introduction

31   Chitosan is a family of linear polysaccharides composed of glucosamine and N-
32 acetylglucosamine units linked together by β (1→4) glycosidic links (Figure 1). Chitosan is obtained
33 by 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 chitosan will have a degree of acetylation (DA) and molecular weight
36 that will depend on the reaction parameters involved [1]. The molecular weight, the DA and even the
37 pattern of acetylation (the distribution of glucosamine and N-acetylglucosamine units along the
38 chitosan chain) will affect its chemical and biological properties [2, 3].
39   The degree of deacetylation (DD = 100 − DA) of chitosan is about 50% or higher. At this stage the
40 polysaccharide becomes soluble in aqueous acid solutions because of the protonation of the free
41 amino groups of the D-glucosamine units. In fact, the solubility of chitosan in 1% or 0.1M acetic acid
42 is a simple practical criterion used to differentiate chitosan from chitin. In a protic solvent chitosan
43 behaves as a cationic polyelectrolyte [4].

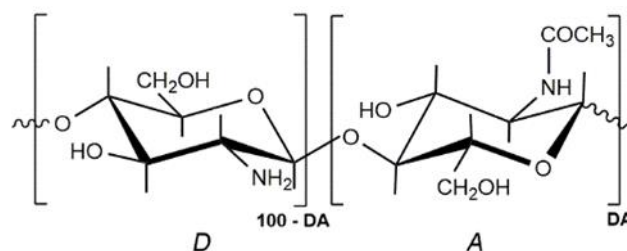



Figure 1. Structural units of chitin and chitosan. (A) N-acetylglucosamine unit; (D) Glucosamine unit. In chitosan DA< 50.

Chitosan is a biocompatible, biodegradable and non-toxic material. It exhibits other significant biological properties, such as wound healing capacity, antimicrobial, antibacterial and hemostatic activities. Chitosan is an excellent film former and can be processed into fibers, gels, microspheres-microcapsules, micro/nanoparticles [5]. Also, because of the free –OH and –NH₂ groups in its structure it is amenable to chemical modifications to potentiate some of its properties for a determined application. All these remarkable physical, chemical and biological properties have made chitosan an excellent candidate for applications in cosmetics, food industry, medicine and pharmacy [4].

Chitosan also shows mucoadhesive and absorption-enhancing properties. It can interact with mucus and epithelial cells resulting in opening of cellular tight junctions [6]. These properties make chitosan also an ideal candidate for the delivery of drugs and bioactive molecules in general. There are numerous reports on the applications of chitosan in drug delivery, with various reviews on the subject [7-9]. Applications include chitosan as excipient in tablets, chitosan hydrogels, films, fibers, micro/nanocapsules and micro/nanoparticles.

Chitosan nanoparticles find applications in drug delivery, not only by the traditional routes of administration (eg. oral and parenteral routes) but also via mucosal (nasal, pulmonary, vaginal) and ocular routes [10]. Chitosan nanoparticles are as well used in designing non-viral vectors for gene delivery and the delivery of vaccines [11].

Chitosan nanoparticles have been produced using diverse approaches. Among them ionotropic gelation [12, 13], spray drying [14], water-in-oil emulsion cross-linking [15], reverse micelle formation [16, 17], emulsion-droplet coalescence [18, 19], nanoprecipitation [20] and by a self-assembling mechanism [21, 22].

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 process of self-assembling can take place at molecular or supramolecular level [23, 24]. It can occur by self-association or by association with other different structures through interactions such as hydrogen bonding, van der Waals forces, ionic or hydrophobic interactions. It can also be caused by an inclusion/complexation mechanism, like the iodine inclusion complex with starch [24].

Chitosan self-assembled (also referred to as self-aggregated) nanoparticles (NPs) are particularly useful for encapsulating hydrophilic as well as lipophilic drugs [25]. Self-assembling can be provoked by the introduction of hydrophobic moieties into chitosan molecules by grafting to generate a hydrophobic-hydrophilic balance. The grafting agent can be a hydrophobic moiety such as cholesterol [26], cholic [27] and deoxycholic acid [28] or 5β-cholanic acid [29], to form micelles that can entrap lipophilic drugs or it can be the drug itself. In many occasions instead of chitosan a soluble chitosan derivative, such as glycol chitosan [30] or succinyl chitosan [31] is used. Another suitable way to generate self-assembled chitosan nanoparticles is through the formation of polyelectrolyte complexes with polyanions [32]. The aim of the present article is to review the main approaches developed for preparing chitosan nanoparticles by self-assembling via 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 [9]. The obtained complexes (also called polysalts) generally precipitate or separate from the solution forming a complex rich liquid phase (coacervate). However, under certain conditions, polyelectrolytes with weak ionic groups and significantly different molecular weights at non-stoichiometric mixing ratios can generate water-soluble PECs on a molecular level [33, 34].

The formation of polyelectrolyte complexes is accompanied by the release of small counter-ions to 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 the responsible one 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 possessing hydrophobic and hydrophilic regions makes PECs a particular class of physically cross-linked hydrogels sensible to pH and to other environmental factors such as temperature and ionic strength.

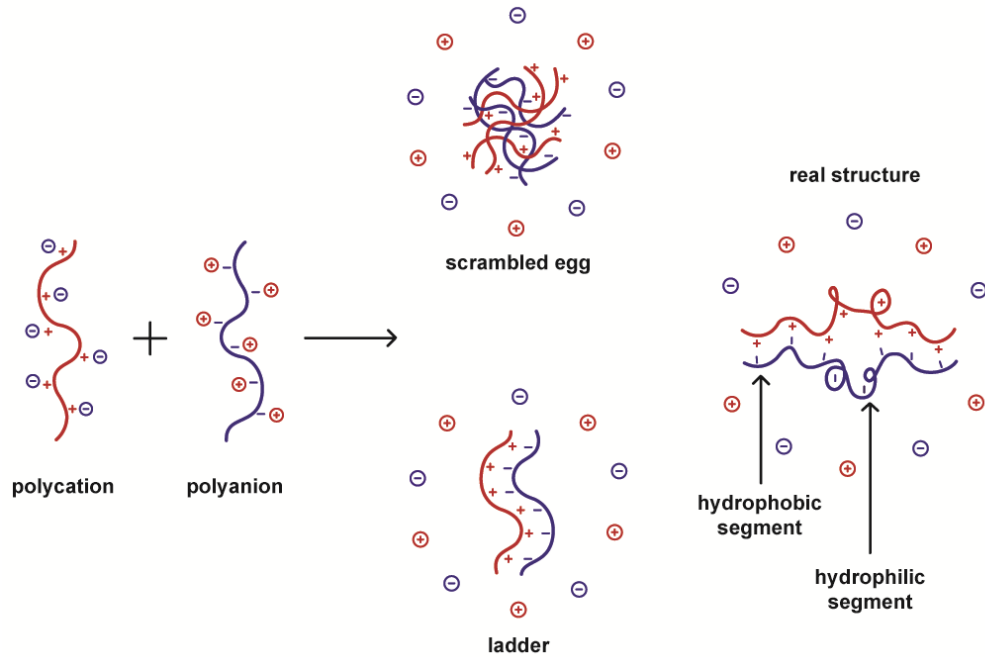
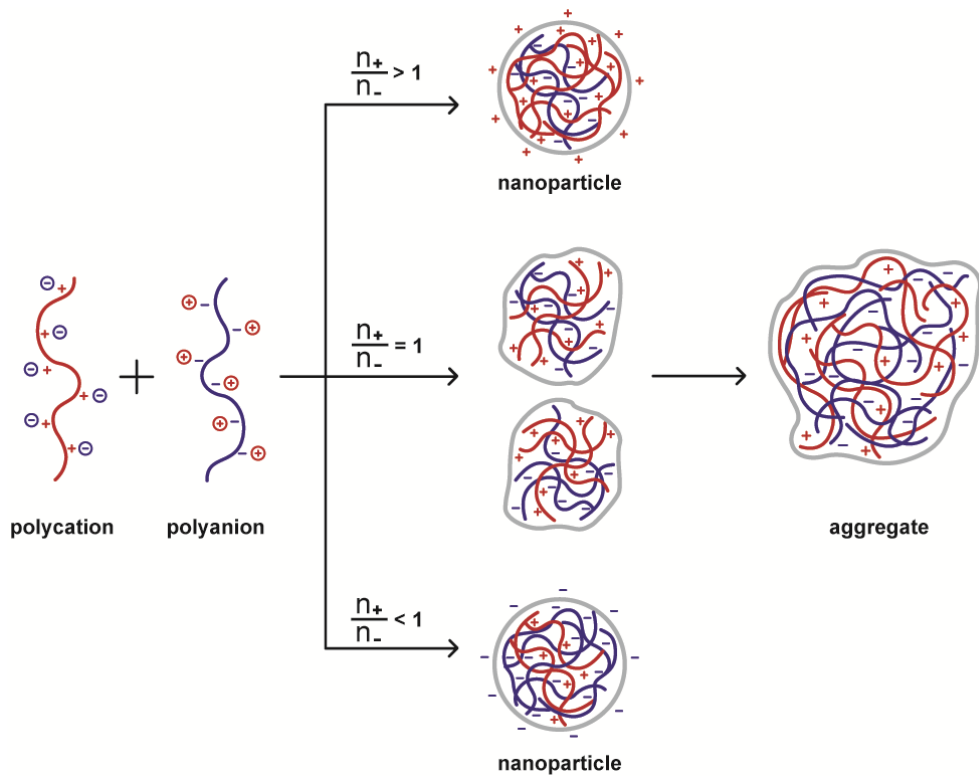



Figure 2. The structure of polyelectrolyte complexes. Scrambled egg and ladder arrangements illustrate extreme situations. The actual structure can be represented an intermediate one combining hydrophobic ladder-like segments coexisting with disordered hydrophilic regions.

