Review

Chitosan Based Self-assembled Nanoparticles in Drug Delivery

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

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

1. Introduction

Chitosan is a family of linear polysaccharides composed of glucosamine and N-acetylglucosamine units linked together by β (1→4) glycosidic links (Figure 1). Chitosan is obtained by partial deacetylation of the naturally occurring polysaccharide, chitin, which is essentially poly(N-acetylglucosamine). Depending on the natural source and the conditions used to isolate and deacetylate chitin, the resulting chitosan will have a degree of acetylation (DA) and molecular weight that will depend on the reaction parameters involved [1]. The molecular weight, the DA and even the pattern of acetylation (the distribution of glucosamine and N-acetylglucosamine units along the chitosan chain) will affect its chemical and biological properties [2, 3].

The degree of deacetylation (DD = 100 – DA) of chitosan is about 50% or higher. At this stage the polysaccharide becomes soluble in aqueous acid solutions because of the protonation of the free amino groups of the D-glucosamine units. In fact, the solubility of chitosan in 1% or 0.1M acetic acid is a simple practical criterion used to differentiate chitosan from chitin. In a protic solvent chitosan behaves as a cationic polyelectrolyte [4].

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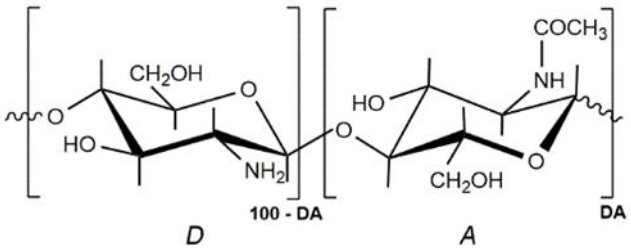

Figure 1. Structural units of chitin and chitosan. (A) N-acetylglucosamine unit; (D) Glucosamine unit. In chitosan DA< 50.

Chitosan is a biocompatible, biodegradable and non-toxic material. It exhibits other significant biological properties, such as wound healing capacity, antimicrobial, 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 $–NH_2$ 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

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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.

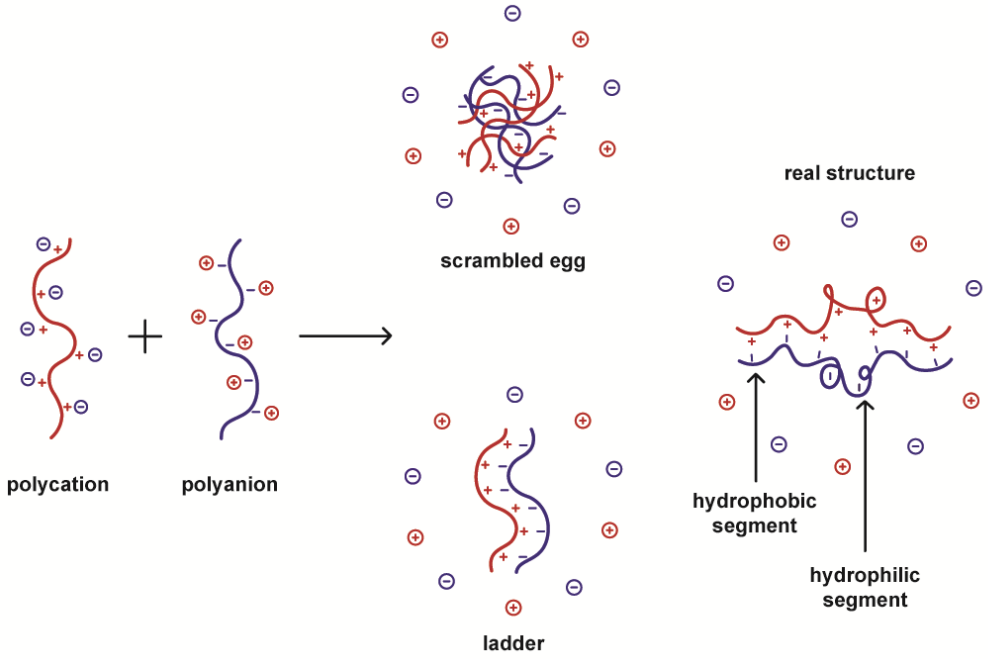

Figure 2. The structure of polyelectrolyte complexes. Scrambled egg and ladder arrangements illustrate extreme situations. The actual structure can be represented an intermediate one combining hydrophobic ladder-like segments coexisting with disordered hydrophilic regions.

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].

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

Because of the recognised biological properties of chitosan already mentioned, many applications of these PECs have been proposed with biomedical purposes, with particular emphasis in drug delivery [61]. In this connection, researchers have shown special interest in the preparation of chitosan PEC nanoparticles for the delivery of drugs, proteins, genes and vaccines [35, 62, 63].

When chitosan PEC particles are formed, they tend to aggregate because of charge neutralization so that in order to avoid aggregation and to obtain nanoparticles at least two conditions are mandatory: the polyelectrolyte solutions must be diluted and one of the polyions must be in appropriate excess so that the charge ratio (n_+/n_-) ≠ 1. (Figure 3).