There are numerous factors affecting the structure and stability of PECs, such as the ionization degree of each one of the polyelectrolytes and their charge density, the charge distribution on the polymer chains, the polyelectrolytes concentration, the mixing ratio (Z), the mixing order, the nature of the ionic groups on the polymer chains, the molecular weight of the polyelectrolytes, the flexibility of the polymer chains, the time of interaction, the temperature and the ionic strength and pH of the medium [35].

As a cationic biopolymer, chitosan may react with negatively charged polyelectrolytes, giving rise to the formation of PECs [36, 37]. There are many reports of PECs between chitosan and carboxymethyl cellulose (CMC) [38, 39], alginate [40-44], poly(acrylic acid) [45, 46], pectin [47-50], carrageenans [51, 52], heparin [53] and various other polyions [54-60].

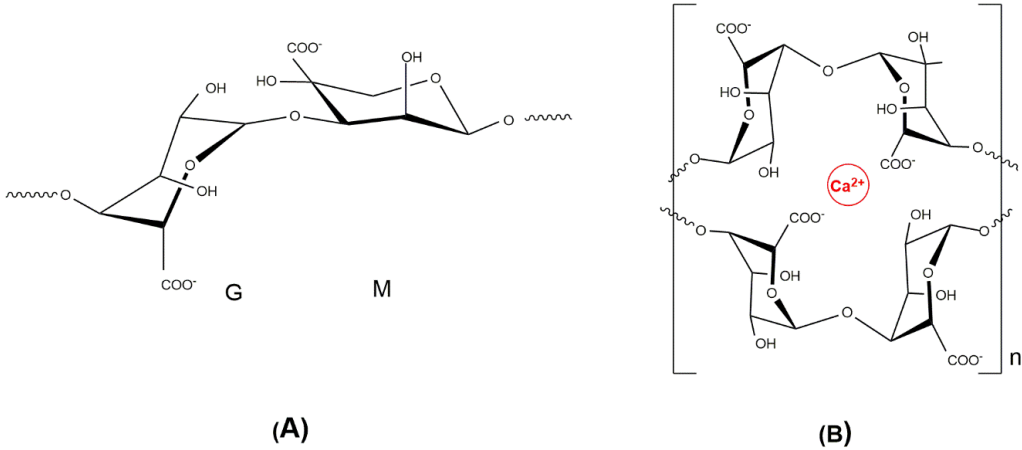
122   *2.1. Chitosan based PEC nanoparticles and their application in drug delivery*

123   Because of the recognised biological properties of chitosan already mentioned, many
124   applications of these PECs have been proposed with biomedical purposes, with particular emphasis
125   in drug delivery [61]. In this connection, researchers have shown special interest in the preparation
126   of chitosan PEC nanoparticles for the delivery of drugs, proteins, genes and vaccines [35, 62, 63].
127   When chitosan PEC particles are formed, they tend to aggregate because of charge neutralization
128   so that in order to avoid aggregation and to obtain nanoparticles at least two conditions are
129   mandatory: the polyelectrolyte solutions must be diluted and one of the polyions must be in
130   appropriate excess so that the charge ratio (n_+/n_-) ≠ 1. (Figure 3).

131
132   Figure 3. Effect of the polyelectrolytes charge ratio on the size and charge of the PEC formed.
133   When the charge ratio is different from unity nanoparticles charged with the same charge as the
134   polyion in excess are formed. If the charge ratio is equal to unity, uncharged particles are formed
135   producing large aggregates.

136   Other conditions such as pH (particularly important in weak polyelectrolyes), ionic strength and
137   rate of mixing should be adjusted to the particular pair chitosan-polyanion system selected, since
138   these variables will also influence the size and charge of nanoparticles.
139   Different preparation methodologies will result in diverse kinds of nanoparticles, which can be
140   classified as nanoaggregates, nanocapsules or nanospheres. The particular procedure selected can be
141   largely determined by the water solubility of the active agent to be encapsulated and the polyanion
142   used.
143
144   2.1.1. Chitosan-alginate PEC nanoparticles
145
146   Alginates are a family of anionic polysaccharides extracted from brown algae. They are
147   composed of α-L-guluronic acid (G) and β-D-mannuronic (M) acid units linearly linked by 1,4-
148   glycosidic bonds (Figure 4). The M/G ratio and their distribution along the chains (chain
149   microstructure) are strongly dependent on the particular species of algae from which it was extracted
150   [64]. Alginate is non-toxic, biocompatible and biodegradable, mucoadhesive and non-inmunogenic.
151   The capacity of alginate to gel in the presence of calcium ions in the so-called "egg-box" model has

152 been extensively exploded to prepare gels, capsules, micro and nanoparticles for drug delivery [65].
153 The guluronic units are the responsible ones for the crosslinking reaction and therefore the properties
154 of the beads formed such as strength and porosity will depend on the alginate source. Other
155 parameters that can have an effect on the characteristics of beads are the alginate molecular weight,
156 and the concentration of CaCl₂ and alginate solutions [64].

157

(A)   **(B)**

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

160   Chitosan-alginate PEC nanoparticles are usually prepared by one of the following three
161 procedures.
162 a) Plain complex coacervation by mixing dilute solutions of CS and ALG. The order of addition of
163 one polysaccharide into the other, the CS/ALG ratio and the pH of the solutions are important factors
164 determining relevant parameters of nanoparticles (size, particle charge, stability, encapsulation
165 efficiency).
166   This procedure was used to prepare negatively charged CS/ALG nanoparticles by dropping a
167 CS solution over the ALG solution. It was found that particle sizes varied from 320 nm to 700 nm,
168 depending on the pH and ionic strength of the solution. The Z-potential of NPs was also dependent
169 on pH and varied from +6.34 mV at pH 3.0 to –44.5 mV at pH 10.0. The loading capacities of NPs for
170 ibuprofen and dipyridamole were 14.18% and 13.03%, respectively. Drug release was governed
171 simultaneously by the solubility of the drug and the permeability of the CS/ALG nanoparticles [66].
172   In a modification of this procedure, a CS solution containing Tween 80 was dropped into a
173 previously prepared solution of an alginate complex with doxorubicin (DOX). The NP suspension
174 was stirred overnight and the doxorubicin loaded CS/ALG NPs were separated by centrifugation.
175 The size of NPs was 100 ± 35 nm, the Z-potential 35 ± 4 mV and the encapsulation efficiency achieved
176 was 95 ± 4% [67].
177   The reverse procedure was used to encapsulate amoxicillin in CS/ALG nanoparticles. Essentially
178 a mixture of chitosan, Pluronic and amoxicillin was prepared in various concentrations of all the
179 components. To this mixture, an aqueous solution of ALG was sprayed with stirring to form NPs.
180 Both solutions were at PH 5.0. The process was optimized for variables such as pH and mixing ratio
181 of polymers, concentrations of polymers, drug and surfactant, using 33 Box-Behnken design. The
182 resulting particle size, surface charge, drug entrapment percent, in-vitro mucoadhesion and in-vivo
183 mucopenetration of nanoparticles on rat models were inspected. The optimized formulation with
184 particle size, zeta potential and encapsulation efficiencies 651 nm, +59.76 mV and 91.23%,
185 respectively, showed comparative low in-vitro mucoadhesion with respect to plain chitosan
186 nanoparticles, but excellent mucopenetration and localization [68].
187   A modified hybrid blending system was developed by Goycoolea *et al.*, which combined
188 complex coacervation of CS and ALG with ionotropic gelation of CS with trisodium tripoliphosphate
189 (TTP). The purpose of this combination was to increase the stability in biological media and achieve
190 better pharmacological performance than conventional CS-TPP nanoparticles. In this method, an
191 ALG solution containing TPP was mixed under rapid stirring with the CS solution and the CS-TPP-

192   ALG nanoparticles were formed. Insulin loaded CS-TPP-ALG nanoparticles were obtained by adding
193   insulin to the ALG-TPP solution prior to mixing with the CS solution. The average size of the insulin-
194   loaded NPs was in the range from ~273 to ~396 nm, somewhat bigger than the unloaded ones (~266
195   to~312 nm) and their Z-potentials were positive and ranged from ~42 to ~49 mV, providing
196   increased stability to nanoparticles. Encapsulation efficiencies as high as 50.7% were attained [69].
197   b) Ionotropic pregelation of alginate (usually with $CaCl_2$, but other divalent ions might also be used)
198   followed by complexation with chitosan.
199   This a very common method in which the active agent can be dissolved or dispersed in the ALG
200   solution or can be loaded into the resulting CS/ALG nanoparticles.   Azevedo *et al.* used this
201   procedure setting the initial pH of the ALG and CS solutions to 4.9 and 4.6, respectively. In their
202   formulation, the average size for CS/ALG NPs was 119.5 ± 49.9 nm with a Z-potential of −30.9 ± 0.5
203   mV. Vitamin B2 loaded NPs were obtained by dissolving the compound in the ALG solution before
204   the pregelation step. The average size of nanoparticles with vitamin B2 was 104.0 ± 67.2 nm with a Z-
205   potential of −29.6 ± 0.1 mV. The nanoparticles showed encapsulation efficiency and loading capacity
206   values of 55.9 ± 5.6% and 2.2 ± 0.6%, respectively [70].
207   c) o/w microemulsion of alginate followed by ionotropic gelation and further complexation with
208   chitosan.
209   Bhunchu *et al.* employed this method to prepare CS/ALG NPs containing curcumin diethyl
210   disuccinate (CDD).   CDD dissolved in acetone (1 ml) was added dropwise into 20 ml of a dilute ALG
211   solution (0.6 mg/ml) containing a non-ionic surfactant (Pluronic F127, Cremophor RH40™ and
212   Tween 80®). Afterwards of 4 ml of $CaCl_2$ solution (0.67 mg/ml) of was added with stirring, followed
213   by sonication. To the resultant pregel 4 ml of CS solution of various concentrations (0.15 - 0.45 mg/ml
214   in 1% (v/v) acetic acid) was added with continuous stirring at 1000 rpm 30 min. After standing
215   overnight for equilibration CDD loaded CS/ALG NPs were obtained as a dispersion in aqueous
216   solution.   Pluronic F127 gave the smallest particle size, 414 ± 16 nm with the highest Z-potential, -22.1
217   ± 1.4 mV. The encapsulation efficiency and loading capacity of these NPs were 54.9 ± 1.3% and 3.33 ±
218   0.08%, respectively. These NPs improved cellular uptake of CDD in Caco-2 cells, in comparison with
219   free CDD [71].
220   A list of some selected examples of CS/ALG PEC nanoparticles based on the different
221   `procedures mentioned above is given in Table 1.
222
223   Table 1. Chitosan-Alginate PEC nanoparticles. The intervals shown generally indicate extreme values
224   obtained with different preparation conditions.

Procedure	Active agent	Particle size, nm	Z-potential, mV	Ref.
Complex coacervation				
CS added into ALG	Ibuprophen Dipyridamole	320 to 700[b]	++6.34[b] to − 44.5[b*]	[66]
	Gatifloxacin[a]	347[c]	+38.6[c]	[72]
CS into ALG-DOX	Doxorubicin	100 ± 28[b] 100 ± 35[c]	36 ± 3[b] 35 ±4[c]	[67]
ALG added into CS	Amoxicillin[a]	264 to > 601	+ 35 to + 61.9	[68]
	Fluorescein isothiocyanate	338.1 ± 15.9[b] 265.7 ± 7.4[c]	+33.8 ± 7.9[b] +29.5 ± 4.1[c]	[73]
ALG into Thiolated CS	Fluorescein isothiocyanate	338.1 ± 15.9[b] 265.7 ± 7.4[c]	+33.8 ± 7.9[b] +29.5 ± 4.1[c]	
ALG+TPP added into CS	Insulin	260 - 525	+41 to +50	[69]

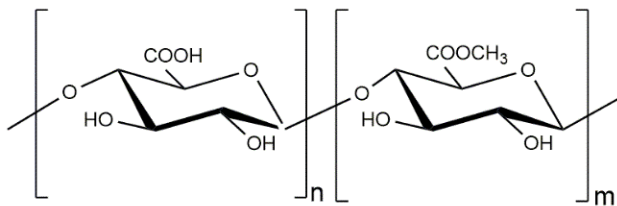
Ionotropic pregelation of alginate plus PEC coating with CS

CS into Ca/(ALG+drug)	Insulin	781 ± 61[b]	-14.5 ± 2.09[b]	[74]
		748 ± 217	-5.6 ± 1.9[c]	
	Vitamin-B2	119.5 ± 49.9[b]	−30.9 ± 0.5[b]	[70]
		104.0 ± 67.2[c]	−29.6 ± 0.1[c]	
	Acetamiprid	201.5	−32.1	[75]
CS+EGF into Ca/ALG	EGF-antisense[a]	194 - 1435	~ +30	[76]
CS+plasmid into Ca/ALG	pEGFP plasmid	161	+29.3	[77]

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

	Turmeric oil	522 - 667	-21.8 to -22.2	[78]
	A.A.	400		[79]
	CDD	414 ± 16	22.1 ± 1.4	[80]
LMWAlg + OligoCS	BSA	134 - 229		[81]

225 [a]Optimization performed; [b]unloaded particle; [c]loaded particle; A.A., aminoacid derivatives; CDD,
226 curcumin dietil disuccinate; *pH 3.0

227
228 2.1.2. Chitosan-Pectin PEC nanoparticles
229
230   Pectin is an anionic hetero-polysaccharide derived from plant cell walls, consisting primarily of
231 1,4 linked α-D-galactopyranosyl uronic acid residues with 1,2-linked α-L-rhamnopyranose residues
232 interspersed with varying frequency (Figure 5). Pectin structure presents also certain amount of
233 neutral sugars (arabinose, galactose, rhamnose, xylose and glucose). A number of the galacturonic
234 acid residues in pectin are methyl or acetyl esterified. The percentage of galacturonic acid residues
235 that are esterified is known as degree of esterification (DE).

236
237   Figure 5. Chemical structure of partially acetylated polygalacturonic acid in pectin.

238   Pectin is hydrophilic, biocompatible and biodegradable with low toxicity. Similarly to alginate,
239 pectin with low metoxyl content (DE <50%) has the ability to gel in the presence of Ca²⁺ ions
240 generating junction zones between chains with an egg-box structure. Pectins with higher DE can also
241 form gels, providing there are a sufficient number of blocks of non-esterified uronic acid residues per
242 molecule to allow the formation of a sufficient number of junction zones to form a network. These
243 properties of pectin have been employed to prepare diverse formulations for applications in drug
244 delivery.
245   Galacturonic acid provides pectin a negative charge in solutions with pH higher than 3.5
246 permitting the formation of polyelectrolyte complexes with chitosan. The strength of the interaction
247 is dependent on the degree of esterification of the pectin, with pectins of a relatively low DE (36%)
248 readily forming PECs with CS [82]. PEC formation is also affected by the ratio of pectin to CS and the
249 pH of the solutions [83].

250   CS-pectin PEC nanoparticles can be prepared by the same methods previously described for CS-
251   ALG PEC nanoparticles. Birch and Schiffman prepared nanoparticles by the complex coacervation
252   technique adding pectin at the appropriate CS-to-pectin ratio to the CS solution. This way they
253   obtained particles sizes ranging from 560 ± 10 nm to 1000 ± 40 nm. The Z-potential varied from +20 ±
254   1 mV to +26 ± 1 mV.   When the addition order was reversed the particle size increased from 460 ± 20
255   to 1110 ± 30 nm and the Z-potential ranged from +19 ± 1 to +28 ± 1 mV [84].
256   Campino *et al*. prepared CS-pectin PEC nanoparticles by two different procedures: a) coating,
257   adding a dispersion of low molecular weight CS NPs previously prepared by ionotropic gelation of
258   CS with TPP to a pectin (from apple and citrus fruit) solution; and b) blending, adding a CS solution
259   to a solution of pectin and TPP. Nanoparticles were charged with ovalbumin (OVA) and bovine
260   serum albumin (BSA) as model proteins. They pointed out that the blending technique can be
261   advantageous because being a one-step preparation, is highly desirable for a scale-up process.
262   Additionally, it brings the possibility to tune the size and Z-potential by properly selecting the ratios
263   of CS, pectin and TPP. However, they found that there was a decrease of the loading of BSA and OVA
264   in the case of the blending technique due to the electrostatic interactions of CS with the protein and
265   pectin, both negatively charged. Therefore, they concluded that the selected technique would depend
266   on the physico-chemical characteristics of the polymer and protein involved [85]. Some of the
267   parameters informed in their work are listed in Table 2, together with some selected examples of CS-
268   pectin preparation procedures reported by other authors.
269
270   Table 2. Chitosan-Pectin PEC nanoparticles. The intervals shown generally indicate extreme values
271   obtained with different preparation conditions.

Procedure	Active agent	Particle size, nm	Z-potential, mV	Ref.
Complex coacervation				
Pectin added into CS	Insulin	441.3 ± 31.6[a]		[86]
		580 – 896[b]	+62.3 ± 2.6[b]	
		*650.8 ± 86.4[b]	+32.7 ± 3.8[b]	
	Curcumin	10-59 (dry NPs)		[87]
	Insulin	1175 – 2618[a]	-22.5 to +35.0[a]	[22]
		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+CaCl$_2$	OVA	419[a]	-30.4[a]	[89]
		302 – 409[b]	−21.9 to −26.0[b]	

272   [a]unloaded particle; [b]loaded particle *The CS solution contained Ca^{2+}ions
273

274   2.1.3. Chitosan-Dextran sulfate PEC nanoparticles
275
276   Dextran sulfate (DS) is a biodegradable and biocompatible negatively charged branched
277   polyanion able to interact with positively charged polymers. It is a high-molecular weight, branched-
278   chain polysaccharide polymer of D-glucose containing 17-20% sulfur. The straight chain consists of
279   approximately 95% α-(1,6) glycosidic linkages. The remaining α- (1,3) linkages account for the
280   branching of dextran (Figure 6).

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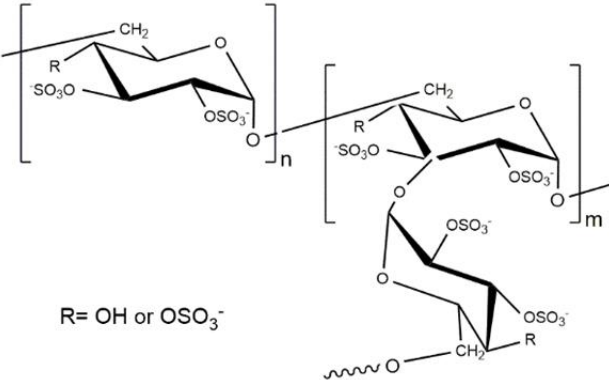

282   Figure 6. The chemical structure of dextran sulfate.

283   DS has been used as an anticoagulant and has found applications in drug delivery. For instance,
284   it was used to mask the positive charge of doxorubicin (DOX) before addition to a CS solution and
285   nanoparticle formation by ionotropic gelation with TPP. This modification doubled DOX
286   encapsulation efficiency relative to controls and enabled reaching loadings up to 4.0 wt-% DOX [90].
287   CS-DS PEC nanoparticles are almost invariably prepared by simple coacervation. The factors
288   affecting the mechanism of formation of these nanoparticles: the mode of addition, charge mixing
289   ratio, pH and ionic strength of the media and the molar mass of both components have been
290   thoroughly revised by Schatz *et al.* [91, 92].
291   There are numerous reports on the preparation of CS-DS PEC nanoparticles with potential
292   application for delivery of proteins (insulin, BSA) growth factors [93-95], immunoglobulin-A [96] and
293   vaccines [97, 98]. Recently fluorescein isothiocyanate loaded CS-DS nanoparticles (FCS-DS NPs; mean
294   size 400 nm and surface charge +48 mV) were applied topically to the porcine ocular surface where it
295   was retained for more than 4 h. After 6 h of topical FCS-DS NPs, particles accumulated in the corneal
296   epithelium but were not found in the corneal stroma. However, when epithelium was removed, FCS-
297   DS NPs penetrated the stroma. These results indicate that FCS-DS NPs are potentially useful for
298   drug/gene delivery to the ocular surface and to stroma when epithelium is damaged [99].
299   Most of nanoparticles formulations reported describe processing factors affecting the
300   characteristics of CS–DS nanoparticles, including their physicochemical properties as well as the
301   optimal conditions for their preparation. Some examples are listed in Table 3.