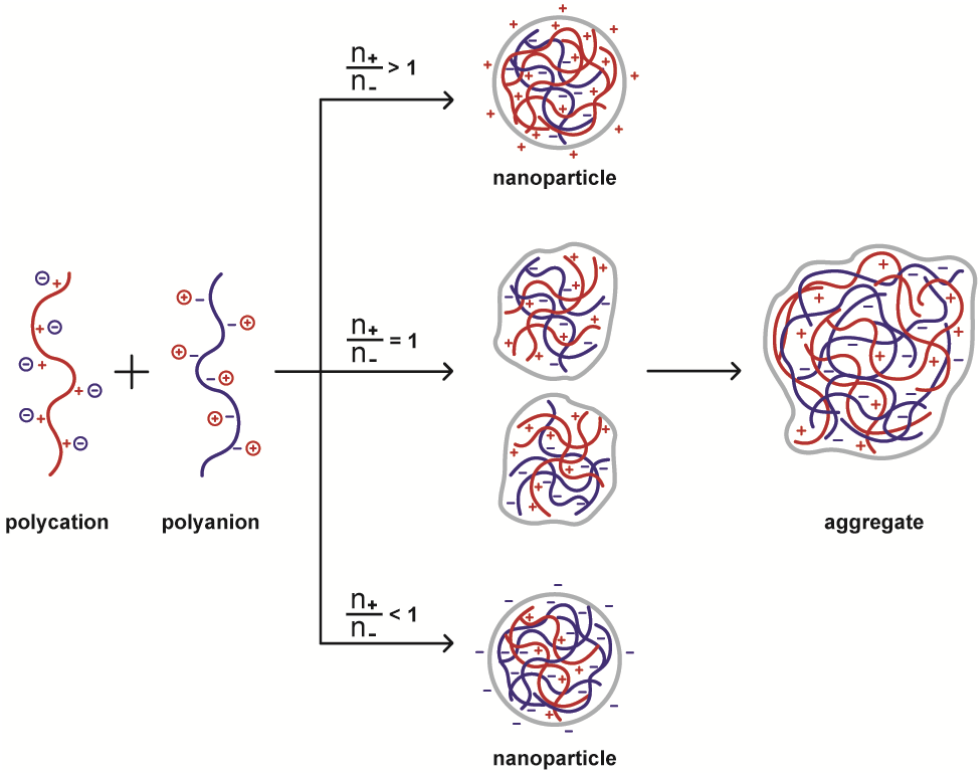

Figure 3. Effect of the polyelectrolytes charge ratio on the size and charge of the PEC formed. When the charge ratio is different from unity nanoparticles charged with the same charge as the polyion in excess are formed. If the charge ratio is equal to unity, uncharged particles are formed producing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

Other conditions such as pH (particularly important in weak polyelectrolyes), ionic strength and rate of mixing should be adjusted to the particular pair chitosan-polyanion system selected, since these variables will also influence the size and charge of nanoparticles.

Different preparation methodologies will result in diverse kinds of nanoparticles, which can be classified as nanoaggregates, nanocapsules or nanospheres. The particular procedure selected can be largely determined by the water solubility of the active agent to be encapsulated and the polyanion used.

2.1.1. Chitosan-alginate PEC nanoparticles

Alginates are a family of anionic polysaccharides extracted from brown algae. They are composed of α-L-guluronic acid (G) and β-D-mannuronic (M) acid units linearly linked by 1,4-glycosidic bonds (Figure 4). The M/G ratio and their distribution along the chains (chain microstructure) are strongly dependent on the particular species of algae from which it was extracted [64]. Alginate is non-toxic, biocompatible and biodegradable, mucoadhesive and non-inmunogenic. The capacity of alginate to gel in the presence of calcium ions in the so-called “egg-box” model has

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been extensively exploded to prepare gels, capsules, micro and nanoparticles for drug delivery [65]. The guluronic units are the responsible ones for the crosslinking reaction and therefore the properties of the beads formed such as strength and porosity will depend on the alginate source. Other parameters that can have an effect on the characteristics of beads are the alginate molecular weight, and the concentration of $CaCl_2$ and alginate solutions [64].

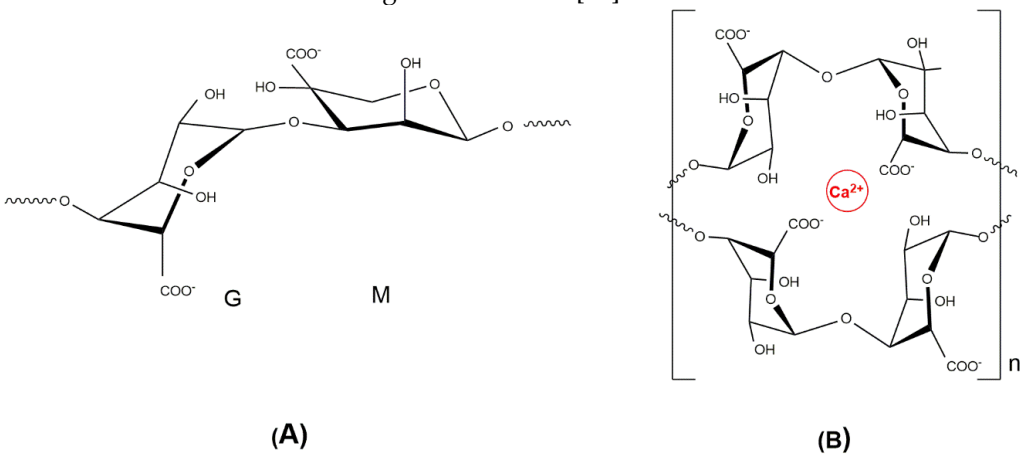

Figure 4. (A) Structural units in alginate. (G) Guluronic acid; (M) Mannuronic acid. (B) Representation of two G-blocks forming an 'egg box' sequence with calcium ion.

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

a) Plain complex coacervation by mixing dilute solutions of CS and ALG. The order of addition of one polysaccharide into the other, the CS/ALG ratio and the pH of the solutions are important factors determining relevant parameters of nanoparticles (size, particle charge, stability, encapsulation efficiency).

This procedure was used to prepare negatively charged CS/ALG nanoparticles by dropping a CS solution over the ALG solution. It was found that particle sizes varied from 320 nm to 700 nm, depending on the pH and ionic strength of the solution. The Z-potential of NPs was also dependent 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 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 [66].

In a modification of this procedure, a CS solution containing Tween 80 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 NPs was 100 ± 35 nm, the Z-potential 35 ± 4 mV and the encapsulation efficiency achieved was 95 ± 4% [67].

The reverse procedure was used to encapsulate amoxicillin in CS/ALG nanoparticles. Essentially a mixture of chitosan, Pluronic and amoxicillin was prepared in various concentrations of all the components. To this mixture, an aqueous solution of ALG was sprayed with stirring to form NPs. Both solutions were at PH 5.0. The process was optimized for variables such as pH and mixing ratio of polymers, concentrations of polymers, drug and surfactant, using 33 Box-Behnken design. The resulting particle size, surface charge, drug entrapment percent, in-vitro mucoadhesion and in-vivo mucopenetration of nanoparticles on rat models were inspected. The optimized formulation with particle size, zeta potential and encapsulation efficiencies 651 nm, +59.76 mV and 91.23%, respectively, showed comparative low in-vitro mucoadhesion with respect to plain chitosan nanoparticles, but excellent mucopenetration and localization [68].

A modified hybrid blending system was developed by Goycoolea *et al.*, which combined complex coacervation of CS and ALG with ionotropic gelation of CS with trisodium tripoliphosphate (TTP). The purpose of this combination was to increase the stability in biological media and achieve better pharmacological performance than conventional CS-TPP nanoparticles. In this method, an ALG solution containing TPP was mixed under rapid stirring with the CS solution and the CS-TPP-

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ALG nanoparticles were formed. Insulin loaded CS-TPP-ALG nanoparticles were obtained by adding insulin to the ALG-TPP solution prior to mixing with the CS solution. The average size of the insulin-loaded NPs was in the range from ~273 to ~396 nm, somewhat bigger than the unloaded ones (~266 to~312 nm) and their Z-potentials were positive and ranged from ~42 to ~49 mV, providing increased stability to nanoparticles. Encapsulation efficiencies as high as 50.7% were attained [69].

b) Ionotropic pregelation of alginate (usually with $CaCl_2$, but other divalent ions might also be used) followed by complexation with chitosan.

This a very common method in which the active agent can be dissolved or dispersed in the ALG solution or can be loaded into the resulting CS/ALG nanoparticles. Azevedo *et al.* used this procedure setting the initial pH of the ALG and CS solutions to 4.9 and 4.6, respectively. In their formulation, the average size for CS/ALG NPs was 119.5 ± 49.9 nm with a Z-potential of −30.9 ± 0.5 mV. Vitamin B2 loaded NPs were obtained by dissolving the compound in the ALG solution before the pregelation step. The average size of nanoparticles with vitamin B2 was 104.0 ± 67.2 nm with a Z-potential of −29.6 ± 0.1 mV. The nanoparticles showed encapsulation efficiency and loading capacity values of 55.9 ± 5.6% and 2.2 ± 0.6%, respectively [70].

c) o/w microemulsion of alginate followed by ionotropic gelation and further complexation with chitosan.

Bhunchu *et al.* employed this method to prepare CS/ALG NPs containing curcumin diethyl disuccinate (CDD). CDD dissolved in acetone (1 ml) was added dropwise into 20 ml of a dilute ALG solution (0.6 mg/ml) containing a non-ionic surfactant (Pluronic F127, Cremophor RH40™ and Tween 80®). Afterwards of 4 ml of $CaCl_2$ solution (0.67 mg/ml) of was added with stirring, followed by sonication. To the resultant pregel 4 ml of CS solution of various concentrations (0.15 - 0.45 mg/ml in 1% (v/v) acetic acid) was added with continuous stirring at 1000 rpm 30 min. After standing overnight for equilibration CDD loaded CS/ALG NPs were obtained as a dispersion in aqueous solution. Pluronic F127 gave the smallest particle size, 414 ± 16 nm with the highest Z-potential, -22.1 ± 1.4 mV. The encapsulation efficiency and loading capacity of these NPs were 54.9 ± 1.3% and 3.33 ± 0.08%, respectively. These NPs improved cellular uptake of CDD in Caco-2 cells, in comparison with free CDD [71].

A list of some selected examples of CS/ALG PEC nanoparticles based on the different `procedures mentioned above is given in Table 1.

Table 1. Chitosan-Alginate PEC nanoparticles. The intervals shown generally indicate extreme values 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]

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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]

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

2.1.2. Chitosan-Pectin PEC nanoparticles

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

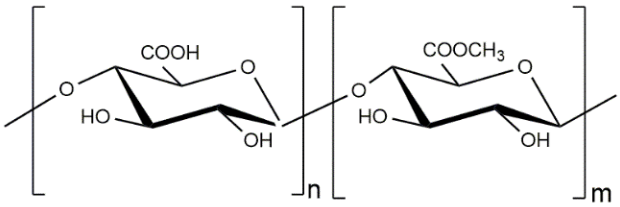

Figure 5. Chemical structure of partially acetylated polygalacturonic acid in pectin.

Pectin is hydrophilic, biocompatible and biodegradable with low toxicity. Similarly to alginate, pectin with low metoxyl content (DE <50%) has the ability to gel in the presence of Ca^{2+} ions generating junction zones between chains with an egg-box structure. Pectins with higher DE can also form gels, providing there are a sufficient number of blocks of non-esterified uronic acid residues per molecule to allow the formation of a sufficient number of junction zones to form a network. These properties of pectin have been employed to prepare diverse formulations for applications in drug delivery.

Galacturonic acid provides pectin a negative charge in solutions with pH higher than 3.5 permitting the formation of polyelectrolyte complexes with chitosan. The strength of the interaction is dependent on the degree of esterification of the pectin, with pectins of a relatively low DE (36%) readily forming PECs with CS [82]. PEC formation is also affected by the ratio of pectin to CS and the pH of the solutions [83].