302   Table 3. Chitosan-Dextran sulfate PEC nanoparticles. The intervals shown generally indicate extreme
303   values obtained with different preparation conditions.

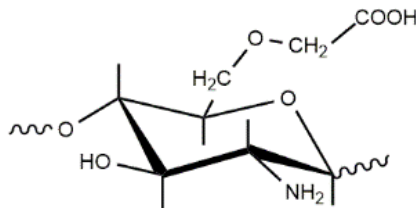
Procedure	Active agent	Particle size, nm	Z-potential, mV	Ref.
Complex coacervation				
DS added into CS		>244[a]	-47.1 to -60[a]	[100]
	BSA	293 - 1138[b]	-26.6 to +56.4[b]	
	Rhodamine 6G	245 - 3521[b]	-31.0 to +34.0[b]	
CS added into DS	Insulin	489 - 665[b]	-0.4 to -21.5[b]	[101]
		527 - 1577[b]	-20.6 to +11.5[b]	[102]
	Amphotericin B	616 - 891[a]		[94]

		644 – 1040[b]	-27 to -37	
	REPIFERMIN®	239	-18.4	[95]
		306	-15.5	
Mixing with agitation	Hydralazine	294,2 ± 57[a]	-7.16 ± 3.69[a]	[103]
		338,1 ± 4,5[b]	-4.84 ± 1.38[b]	

304   [a]unloaded particle; [b]loaded particle

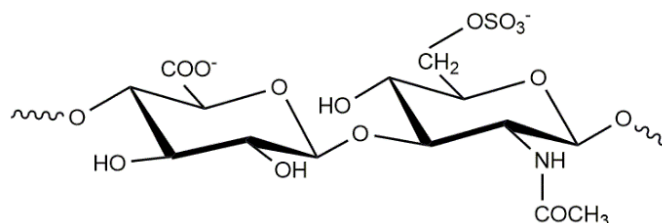
305   PECs of soluble chitosan derivatives with DS have also been formulated to overcome the
306 insolubility of chitosan in neutral and basic media. Glycol chitosan (GC) and DS solutions were mixed
307 together to prepare GC-DS PEC nanoparticles loaded the antifolic agent methotrexate (MTX) aiming
308 to increase its efficacy for the treatment of brain tumours.   The encapsulation efficiency was as high
309 as 87%. *In vitro* experiments indicated the potentiality for the controlled delivery of the drug to the
310 brain [104].

311   PEC nanoparticles of water soluble N,N,N-Trimethyl chitosan (TMC) and DS were prepared by
312 adding DS solutions to TMC solutions at desired pH values (5, 8, 10, and 12). The optimized
313 formulation (particle size, 255.2 ± 12.42 nm; Z-potential −3.9 ± 1.22 mV; drug load, 81.6 ± 2.21 %;
314 encapsulation efficiency, 87.89 ± 0.57 %) was attained in alkaline conditions (pH 10), where the more
315 stable PECs were formed. The release efficiency and ex-vivo nasal toxicity evaluation were assessed
316 after loading a model drug, ropinirole hydrochloride into optimized PEC formulation (particle size,
317 255.2 ± 12.42 nm; Z-potential, −3.9 ± 1.22 mV; DL, 81.6 ± 2.21 %; EE, 87.89 ± 0.57 %). Data indicated
318 that the PECs fabricated at alkaline pH presents a reliable formulation for nasal administration and
319 is biologically compatible with the mucosal surface, being potentially applicable as carriers for nose
320 to brain drug delivery [105].

321
322 2.1.4. Chitosan-Carboxymethyl chitosan PEC nanoparticles
323
324   O-Carboxymethyl chitosan (CMCS) is a water soluble amphiphilic derivative of chitosan that
325 conserves the biological properties of native chitosan with increased antibacterial activity [106]. The
326 structural unit of CMCS is shown in Figure 7. CMCS has found applications in biomedicine,
327 especially in drug delivery where CMCS nanoparticles prepared by ionotropic gelation have
328 demonstrated promising for drug [107, 108] and antigen delivery [109].

329
330   Figure 7. The structural unit of carboxymethyl chitosan.

331   The pKa of CMCS is 2.0-4.0, so that at pH above 4 it is negatively charged and forms
332 polyelectrolytes complexes with chitosan [110]. CS-CMCS PEC nanoparticles were produced by
333 complex coacervation. Wang *et al.* developed insulin-loaded nanogels with opposite zeta potential
334 by adding a previously prepared insulin-CMCS solution into a CS solution (particle size 260 ± 4.47
335 nm, Z-potential +17.2 ± 0.49 mV for insulin:CMCS/CS-NGs(+)) or inversing the order of addition
336 (particle size 243 ± 3.85 nm, Z-potential −15.9 ± 0.45 for insulin:CMCS/CS-NGs(-)), respectively.
337 Encapsulation efficiencies around 75 % and loading capacities near 30 % were attained in both cases.
338 They observed that negatively charged particles exhibited enhanced adhesion and permeation
339 indicating the better performance of insulin:CMCS/CS-NGs(-) for blood glucose management than
340 positive ones [111, 112].

341   CS-CMCS nanoparticles have also been prepared by combining ionotropic gelation and complex
342   coacervation. CMCS and TPP at varying concentrations were blended with a previously prepared
343   mixture of DOX and CS solutions. Nanoparticles within the range of 248 to 363 nm size and -27.6 to
344   -42.2 mV with encapsulation efficiencies and loading capacities around 70.5 and 20 % respectively
345   were obtained depending on the preparation conditions. Results from *in vivo* experiments indicated
346   CS/CMCS-NPs were efficient and safe for oral delivery of DOX [113]. After some modification of the
347   preparation procedure, positively charged CS/CMCS-NPs were obtained. Now the DOX aqueous
348   solution was premixed with CMCS and subsequently, CS solution and TPP were blended with the
349   mixture under agitation. Nanoparticles sizes were between 197 and 443 nm and the Z-potential
350   varied from +12.2 to +37.6 mV, depending on the pH of the media. *In vivo* studies revealed that
351   CS/CMCS-NGs had a high transport capacity by paracellular and transcellular pathways, which
352   guaranteed excellent absorption of encapsulated DOX throughout the entire small intestine [114].
353
354   2.1.5. Chitosan- Chondroitin sulfate PEC nanoparticles
355
356   Chitosan-chondroitin sulfate PEC NPs have been prepared by complex coacervation and the
357   influence of the preparation conditions on the properties of nanoparticles was reported [115, 116].
358   Chondroitin sulphate is a linear glycosaminoglycan (GAG) composed of alternating D-glucuronate
359   and N-acetyl-d-galactosamine-4- or 6-sulfate β(1,3) linked (Figure 8). It is found in cartilage, bone and
360   connective mammalian tissue. Chondroitin sulphate (CHOS) has shown *in vivo* anti-inflammatory
361   properties in animal models and *in vitro* regulation of chondrocyte metabolism, such as stimulation
362   of proteoglycan and collagen synthesis, and inhibition of the production of cytokines involved in
363   cartilage degradation [117]. Its biological properties have stimulated the preparation and evaluation
364   of CS-CHOS nanoparticles for drug/gen delivery [118, 119] and delivery of platelet lysates [120]. CS-
365   CHOS nanoparticles have been suggested as a novel delivery system for the transport of hydrophilic
366   macromolecules [121].

367

368   Figure 8. Chemical structure of chondroitin sulfate.

369   2.1.6. Chitosan-Heparin and Chitosan-Hyaluronan PEC nanoparticles
370
371   CS PECs with other two glycosaminoglycans, hyaluronic acid (hyaluronan, HA) and heparin
372   (HEP) have also been used to prepare nanoparticles. HA is a high molecular weight linear
373   polysaccharide composed of β(1, 3) linked D-glucuronate and N-acetyl-D-glucosamine units. It is
374   present in all soft tissues of higher organisms and in particularly high concentrations in the synovial
375   fluid and vitreous humor of the eye. It plays a vital role in many biological processes such as tissue
376   hydration, proteoglycan organization, cell differentiation, angiogenesis, and acts as a protective
377   coating around the cell membrane. For its part HEP has a more heterogeneous composition, but its
378   main disaccharide unit is composed of D-glucuronate-2-sulfate (or iduronate-2-sulfate) and N-sulfo-
379   D-glucosamine-6-sulfate $\alpha(1,3)$ linked, which provides it with the highest negative charge density of
380   any known biological macromolecule (Figure 9). HEP can be found primarily on the cell surface or
381   in the extracellular matrix, attached to a protein core. Heparin is a well-known anticoagulant drug
382   and is extensively used in medical practice [122]. The important bioactivity of both GAGs has
383   stimulated the preparation of CS-HA and CS-HEP PEC nanoparticles for their high potential of
384   applications as delivery systems for these macromolecules, particularly in tissue engineering [59, 123-
385   125].