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CS-pectin PEC nanoparticles can be prepared by the same methods previously described for CS-ALG PEC nanoparticles. Birch and Schiffman prepared nanoparticles by the complex coacervation technique adding pectin at the appropriate CS-to-pectin ratio to the CS solution. This way they obtained particles sizes ranging from 560 ± 10 nm to 1000 ± 40 nm. The Z-potential varied from +20 ± 1 mV to +26 ± 1 mV. When the addition order was reversed the particle size increased from 460 ± 20 to 1110 ± 30 nm and the Z-potential ranged from +19 ± 1 to +28 ± 1 mV [84].

Campino *et al*. prepared CS-pectin PEC nanoparticles by two different procedures: a) coating, adding a dispersion of low molecular weight CS NPs previously prepared by ionotropic gelation of CS with TPP to a pectin (from apple and citrus fruit) solution; and b) blending, adding a CS solution to a solution of pectin and TPP. Nanoparticles were charged with ovalbumin (OVA) and bovine serum albumin (BSA) as model proteins. They pointed out that the blending technique can be advantageous because being a one-step preparation, is highly desirable for a scale-up process. Additionally, it brings the possibility to tune the size and Z-potential by properly selecting the ratios of CS, pectin and TPP. However, they found that there was a decrease of the loading of BSA and OVA in the case of the blending technique due to the electrostatic interactions of CS with the protein and pectin, both negatively charged. Therefore, they concluded that the selected technique would depend on the physico-chemical characteristics of the polymer and protein involved [85]. Some of the parameters informed in their work are listed in Table 2, together with some selected examples of CS-pectin preparation procedures reported by other authors.

Table 2. Chitosan-Pectin PEC nanoparticles. The intervals shown generally indicate extreme values 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]	

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

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2.1.3. Chitosan-Dextran sulfate PEC nanoparticles

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

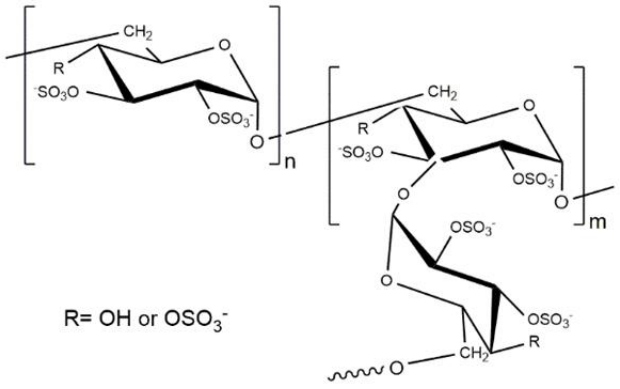

Figure 6. The chemical structure of dextran sulfate.

DS has been used as an anticoagulant and has found applications in drug delivery. For instance, it was used to mask the positive charge of doxorubicin (DOX) before addition to a CS solution and nanoparticle formation by ionotropic gelation with TPP. This modification doubled DOX encapsulation efficiency relative to controls and enabled reaching loadings up to 4.0 wt-% DOX [90].

CS-DS PEC nanoparticles are almost invariably prepared by simple coacervation. The factors affecting the mechanism of formation of these nanoparticles: the mode of addition, charge mixing ratio, pH and ionic strength of the media and the molar mass of both components have been thoroughly revised by Schatz *et al.* [91, 92].

There are numerous reports on the preparation of CS-DS PEC nanoparticles with potential application for delivery of proteins (insulin, BSA) growth factors [93-95], immunoglobulin-A [96] and vaccines [97, 98]. Recently fluorescein isothiocyanate loaded CS-DS nanoparticles (FCS-DS NPs; mean size 400 nm and surface charge +48 mV) were applied topically to the porcine ocular surface where it was retained for more than 4 h. After 6 h of topical FCS-DS NPs, particles accumulated in the corneal epithelium but were not found in the corneal stroma. However, when epithelium was removed, FCS-DS NPs penetrated the stroma. These results indicate that FCS-DS NPs are potentially useful for drug/gene delivery to the ocular surface and to stroma when epithelium is damaged [99].

Most of nanoparticles formulations reported describe processing factors affecting the characteristics of CS–DS nanoparticles, including their physicochemical properties as well as the optimal conditions for their preparation. Some examples are listed in Table 3.

Table 3. Chitosan-Dextran sulfate PEC nanoparticles. The intervals shown generally indicate extreme 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]

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		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]	

[a]unloaded particle; [b]loaded particle

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

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

2.1.4. Chitosan-Carboxymethyl chitosan PEC nanoparticles

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

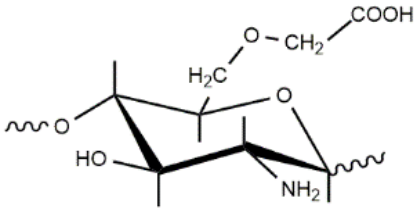

Figure 7. The structural unit of carboxymethyl chitosan.

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

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CS-CMCS nanoparticles have also been prepared by combining ionotropic gelation and complex coacervation. CMCS and TPP at varying concentrations were blended with a previously prepared mixture of DOX and CS solutions. Nanoparticles within the range of 248 to 363 nm size and -27.6 to -42.2 mV with encapsulation efficiencies and loading capacities around 70.5 and 20 % respectively were obtained depending on the preparation conditions. Results from *in vivo* experiments indicated CS/CMCS-NPs were efficient and safe for oral delivery of DOX [113]. After some modification of the preparation procedure, positively charged CS/CMCS-NPs were obtained. Now the DOX aqueous solution was premixed with CMCS and subsequently, CS solution and TPP were blended with the mixture under agitation. Nanoparticles sizes were between 197 and 443 nm and the Z-potential varied from +12.2 to +37.6 mV, depending on the pH of the media. *In vivo* studies revealed that CS/CMCS-NGs had a high transport capacity by paracellular and transcellular pathways, which guaranteed excellent absorption of encapsulated DOX throughout the entire small intestine [114].

2.1.5. Chitosan- Chondroitin sulfate PEC nanoparticles

Chitosan-chondroitin sulfate PEC NPs have been prepared by complex coacervation and the influence of the preparation conditions on the properties of nanoparticles was reported [115, 116]. Chondroitin sulphate is a linear glycosaminoglycan (GAG) composed of alternating D-glucuronate and N-acetyl-d-galactosamine-4- or 6-sulfate β(1,3) linked (Figure 8). It is found in cartilage, bone and connective mammalian tissue. Chondroitin sulphate (CHOS) has shown *in vivo* anti-inflammatory properties in animal models and *in vitro* regulation of chondrocyte metabolism, such as stimulation of proteoglycan and collagen synthesis, and inhibition of the production of cytokines involved in cartilage degradation [117]. Its biological properties have stimulated the preparation and evaluation of CS-CHOS nanoparticles for drug/gen delivery [118, 119] and delivery of platelet lysates [120]. CS-CHOS nanoparticles have been suggested as a novel delivery system for the transport of hydrophilic macromolecules [121].

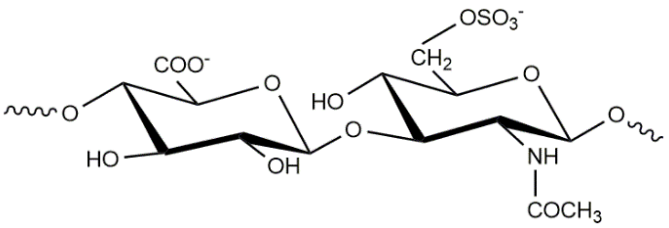

Figure 8. Chemical structure of chondroitin sulfate.

2.1.6. Chitosan-Heparin and Chitosan-Hyaluronan PEC nanoparticles

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

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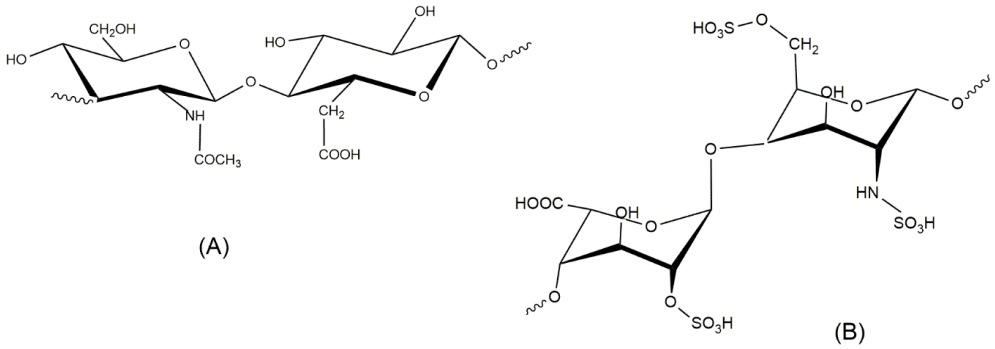

Figure 9. Chemical structures of (A) Hyaluronic acid and (B) Heparin

2.1.7. Chitosan and γ-Polyglutamic acid PEC nanoparticles

γ-Poly(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].

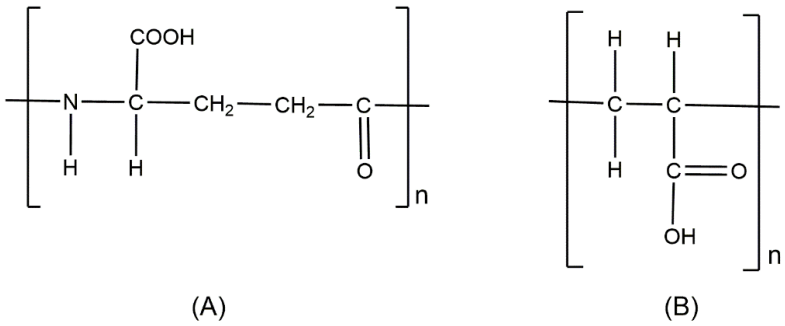

Figure 10. Chemical structures of (A) γ-Polyglutamic acid and (B) Poly(acrylic acid)

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].

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More recently Pereira *et al.* used the pregelation method to prepare CS/γ-PGA PEC nanoparticles to be used as a nanocarrier system for the plant growth regulator gibberellic acid (GA3). To this end, 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 to the γ-PGA/$CaCl_2$ solution under stirring, using a peristaltic pump. To prepare GA3 loaded NPs, the plant hormone was added to the γ-PGA/CaCl2 before addition of the CS solution. The unloaded γ-PGA/CS nanoparticles presented an average size of 117 ± 9 nm and Z-potential of −29 ± 0.5 mV at pH 4.4. The corresponding values for the GA3 loaded γ-PGA/CS nanoparticles were 134 ± 9 nm and 0.35 ± 0.05, and −27.8 ± 0.5 mV at pH 4.4, respectively. The encapsulation efficiency of GA3 the particles was 61%. In laboratory experiments using *Phaseolus vulgaris* seeds, the γ-PGA/CS-GA3 NPs showed high biological activity, with enhanced rate of germination when compared with the free hormone. The encapsulated GA3 was also more efficient than the free GA3 in the increase of leaf area and the induction of root development, demonstrating the considerable potential of this system for use in the field [132].