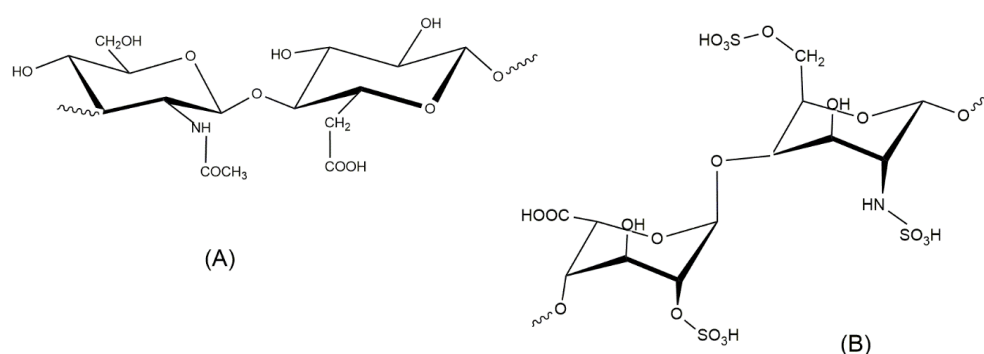



Figure 9. Chemical structures of (A) Hyaluronic acid and (B) Heparin

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

Poly(γ-glutamic acid) (γ-PGA) is an anionic, natural polypeptide made of D- and L-glutamic acid units, joined together by amide linkages between the α-amino and γ-carboxylic acid groups (Figure 10). PEC formation between CS and γ-PGA has been evaluated in terms of physical and chemical properties. In experimental trials, it has shown wound-healing efficacy with potential application as wound dressing material [126].

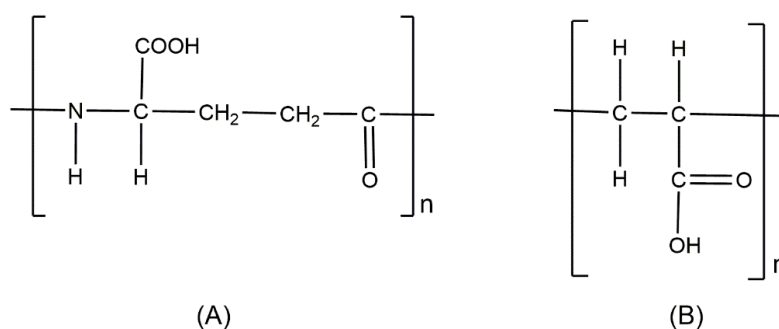



Figure 10. Chemical structures of (A) Poly(γ-glutamic acid) and (B) Poly(acrylic acid)

PEC nanoparticles of γ–PGA and low molecular weight CS were obtained by complex coacervation by Lee *et al.* by adding an aqueous γ–PGA solution at pH 7.4 to a low molecular weight 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) had a zeta potential of +32.1 ± 1.6 mV with a particle size of 145.6 ± 1.9 nm. Insulin loaded NPs were obtained by including insulin in the γ–PGA solution before its addition to the CS solution. Nanoparticles with mean sizes around 195 nm and Z-potential of +30 mV were obtained when the amount of insulin added was ≥ 84 µg/ml. The maximum loading efficiency and loading content were 55.1 and 14.1%, respectively. Animal studies indicated that the insulin loaded NPs enhanced insulin adsorption and reduced the blood glucose level in diabetic rats [127]. Hajdu *et al.* [128] reported the effect of pH, polymer ratios, concentrations, and orders of addition on the physicochemical properties of NPs

The same procedure was used to prepare exendin-4 loaded NPs, only that in this case the CS solution contained distinct metal ions (Cu^{2+}, Fe^{2+}, Zn^{2+} or Fe^{3+}) to enhance the drug loading efficiency. Loading efficiency of 60.9 ± 2.0% was achieved for exendin-4 loaded NPs formed with Fe^{3+}. Their particle size was 260.6 ± 26.4 nm [129].

Nanoparticles of γ–PGA and CS have also been prepared by the combination of ionotropic gelation and complex coacervation. To this end, the insulin and γ-PGA solutions were premixed. Afterwards, TPP and $MgSO_4$ solutions were mixed together and added to the insulin and γ-PGA mixture. The resultant solution was then added by flush mixing with a pipette tip into the aqueous CS solution and the nanoparticles were formed. These NPs also resulted a promising carrier for improved trans mucosal delivery of insulin in the small intestine [130, 131].

418   More recently Pereira *et al.* used the pregelation method to prepare CS/γ-PGA PEC nanoparticles
419   to be used as a nanocarrier system for the plant growth regulator gibberellic acid (GA3). To this end,
420   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
421   to the γ-PGA/CaCl$_2$ solution under stirring, using a peristaltic pump. To prepare GA3 loaded NPs,
422   the plant hormone was added to the γ-PGA/CaCl2 before addition of the CS solution. The unloaded
423   γ-PGA/CS nanoparticles presented an average size of 117 ± 9 nm and Z-potential of −29 ± 0.5 mV at
424   pH 4.4. The corresponding values for the GA3 loaded γ-PGA/CS nanoparticles were 134 ± 9 nm and
425   0.35 ± 0.05, and −27.8 ± 0.5 mV at pH 4.4, respectively. The encapsulation efficiency of GA3 the
426   particles was 61%. In laboratory experiments using *Phaseolus vulgaris* seeds, the γ-PGA/CS-GA3 NPs
427   showed high biological activity, with enhanced rate of germination when compared with the free
428   hormone. The encapsulated GA3 was also more efficient than the free GA3 in the increase of leaf area
429   and the induction of root development, demonstrating the considerable potential of this system for
430   use in the field [132].

431

432   2.1.8. Chitosan-Poly(acrylic acid) PEC nanoparticles

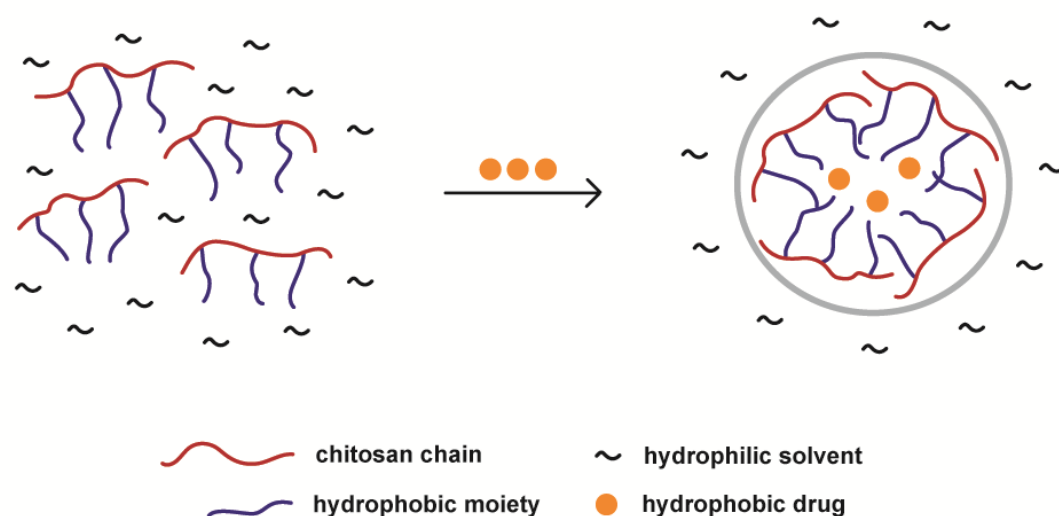
433

434   Poly(acrylic acid) (PAA) is a biocompatible linear anionic polyelectrolyte that readily reacts with
435   CS generating polyelectrolyte complexes by the electrostatic interaction between its COO$^-$ groups and
436   the NH$_3^+$ groups of chitosan [32, 37].
437   Hu *et al.* prepared CS-PAA PEC nanoparticles by template polymerization of acrylic acid in
438   chitosan solution using chitosan as the template. Positively charged NPs with mean size and Z-
439   potential of 206 ± 22 nm and +25.3 ± 3.2 mV, respectively were obtained with 70 % yield.   These NPs
440   were loaded with silk peptide powder (SP) with an encapsulation efficiency of 82 %. Release
441   experiments showed a marked pH dependence of the peptide release profile.   They also obtained
442   CS-PAA PEC NPs by complex coacervation dropping the CS solution into the solution of PAA and
443   vice versa, to study the effect of reversing the order of addition on the resulting nanoparticles. When
444   CS was added to PAA, negatively charged particles were obtained with mean size 436 ± 78 nm and a
445   Z-potential of -22.2 ± 3.6 mV. On the other hand, adding PAA solution into CS solution produced
446   positively charged NPs with mean size and Z-potential of 358 ± 46 nm and +47.2 ± 2.8 mV,
447   respectively. The order of addition also influenced the microstructure of NPs. Transmission electron
448   micrographs of dry nanoparticles showed that NPs obtained by adding the CS solution over the
449   solution of PAA had a hollow core, in contrast with nanoparticles obtained with the reverse addition
450   method, which presented a compact core [133]. In a further study it was found that the nanoparticle
451   size was affected by the molecular weight of CS and PAA, the ratio of amino group to carboxyl group
452   (n$_a$/n$_c$) and the incubation temperature [134].
453   Davidenko *et al.* examined the influence of some experimental parameters such as the pH of the
454   polyelectrolyte solutions, their concentrations and the purification procedure on the dimensions of
455   nanoparticles and their size distribution. NPs were formed by dropwise addition of an aqueous
456   solution of PAA into the corresponding volume of an aqueous solution of CS of a determined
457   concentration with high-speed magnetic stirring (ca. 1300 rpm). The ratio of primary amino groups
458   in CS to carboxylic groups in PAA was fixed at 1.25. They showed that at concentrations below 0.1%
459   it was possible to obtain nanometric particle suspensions. The most convenient pH values for
460   obtaining CHI–PAA NPs with an optimum yield (nearly 90 %) are 4.5–5.5 for CS and 3.2 for PAA.
461   With these conditions, the size of NPs was 0.477 ± 0.008 nm. Particle sizes of approximately 130-140
462   nm were obtained at other pH values, but with yields lower than 45 %. It was found that purification
463   by dialysis can provoke a drastic change both in the distribution profile and in the particle size of the
464   complex. To avoid this the pH of the NPs dispersion should be as near as possible to the pH of the
465   outer dialysis solution [135]. CS-PAA PEC nanoparticles obtained by this procedure were loaded
466   with 5-fluoruracil (5-Fu) and the release profiles at pH 2 and 7.4 were obtained. At pH 2 almost 100%
467   release was achieved after 2 hours, whereas at pH 7,4 only 65% of the loaded drug was released after
468   9 hours. At this pH constant release was observed after the first 90 minutes [136].