2.1.8. Chitosan-Poly(acrylic acid) PEC nanoparticles

Poly(acrylic acid) (PAA) is a biocompatible linear anionic polyelectrolyte that readily reacts with CS generating polyelectrolyte complexes by the electrostatic interaction between its COO^- groups and the NH_3^+ groups of chitosan [32, 37].

Hu *et al.* prepared CS-PAA PEC nanoparticles by template polymerization of acrylic acid in chitosan solution using chitosan as the template. Positively charged NPs with mean size and Z-potential of 206 ± 22 nm and +25.3 ± 3.2 mV, respectively were obtained with 70 % yield. These NPs were loaded with silk peptide powder (SP) with an encapsulation efficiency of 82 %. Release experiments showed a marked pH dependence of the peptide release profile. They also obtained CS-PAA PEC NPs by complex coacervation dropping the CS solution into the solution of PAA and vice versa, to study the effect of reversing the order of addition on the resulting nanoparticles. When CS was added to PAA, negatively charged particles were obtained with mean size 436 ± 78 nm and a Z-potential of -22.2 ± 3.6 mV. On the other hand, adding PAA solution into CS solution produced positively charged NPs with mean size and Z-potential of 358 ± 46 nm and +47.2 ± 2.8 mV, respectively. The order of addition also influenced the microstructure of NPs. Transmission electron micrographs of dry nanoparticles showed that NPs obtained by adding the CS solution over the solution of PAA had a hollow core, in contrast with nanoparticles obtained with the reverse addition method, which presented a compact core [133]. In a further study it was found that the nanoparticle size was affected by the molecular weight of CS and PAA, the ratio of amino group to carboxyl group (n_a/n_c) and the incubation temperature [134].

Davidenko *et al.* examined the influence of some experimental parameters such as the pH of the polyelectrolyte solutions, their concentrations and the purification procedure on the dimensions of nanoparticles and their size distribution. NPs were formed by dropwise addition of an aqueous solution of PAA into the corresponding volume of an aqueous solution of CS of a determined concentration with high-speed magnetic stirring (ca. 1300 rpm). The ratio of primary amino groups in CS to carboxylic groups in PAA was fixed at 1.25. They showed that at concentrations below 0.1% it was possible to obtain nanometric particle suspensions. The most convenient pH values for obtaining CHI–PAA NPs with an optimum yield (nearly 90 %) are 4.5–5.5 for CS and 3.2 for PAA. With these conditions, the size of NPs was 0.477 ± 0.008 nm. Particle sizes of approximately 130-140 nm were obtained at other pH values, but with yields lower than 45 %. It was found that purification by dialysis can provoke a drastic change both in the distribution profile and in the particle size of the complex. To avoid this the pH of the NPs dispersion should be as near as possible to the pH of the outer dialysis solution [135]. CS-PAA PEC nanoparticles obtained by this procedure were loaded with 5-fluoruracil (5-Fu) and the release profiles at pH 2 and 7.4 were obtained. At pH 2 almost 100% release was achieved after 2 hours, whereas at pH 7,4 only 65% of the loaded drug was released after 9 hours. At this pH constant release was observed after the first 90 minutes [136].

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The complex coacervation procedure has also been used for preparing CS-PAA PECs nanofiber structures with fibre average diameters of 210 nm to 910 nm and Z-potentials of 39.1 ± 1.3 mV to -21.5 ± 3.1 mV, respectively. These parameters vary the preparation conditions (volume ratio of CS to PAA, final suspension pH, concentration and molecular weight of CS, incubation time and reaction temperature). Nanofibers can bind plasmid DNA very well and show potential to enhance gene transfer in tissue engineering applications [137, 138].

2.1.8. Chitosan PEC nanoparticles with other polyanions

The preparation of CS PEC nanoparticles for the delivery of drug and therapeutic proteins is continuously increasing. They include other polyanions of natural origin, like carrageenan [139, 140], carboxymethyl gum kondagogu [141], and gum arabic [142], as well as synthetic ones. Examples of the latter are poly(malic acid) [143], poly(2-acrylamido-2-methylpropanesulfonic acid) [144], and polystyrene-block-poly(acrylic acid) [145]. The methods used for the preparation of these nanoparticles are based on the general techniques already described and therefore will not be discussed here.

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

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

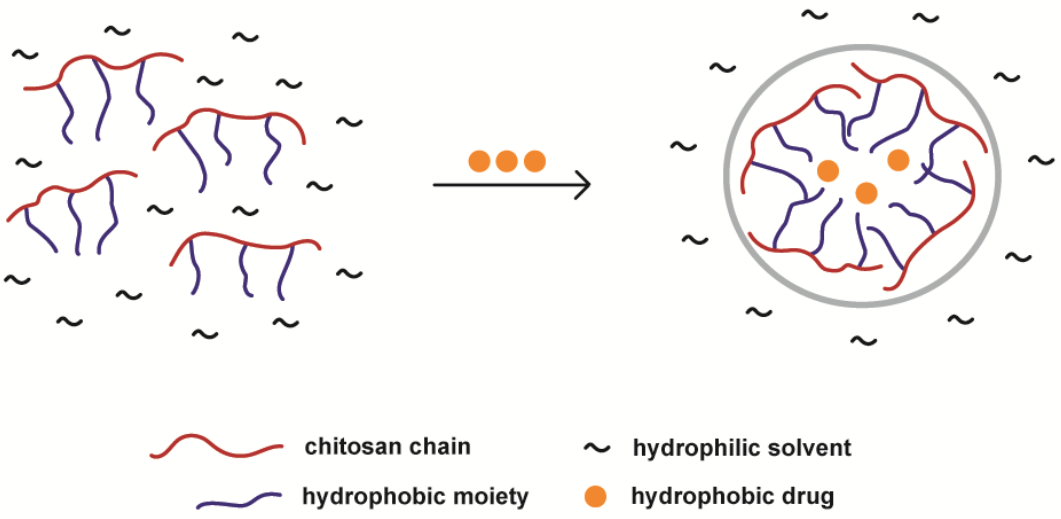

Figure 11. Schematic representation of hydrophobically modified chitosan self-assembling. The aggregates can entrap hydrophobic drugs in their hydrophobic core.

3.1 Chitosan and Chitosan Oligosaccharides hydrophobically modified.

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

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

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

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

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

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

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

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

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remained ca. +13 to +15 mV for the other acyl chitosans. Drug loading content and drug release rate were also influenced by the CS substitution degree or the chain length of the acyl substituents of CS. Rifampin loading content increased and drug release rate decreased with the increase of CS substitution with PLA units [154]. Vitamin C loading content increased and drug release rate decreased with the chain length of the acyl group modifying CS [155].

Water soluble chitosan N,O6-acetyl chitosan was prepared for future hydrophobic modification with different steroids and DL-α-tocopherol for the sustained release of agrochemicals, testosterone and vitamin E [156]. Drug content achieved values between 11.8 and 56.4 wt-%. The formed CS-steroid and CS-tocopherol micelles showed hydrodynamic sizes of ca. 200 to 360 nm in phosphate buffered saline solution with zeta potential values varying from +7 to +22.7 mV in bi-distilled water. Sustained releases were achieved for the steroids and tocopherol from the CS particles and biological activity of released drug appeared unaffected [156].

Amphiphilic block or graft copolymers of phthaloyl chitosan with different materials as poly(ethylene glycol), N-vinyl-2-pyrrolidone and ε-caprolactone are materials with a wide range of pharmaceutical applications [157-163]. For example, N-phthaloylchitosan-g-mPEG micelles have been physically loaded with camptothecin and all-trans retinoic acid for their controlled release [157-159]. These micelles exerted a protective effect on the loaded drug from hydrolysis (camptothecin, which is sensitive to hydrolysis of the lactone group) or photodegradation (all-trans retinoic acid). Furthermore, continuous release without initial burst of prednisone acetate from N-phthaloylchitosan-g-polyvinylpyrrolidone micelles was achieved [160].

There are also various reports showing that chitosan-graft-polycaprolactone nanomicelles have been physically loaded with 7-ethyl-10-hydroxy-camptothecin, BSA, paclitaxel and 5-fluorouracil [161-164].

Another amphiphilic copolymer of CS was synthesized from N-acetyl histidine as the hydrophobic segment and arginine-grafted chitosan by EDC carbodiimide-mediated coupling for controlled delivery of doxorubicin [165]. The key finding was the effectivity of doxorubicin loaded N-acetyl histidine and arginine-grafted CS for suppression of both sensitive and resistant human breast tumor cell line (MCF-7) in a dose- and time-dependent pattern.

More details of prepared chitosan and chitosan oligosaccharide hydrophobically modified conjugates can be found in Table 4.

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]		

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

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

3.2 Glycol chitosan hydrophobically modified

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

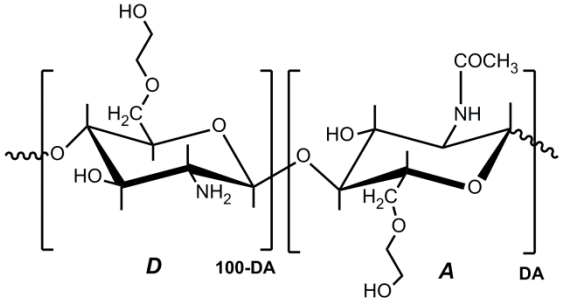

Figure 12. The chemical structure of glycol chitosan.

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

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

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

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

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

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

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

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

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]		

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	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]		

[a]unloaded particle; [b]loaded particle

3.3 Carboxymethyl chitosan hydrophobically modified

O-Carboxymethyl chitosan, typically named carboxymethyl chitosan (CMCS), has been hydrophobically modified with oleoyl chloride in pyridine/dichloromethane or with linoleic acid using an EDC-mediated amide linkage reaction [178-180].

Oleoyl-modified CMCS formed self-aggregates in aqueous medium with average hydrodynamic diameters that were dependent on the molecular weight of chitosan used to prepare the CMCS [178, 179]. Hydrodynamic diameters of 157.4 nm (CS with molecular weight of 50 kDa), 161.8 nm (CS with molecular weight of 38 kDa), 274.1 nm (CS with molecular weight of 170 kDa) and 396.7 nm (CS with molecular weight of 820 kDa) have been reported for different oleoyl-modified CMCS. The zeta potential values observed for blank oleoyl-modified CMCS particles were +15.6 ± 1.1 mV, +17.2 ± 0.9 mV and +19.6 ± 1.4 mV. Rifampicin and microbial antigens were physically entrapped in the oleoyl-modified CMCS micelles with drug loading efficiency of 20% for rifampicin and ca. 52 to 62.5% for microbial antigens. Sustained release of encapsulated drugs was extended until 40-48 h [178, 179].

Linoleic acid modified CMCS self-aggregated micelles were loaded with the anticancer drug adriamycin for sustained release [180]. The average hydrodynamic diameter of blank linoleic-modified CMCS was 417.8 ± 17.8 nm. Adriamycin was slowly released from the micelles for about 3 days. Results are summarized in the Table 6.

Table 6. Carboxymethyl chitosan hydrophobically modified.