469   The complex coacervation procedure has also been used for preparing CS-PAA PECs nanofiber
470 structures with fibre average diameters of 210 nm to 910 nm and Z-potentials of 39.1 ± 1.3 mV to -21.5
471 ± 3.1 mV, respectively. These parameters vary the preparation conditions (volume ratio of CS to PAA,
472 final suspension pH, concentration and molecular weight of CS, incubation time and reaction
473 temperature). Nanofibers can bind plasmid DNA very well and show potential to enhance gene
474 transfer in tissue engineering applications [137, 138].
475
476 2.1.8. Chitosan PEC nanoparticles with other polyanions
477
478   The preparation of CS PEC nanoparticles for the delivery of drug and therapeutic proteins is
479 continuously increasing. They include other polyanions of natural origin, like carrageenan [139, 140],
480 carboxymethyl gum kondagogu [141], and gum arabic [142], as well as synthetic ones. Examples of
481 the latter are poly(malic acid) [143], poly(2-acrylamido-2-methylpropanesulfonic acid) [144], and
482 polystyrene-block-poly(acrylic acid) [145]. The methods used for the preparation of these
483 nanoparticles are based on the general techniques already described and therefore will not be
484 discussed here.

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

486   Hydrophobic modification of chitosan and chitosan derivatives allows achieving a proper
487 hydrophilic/hydrophobic balance to promote self-assembly in aqueous or polar medium. This
488 modification is usually achieved by grafting hydrophobic moieties to the polysaccharide chains. The
489 hydrophobically modified chitosan chains self-aggregate in hydrophilic media as illustrated in Figure
490 11. The following sections are devoted to illustration the state of the art of this method of chitosan
491 and chitosan derivatives NPs preparation.

492

493   Figure 11. Schematic representation of hydrophobically modified chitosan self-assembling. The
494   aggregates can entrap hydrophobic drugs in their hydrophobic core.

495 *3.1 Chitosan and Chitosan Oligosaccharides hydrophobically modified.*

496

497   Deoxycholic acid-modified chitosan self-aggregates have been proposed as a gene delivery
498 system for DNA transfection in cells [146, 147]. This system is based on complex formation between
499 plasmid DNA and positively charged chitosan self-aggregates, which produces micelle-like
500 nanoparticles with controlled dimensions for effective gene delivery to cells. The hydrophobic
501 modification of chitosan was accomplished with deoxycholic acid mediated by carbodiimide
502 coupling (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC) for amide bond formation. Self-
503 aggregates obtained by varying the chitosan/deoxycholic acid ratio (degree of substitution of
504 chitosan, DS from 0.02 to 0.1) and the molecular weight of reacting CS (molecular weight, MW from

505 5 to 200 kDa). They exhibited hydrodynamic sizes ranging from 132 to 300 nm. For CS molecular
506 weights higher than 40 kDa a transition from a bamboo-like cylindrical structure to a poorly
507 organized bird nest-like structure of self-aggregates was proposed. The DNA-CS complex formation
508 had strong dependency on the size and structure of CS self-aggregates and significantly influenced
509 the gene transfection efficiency (up to a factor of 10) [147].

510   Similarly, Wang et al. prepared cholesterol-modified chitosan self-aggregates with succinyl
511 linkages mediated by EDC coupling amidation of CS, attaining a DS of 0.073 and hydrodynamic
512 diameters of 417.2 nm. Epirubicin was used as a model anticancer drug. It was physically entrapped
513 into the cholesterol-CS self-aggregates forming almost spherical nanoparticles of 338.2 to 472.9 nm
514 with the epirubicin loading content increasing from ca. 8 to 14%. Controlled release of epirubicin
515 from the loaded nanoparticles was slow, reaching a total release of 24.9% in 48 h [148].

516   CS-cholesterol self-aggregates were also synthesized with another approach. Prior
517 phthaloylation of CS allowed achieving the esterification of the primary –OH group at C6 with
518 EDC/N-Hydroxysuccinimide pre-activated cholesterol succinate. Later, CS deprotection afforded 6-
519 O-cholesterol-modified chitosans (DS of 0.017, 0.04 and 0.059) which self-assembled forming
520 nanoparticles of 100-240 nm size. These NPs were capable to physically entrap all-trans retinoic acid
521 with different drug loading contents, encapsulation efficiencies and particle sizes. Sustained release
522 of all-trans retinoic acid extended over 72 h [149].

523   Chitosan oligosaccharides (low molecular weight CS produced by depolymerization) are
524 usually preferred over high molecular weight CS for pharmaceutical applications [150]. Thus, Hu et
525 al. prepared a CS oligosaccharide (ca. 19 kDa weight average molecular weight) hydrophobically
526 modified with stearic acid and encapsulated paclitaxel or doxorubicin for their controlled delivery
527 [150-153]. CS oligosaccharide (COS) modification was conducted with stearic acid by an EDC
528 mediated amide linkage reaction achieving COS substitution degrees of 0.035, 0.05, 0.12, 0.255 and
529 0.42 [150-153]. Further glutaraldehyde cross-linking of COS micelle shells prior and after paclitaxel
530 physical entrapping allowed achieving drug loading contents of up to 94% and to control the micelle
531 size and paclitaxel release rate [150]. It was observed a reduction of micelle diameters from 322.2 nm
532 to 272.0 nm after glutaraldehyde cross-linking for the blank COS-stearic acid particles and from 355.0
533 to 305.3 nm for the doxorubicin-loaded COS-stearic acid particles. Zeta potential of particles was
534 reduced from +57.1 to +34.2 mV and from +69.1 to +51.8 mV, respectively [151]. Shell cross-linking of
535 doxorubicin-loaded COS-stearic acid micelles also showed enhanced cytotoxicity to A549, LLC and
536 SKOV3 cancer cell lines [151].

537   To reduce the observed initial burst release during dilution of doxorubicin-loaded COS-stearic
538 acid micelles by body fluid, stearic acid was also physically encapsulated into the micelle core [152].
539 Hydrodynamic diameter of stearic acid-loaded COS-stearic acid micelles increased significantly from
540 27.4 nm up to ca. 60 nm for a 10 wt-% of stearic acid/COS-g-stearic acid micelles, while zeta potential
541 decreased from +51.7 mV to ca. +35 mV [152]. The incorporation of stearic acid physically entrapped
542 in the core of doxorubicin-loaded COS-g-stearic acid micelle significantly reduced the drug release
543 rate.

544   Hu et al. also studied the dual functionalization of COS with stearic acid and doxorubicin cis-
545 aconitate [153]. To this end, previously prepared COS-stearic acid conjugate (DS in stearic acid of ca.
546 0.06) was further reacted with doxorubicin cis-aconitate by EDC mediated amidation. This afforded
547 COS conjugates with doxorubicin contents of 3, 6 and 10%. DOX-g-COS-g-stearic acid self-aggregated
548 in aqueous medium giving micelle sizes of 40.1, 70.7 and 105.8 nm respectively, and zeta potential
549 values of +43.7, +40.2 and +32.0 mV, respectively [153].

550   Chitosan has also been hydrophobically modified with different acyl groups mediated by amide
551 linkage formation with different anhydrides and acyl chlorides such as DL-Lactide (PLA unit
552 modifying the CS), propionic and hexanoic anhydrides, nonaoyl chloride, lauroyl chloride,
553 pentadecanoyl chloride, stearoyl chloride [154, 155]. It was observed that micelle size of blank CS-
554 PLA increased with the increase of substitution degree with PLA units or with the increase of side
555 chain length for the different acyl groups (propionate, hexanoate, nonanoate, etc.) while the zeta
556 potential changed from +26.0 mV for propionyl chitosan to +10.2 mV for hexanoyl chitosan and

557 remained ca. +13 to +15 mV for the other acyl chitosans. Drug loading content and drug release rate
558 were also influenced by the CS substitution degree or the chain length of the acyl substituents of CS.
559 Rifampin loading content increased and drug release rate decreased with the increase of CS
560 substitution with PLA units [154]. Vitamin C loading content increased and drug release rate
561 decreased with the chain length of the acyl group modifying CS [155].
562 Water soluble chitosan N,O6-acetyl chitosan was prepared for future hydrophobic modification
563 with different steroids and DL-α-tocopherol for the sustained release of agrochemicals, testosterone
564 and vitamin E [156]. Drug content achieved values between 11.8 and 56.4 wt-%. The formed CS-
565 steroid and CS-tocopherol micelles showed hydrodynamic sizes of ca. 200 to 360 nm in phosphate
566 buffered saline solution with zeta potential values varying from +7 to +22.7 mV in bi-distilled water.
567 Sustained releases were achieved for the steroids and tocopherol from the CS particles and biological
568 activity of released drug appeared unaffected [156].
569 Amphiphilic block or graft copolymers of phthaloyl chitosan with different materials as
570 poly(ethylene glycol), N-vinyl-2-pyrrolidone and ε-caprolactone are materials with a wide range of
571 pharmaceutical applications [157-163]. For example, N-phthaloylchitosan-g-mPEG micelles have
572 been physically loaded with camptothecin and all-trans retinoic acid for their controlled release [157-
573 159]. These micelles exerted a protective effect on the loaded drug from hydrolysis (camptothecin,
574 which is sensitive to hydrolysis of the lactone group) or photodegradation (all-trans retinoic acid).
575 Furthermore, continuous release without initial burst of prednisone acetate from N-
576 phthaloylchitosan-g-polyvinylpyrrolidone micelles was achieved [160].
577 There are also various reports showing that chitosan-graft-polycaprolactone nanomicelles have
578 been physically loaded with 7-ethyl-10-hydroxy-camptothecin, BSA, paclitaxel and 5-fluorouracil
579 [161-164].
580 Another amphiphilic copolymer of CS was synthesized from N-acetyl histidine as the
581 hydrophobic segment and arginine-grafted chitosan by EDC carbodiimide-mediated coupling for
582 controlled delivery of doxorubicin [165]. The key finding was the effectivity of doxorubicin loaded
583 N-acetyl histidine and arginine-grafted CS for suppression of both sensitive and resistant human
584 breast tumor cell line (MCF-7) in a dose- and time-dependent pattern.
585 More details of prepared chitosan and chitosan oligosaccharide hydrophobically modified
586 conjugates can be found in Table 4.