Hydrophobic moiety	**Active agent**	**Particle size, nm**	**Z-potential, mV**	**Ref.**
Oleoyl	Rifampicin	161.8[a]		[178]
	Microbial	157.4 – 396.7[a]	+15.6 to +19.6[a]	[179]
	antigen	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]

[a]unloaded particle; [b]loaded particle

3.4 Succinyl chitosan hydrophobically modified

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

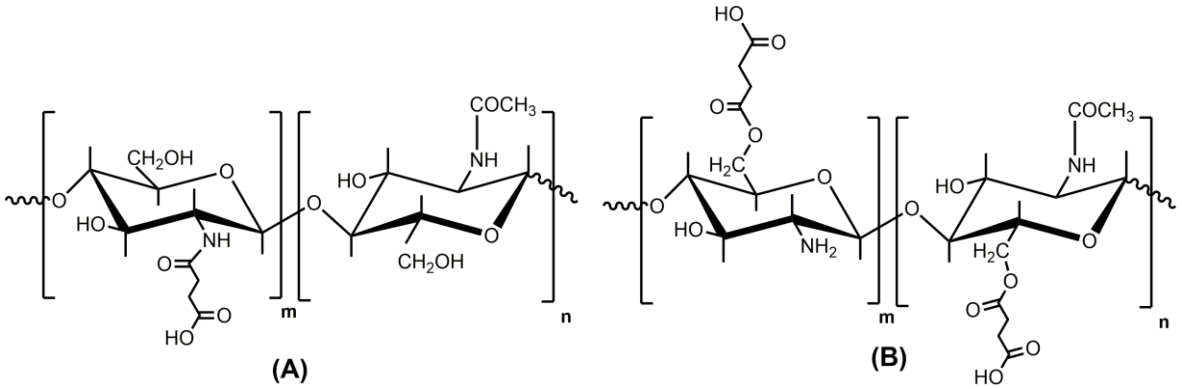

Figure 13. The chemical structure of N-succinyl chitosan (A) and O6-succinyl chitosan (B).

Xiangyang et al. reported the preparation of N-succinyl-N'-octyl chitosan micelles as doxorubicin carriers for effective anti-tumor activity [181]. Average hydrodynamic sizes of doxorubicin loaded modified succinyl chitosan (SCS), which was dependent on the amount of octyl chain and drug loading content, was between 100 to 200 nm. Doxorubicin loaded SCS particles showed sustained release and more cytotoxic against HepG2, A549, BGC and K562 cancer cell lines than parent doxorubicin.

In another study on SCS, the interactions between the polymer and BSA in the formed nanoaggregates are studied using different techniques [182]. The authors concluded that no significant change on the conformation of BSA occurred during the chain entanglements between the protein and N-succinyl chitosan. The hydrodynamic sizes of the formed micelles are reported in Table 7.

The synthesis of O6-succinyl chitosan involves phthaloyl protection of chitosan, reaction with succinic anhydride and deprotection (removal of phthaloyl groups). Further hydrophobic modification of free amine groups of O6-succinyl chitosan with tocopherol succinate mediated by an EDC activated coupling reaction, allowed the preparation of cationic self-assembled SCS nanoparticles with hydrodynamic diameters of 254 ± 4 nm and zeta potential value of +36.3 ± 0.9 mV [173]. Sustained release of covalently linked vitamin E (tocopherol) was extended up to 96 h. The results are shown in Table 7.

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 ± 0.9	[173]

[a]unloaded particle; [b]loaded particle

3.5 Trimethyl chitosan hydrophobically modified

N,N,N-Trimethyl chitosan (TMC) is a water soluble derivative of chitosan prepared by exhaustive N-methylation of some free amine groups of CS using iodomethane.

TMC has been hydrophobically modified with octyl, decanoyl, lauryl, lactose and palmitoyl substituents for hydroxycamptothecin and harmine encapsulation in the hydrophobic core [183-85]. N-octyl-N-trimethyl chitosan and N-lauryl-N-trimethyl chitosan self-assembled in aqueous medium

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as micelles of 23.5 nm and 20.8 nm, while N-decanyl-N-trimethyl chitosan formed micelles with hydrodynamic diameter of 277.2 nm.

Hydroxycamptothecin loaded N-alkyl-N-trimethyl chitosan micelles showed sustained release of the anticancer drug with improved pharmacokinetic properties and stability of the camptothecin lactone ring *in vivo* [183]. On the other hand, harmine loaded hydrophobically modified TMC released a 65.3% of encapsulated drug in 3 days at pH 7.4 [184].

Mi et al. investigated the preparation of self-assembled NPs by TMC and poly(γ-glutamic acid) for oral delivery of insulin [186]. The hydrodynamic diameters and zeta potential values of blank and insulin loaded TMC/poly(γ-glutamic acid) NPs are presented in Table 8.

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]

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

3.6 Other chitosan derivatives hydrophobically modified

N-octyl-O-sulfate chitosan (NOSC) micelles have been prepared from chitosan for the sustained release of physically entrapped paclitaxel for cancer therapy [187-189]. Paclitaxel loaded N-octyl-O-sulfate chitosan micelles showed hydrodynamic diameters of ca. 200 nm and zeta potential values of ca. -30 mV [187, 188]. On the other hand, the additional modification of N-octyl-O-sulfate chitosan with polyethylene glycol monomethyl ether, reduced the hydrodynamic sizes of paclitaxel loaded NOSC until ca. 100 nm [189]. The anticancer drug loaded NPs exhibited reduced toxicity and improved bioavailability of encapsulated paclitaxel [187-189].

Pedro et al. synthesized N-dodecyl-N'-glycidyl(chitosan) for delivery of quercetin [190]. The hydrodynamic parameters of quercetin loaded hydrophobically modified CS micelles were measured by dynamic light scattering showing sizes from 140 to 260 nm and zeta potential values 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 values varied from +14.1 to +29.9 mV, showing both parameters dependence on sample concentration at both pHs. pH was also found to play a key role on the quercetin release from the micelles. The results are summarized in Table 9.

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]

[a]unloaded particle; [b]loaded particle

Conclusions

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

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

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