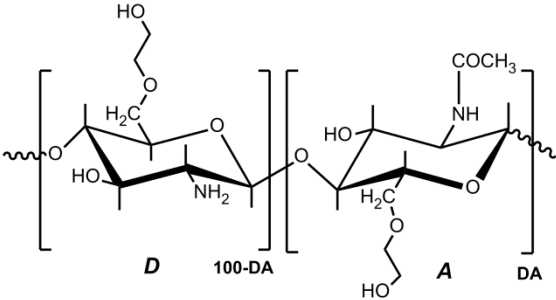
587 Table 4. Chitosan and Chitosan Oligosaccharides hydrophobically modified.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
deoxycholic acid	DNA	162 ± 18[a]		[146]
		~ 300[b]		
		130 – 300[a]		[147]
cholesterol	Epirubicin	417 ± 18[a]		[148]
		338 – 473[b]		
6-O-cholesterol	All-trans	100 – 240[a]	+24.5 to +25.9[a]	[149]
	retinoic acid	192 – 222[b]		
stearyl	Paclitaxel	28.1 – 74.6[a]	+39.0 to +53.2[a]	[150]
		35.8 – 175.1[b]	+44.0 to +58.7[b]	
	Doxorubicin	272 – 322[a]	+34.2 to +57.1[a]	[151]
		305 – 355[b]	+51.8 to +69.1[b]	
		27.4 ± 2.4[a]	+51.7 ± 3.0[a]	[152]
		20.4 ± 1.1[b]	+53.1 ± 14.4[b]	
stearyl+doxorubicin	Doxorubicin	40.1 – 105.8[b]	+32.0 to +43.7[b]	[153]
Acyl	Rifampin	154 – 181[a]		[154]
		163 – 210[b]		

	Vitamin C	444 – 487[a]	+10.2 to +28.9[a]	[155]
		216 – 288[b]	+5.9 to +18.4[b]	
N,O6-acetyl+steroid	Steroids	197 – 358[b]	+7 to +22.7[b]	[156]
N,O6-acetyl+tocopherol	Vitamin E	275 ± 5[b]	+14.9 ± 0.7[b]	
phthaloyl	Camptothecin	~ 170[a]		[157]
		~ 200 – 267[b]		
		~ 50 – 100[a]		[158]
		~ 100 – 250[b]		
	All-trans	~ 50 – 100[a]		[159]
	retinoic acid	~ 80 – 160[b]		
	Prednisone	89.8[a]		[160]
	acetate	143.3[b]		
polycaprolactone,	7-Ethyl-10-	47 – 113[a]	+26.7 to +50.8[a]	[161]
(Chitosan-grafted)	hydroxy-	63 – 152[b]	+25.6 to +48.8[b]	
	camptothecin			
	BSA	168.44[b]		[162]
		200.7[b]		
		435 ± 25[a]		
	Paclitaxel	408 – 529[b]	+27.5 ± 1.1[a]	[163]
		61.4 – 108.6[a]	+30.9 to +33.3[b]	
	5-Fluorouracil	67.9 – 96.7[b]	+18.9 to +43.1[b]	[164]
N-acetyl histidine	Doxorubicin	218[a]	+40.1 ± 2.8[a]	[165]
		185.3 – 218.3[b]	+36.3 to +40.1[b]	

588   [a]unloaded particle; [b]loaded particle; * 5 mg/mL

589   *3.2 Glycol chitosan hydrophobically modified*

590
591   The limited water solubility of chitosan and the precipitation of some self-aggregated chitosan
592   conjugates, restricts its application in medical practice as a drug delivery system. In contrast, glycol
593   chitosan (GCS) exhibits good water solubility at all pHs, biocompatibility and is widely applied as
594   hydrophobic drug and gene carrier [166-173]. The structural units of GCS are shown in Figure 12.

595
596   Figure 12. The chemical structure of glycol chitosan.

597   GCS has been functionalized with cholanic acid, cholesterol, deoxycholic acid, vitamins,
598   testosterone, doxorubicin and other hydrophobic compounds using mostly an EDC-mediated
599   coupling reaction to achieve the amidation of CS amine groups with the desired carboxylic acid or
600   acyl chloride of the hydrophobic substituent. Further physical encapsulation of anticancer drugs or

601 bioactive compounds in the core of self-assembled GCS hydrophobically-modified micelles is usually
602 performed.

603   Hwang et al. introduced cholanic acid in GCS. The resulting GCS-cholanic acid micelles can be
604 easily loaded with the anticancer drug docetaxel [166]. Docetaxel loaded GCS-cholanic acid
605 synthesized spontaneously self-assembled as 350 nm aggregates in aqueous medium. These
606 docetaxel loaded nanoaggregates showed higher anticancer efficacy during *in vivo* experiments to
607 A549 lung cancer cells-bearing mice and reduced the toxicity when compared to the free drug.

608   The anticancer drug camptothecin has also been encapsulated into self-aggregates of GCS-
609 cholanic acid, with drug loading efficiency above 80% [167]. GCS-cholanic acid micelles protected the
610 lactone ring of camptothecin from hydrolysis and camptothecin loaded micelles showed significant
611 antitumor activity towards MDA-MB231 human breast cancer cells implanted in nude mice. 5β-
612 cholanic hydrophobic functionalization of both GCS and polyethylenimine and later mixing of both
613 modified polymers allowed obtaining self-assembled nanoparticles of ca. 350 nm with zeta potential
614 of +23.8 mV, for delivery of siRNA in tumor-bearing mice [168]. The siRNA-GCS-polyethylenimine
615 complex transfected the B16F10 tumor cells, efficiently inhibiting the RFP gene expression of
616 RFP/B16F10-bearing mice. Thus, GCS-polyethylenimine self-aggregates revealed as promising gene
617 carrier for cancer treatment [168]. GCS-cholanic acid self-aggregates have also been proposed for
618 delivery of RGD peptide and indomethacin [169, 170].

619   Hydrophobic modification of GCS with deoxycholic acid and later physical encapsulation of
620 palmityl-acylated exendin-4 peptide in formed self-assembled nanogels for long-acting anti-diabetic
621 inhalation system was studied by Lee et al. [171]. The results achieved were promising, with ca. 72 h
622 residence of administered anti-diabetic drug (palmityl-acylated exendin-4 peptide) in the lungs, good
623 hypoglycemic response and acceptable toxicity.

624   On another approach, the hydrophobic modification of GCS with the drug to be delivered has
625 been explored. Quiñones et al. synthesized GCS hydrophobically-modified with ergocalciferol
626 hemisuccinate, tocopherol hemiesters and testosterone 17β-hemisuccinate for controlled release of
627 vitamin D2, vitamin E and testosterone [172-174]. Substitution degrees of GCS with the studied
628 vitamins and testosterone achieved a value of 0.039 for vitamin D2, 0.21 to 0.36 for vitamin E and
629 0.015 for testosterone. The GCS-vitamin and GCS-testosterone conjugates formed self-assembled NPs
630 in aqueous medium with hydrodynamic sizes from 280 to 500 nm and zeta potential values of +7.7 to
631 +36.5 mV. Sustained release of covalently linked vitamins and testosterone from the GCS self-
632 aggregates was observed in acidic medium for 3 to 4 days.

633   The hydrophobic modification of GCS with N,N-diethylnicotinamide-based oligomer allowed a
634 high paclitaxel loading content with encapsulation efficiency of up to 98% [175]. The hydrodynamic
635 diameters of blank hydrophobically modified GCS was 313 ± 20 nm in PBS. Paclitaxel loaded
636 modified GCS particles with a drug loading content of 9.8, 18.9 and 23.9 wt-% exhibited
637 hydrodynamic sizes of 331 ± 25 nm, 354 ± 23 nm and 363 ± 32 nm respectively. Sustained release of
638 paclitaxel from the GCS self-aggregates was observed. Overall, anticancer assessment of prepared
639 paclitaxel loaded GCS particles appears promising in cancer therapy.

640   Doxorubicin encapsulation in GCS-3-diethylaminopropyl self-aggregates and hydrophobic
641 functionalization of GCS with doxorubicin was also accomplished for the evaluation of doxorubicin
642 delivery systems for cancer therapy [176, 177]. The hydrodynamic parameters of GCS-based self-
643 aggregates discussed are summarized in the Table 5.

644 Table 5. Glycol chitosan hydrophobically modified.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
Cholanic acid	Docetaxel	350[b]		[166]
	Camptothecin	254[a]		[167]
		279 – 328[b]	+23.8 ± 0.9[a]	
	siRNA	350[a]	+10.0 ± 0.8[b]	[168]
		250[b]		

	RGD peptide	224[a]		[169]
		189 – 265[b]		
Cholesterol	Indomethacin	228[a]		[170]
		275 – 384[b]		
Deoxycholic acid	Palmityl-acylated exendin-4	~ 52 – 250[a]		[171]
Ergocalciferol	Vitamin D2	279 ± 7 (PBS)	+7.7 ± 0.1	[172]
DL-⊚-tocopherol	Vitamin E	284 – 496 (PBS)	+11.7 to +36.5	[173]
Testosterone	Testosterone	332 ± 4 (PBS)	+9.7 ± 0.6	[174]
N,N-diethylnicotinamide-based oligomer	Paclitaxel	313 ± 20[a]		[175]
		331 – 363[b]		
3-Diethylaminopropyl	Doxorubicin	102[a]	-0.9[a]	[176]
Doxorubicin	Doxorubicin	238[a]		[177]
		342[b]		

645   [a]unloaded particle; [b]loaded particle

646   *3.3 Carboxymethyl chitosan hydrophobically modified*

647

648   O-Carboxymethyl chitosan, typically named carboxymethyl chitosan (CMCS), has been
649   hydrophobically modified with oleoyl chloride in pyridine/dichloromethane or with linoleic acid
650   using an EDC-mediated amide linkage reaction [178-180].
651   Oleoyl-modified CMCS formed self-aggregates in aqueous medium with average
652   hydrodynamic diameters that were dependent on the molecular weight of chitosan used to prepare
653   the CMCS [178, 179]. Hydrodynamic diameters of 157.4 nm (CS with molecular weight of 50 kDa),
654   161.8 nm (CS with molecular weight of 38 kDa), 274.1 nm (CS with molecular weight of 170 kDa) and
655   396.7 nm (CS with molecular weight of 820 kDa) have been reported for different oleoyl-modified
656   CMCS. The zeta potential values observed for blank oleoyl-modified CMCS particles were +15.6 ± 1.1
657   mV, +17.2 ± 0.9 mV and +19.6 ± 1.4 mV. Rifampicin and microbial antigens were physically entrapped
658   in the oleoyl-modified CMCS micelles with drug loading efficiency of 20% for rifampicin and ca. 52
659   to 62.5% for microbial antigens. Sustained release of encapsulated drugs was extended until 40-48 h
660   [178, 179].
661   Linoleic acid modified CMCS self-aggregated micelles were loaded with the anticancer drug
662   adriamycin for sustained release [180]. The average hydrodynamic diameter of blank linoleic-
663   modified CMCS was 417.8 ± 17.8 nm. Adriamycin was slowly released from the micelles for about 3
664   days. Results are summarized in the Table 6.

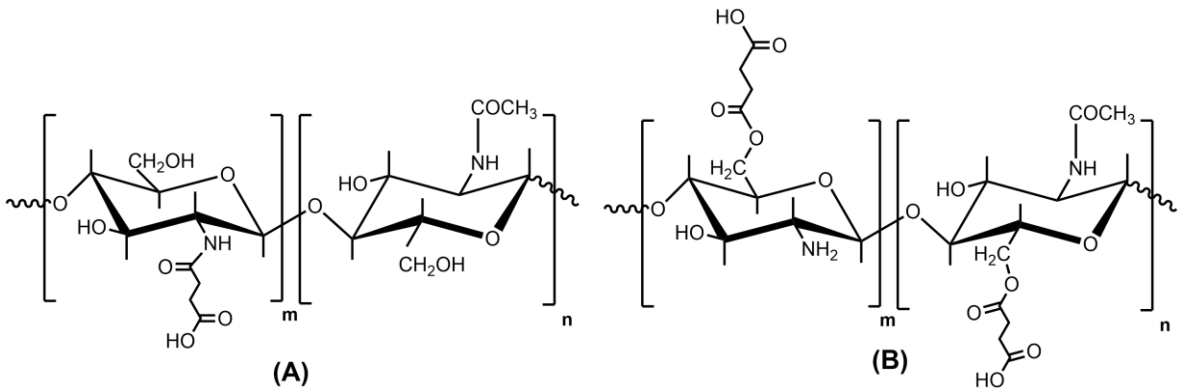
665   Table 6. Carboxymethyl chitosan hydrophobically modified.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
Oleoyl	Rifampicin	161.8[a]		[178]
	Microbial antigen	157.4 – 396.7[a]	+15.6 to +19.6[a]	[179]
		237.6 – 482.3[b]	+14.2 to +17.1[b]	
		331.6 – 573.9[b]	+12.8 to +16.3[b]	
Acyl	Adriamycin	417.8 ± 17.8[a]		[180]

666   [a]unloaded particle; [b]loaded particle

667   *3.4 Succinyl chitosan hydrophobically modified*

668

669 Water soluble succinyl chitosans have been prepared by amidation (N-succinyl chitosan) and
670 esterification (O6-succinyl chitosan) of chitosan by reaction with succinic anhydride (Figure 13).
671

672
673   Figure 13. The chemical structure of N-succinyl chitosan (A) and O6-succinyl chitosan (B).

674 Xiangyang et al. reported the preparation of N-succinyl-N'-octyl chitosan micelles as
675 doxorubicin carriers for effective anti-tumor activity [181]. Average hydrodynamic sizes of
676 doxorubicin loaded modified succinyl chitosan (SCS), which was dependent on the amount of octyl
677 chain and drug loading content, was between 100 to 200 nm. Doxorubicin loaded SCS particles
678 showed sustained release and more cytotoxic against HepG2, A549, BGC and K562 cancer cell lines
679 than parent doxorubicin.
680 In another study on SCS, the interactions between the polymer and BSA in the formed
681 nanoaggregates are studied using different techniques [182]. The authors concluded that no
682 significant change on the conformation of BSA occurred during the chain entanglements between the
683 protein and N-succinyl chitosan. The hydrodynamic sizes of the formed micelles are reported in
684 Table 7.
685 The synthesis of O6-succinyl chitosan involves phthaloyl protection of chitosan, reaction with
686 succinic anhydride and deprotection (removal of phthaloyl groups). Further hydrophobic
687 modification of free amine groups of O6-succinyl chitosan with tocopherol succinate mediated by an
688 EDC activated coupling reaction, allowed the preparation of cationic self-assembled SCS
689 nanoparticles with hydrodynamic diameters of 254 ± 4 nm and zeta potential value of $+36.3 \pm 0.9$ mV
690 [173]. Sustained release of covalently linked vitamin E (tocopherol) was extended up to 96 h. The
691 results are shown in Table 7.

692 Table 7. Succinyl chitosan hidrofobically modified.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
Octyl	Doxorubicin	130.4 – 150.1[a]		[181]
		155.4 – 170.1[b]		
Acyl	BSA	~ 50 – 100[a]		[182]
		~ 100 – 200[b]		
DL-α-tocopherol	Vitamin E	254 ± 4	$+36.3 \pm 0.9$	[173]

693 [a]unloaded particle; [b]loaded particle

694 *3.5 Trimethyl chitosan hydrophobically modified*

695

696 N,N,N-Trimethyl chitosan (TMC) is a water soluble derivative of chitosan prepared by
697 exhaustive N-methylation of some free amine groups of CS using iodomethane.
698 TMC has been hydrophobically modified with octyl, decanoyl, lauryl, lactose and palmitoyl
699 substituents for hydroxycamptothecin and harmine encapsulation in the hydrophobic core [183-85].
700 N-octyl-N-trimethyl chitosan and N-lauryl-N-trimethyl chitosan self-assembled in aqueous medium

701　as micelles of 23.5 nm and 20.8 nm, while N-decanyl-N-trimethyl chitosan formed micelles with
702　hydrodynamic diameter of 277.2 nm.
703　　　　Hydroxycamptothecin loaded N-alkyl-N-trimethyl chitosan micelles showed sustained release
704　of the anticancer drug with improved pharmacokinetic properties and stability of the camptothecin
705　lactone ring *in vivo* [183]. On the other hand, harmine loaded hydrophobically modified TMC
706　released a 65.3% of encapsulated drug in 3 days at pH 7.4 [184].
707　　　　Mi et al. investigated the preparation of self-assembled NPs by TMC and poly(γ-glutamic acid)
708　for oral delivery of insulin [186]. The hydrodynamic diameters and zeta potential values of blank and
709　insulin loaded TMC/poly(γ-glutamic acid) NPs are presented in Table 8.

710　Table 8. Trimethyl chitosan hydrophobically modified.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
Alkyl	Hydroxy-camptothecin	20.8 – 277.2[a] 26.0 – 273.1[b]		[183]
Palmitoyl	Harmine	193.4 ± 3.1[b]	+26.67[b]	[184] [185]
Acyl	Peptide drugs	101.3 – 106.3[a] 522.4 ± 5.9[b]*	+30.6 to +36.2[a] +14.2 ± 0.6[b]*	[186]

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

712　*3.6 Other chitosan derivatives hydrophobically modified*

713

714　　　　N-octyl-O-sulfate chitosan (NOSC) micelles have been prepared from chitosan for the sustained
715　release of physically entrapped paclitaxel for cancer therapy [187-189]. Paclitaxel loaded N-octyl-O-
716　sulfate chitosan micelles showed hydrodynamic diameters of ca. 200 nm and zeta potential values of
717　ca. -30 mV [187, 188]. On the other hand, the additional modification of N-octyl-O-sulfate chitosan
718　with polyethylene glycol monomethyl ether, reduced the hydrodynamic sizes of paclitaxel loaded
719　NOSC until ca. 100 nm [189]. The anticancer drug loaded NPs exhibited reduced toxicity and
720　improved bioavailability of encapsulated paclitaxel [187-189].
721　　　　Pedro et al. synthesized N-dodecyl-N'-glycidyl(chitosan) for delivery of quercetin [190]. The
722　hydrodynamic parameters of quercetin loaded hydrophobically modified CS micelles were
723　measured by dynamic light scattering showing sizes from 140 to 260 nm and zeta potential values
724　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
725　values varied from +14.1 to +29.9 mV, showing both parameters dependence on sample concentration
726　at both pHs. pH was also found to play a key role on the quercetin release from the micelles. The
727　results are summarized in Table 9.

728　Table 9. Other chitosan derivatives.

Hydrophobic moiety	Active agent	Particle size, nm	Z-potential, mV	Ref.
Octyl	Paclitaxel	~ 200[b]		[187]
		200.8[b]	-31.1[a] -28.8[b]	[188]
		104.3 – 133.4[b]		[189]
Acyl	Quercetin	140 – 300[a]	+14.1 to +30.4[a]	[190]

729　[a]unloaded particle; [b]loaded particle

730　**Conclusions**

731　A considerable amount of research is going on the preparation of chitosan nanoparticles by self-
732　assembling for applications in drug delivery. In particular, nanoparticle preparations by

733 polyelectrolyte complexation and by self-assembling of hydrophobically modified chitosan are able
734 to encapsulate the drug under mild conditions without losing their stability and biocompatibility.
735 Therefore chitosan based self-assembled nanoparticles have great potential and multiple application
736 in future in the design of novel drug delivery systems.

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

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