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Nanoparticles Based on Chitosan Derivatives

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1. Introduction

A tremendous effort has been and is currently being devoted to the research in the field of pharmaceutical nanotechnology. Several peculiar properties of gelled polymeric nanosize ($<1\mu\text{m}$) particulate systems have been reported, among which the ability to encapsulate either small molecular weight or macromolecular active principles in mild conditions and protect them from degradation by the harsh pH conditions or enzymes they may encounter in the organism, promote transport of actives across mucosal barriers, undergo internalization by cells thereby carrying actives into them. Chitosan, a copolymer of glucosamine and *N*-acetylglucosamine, obtained by deacetylation of the naturally-occurring chitin, has been studied as a basic biomaterial for preparing pharmaceutical nanoparticles, because it is biodegradable and has a very low toxicity [1-5] besides an ability to promote transport of drugs, peptides and proteins across mucosal barriers [6-10]. The preparation procedures of chitosan nanoparticles, their characterization for drug encapsulation efficiency, physical and biopharmaceutical properties, and toxicity have been covered by recent reviews (11,12). Chitosan has been subjected to derivatization, taking advantage of the reactivity of the primary amino group in position 2, or the hydroxyl group in position 6 of its repeating unit, glucosamine. The derivatization changed the physicochemical, biopharmaceutical and biological properties of the parent chitosan and each derivative type lent itself to preparing nanoparticles with their own physical properties (size, shape, surface charge), drug encapsulation and release capability, biopharmaceutical and biological properties (mucoadhesivity, ability to promote drug transport across biological barriers, aptitude for internalization within cells, cytotoxicity).

In the following sections the nanoparticles obtained from chitosan derivatives will be surveyed in respect to preparation procedures, interactions with cells and tissues, factors influencing biological properties, pharmaceutical applications.

2. Preparation procedures

2.1. Ionotropic gelation

Ionotropic gelation, that is by far the most used technique for preparation of nanoparticles from chitosan derivatives, was first reported in 1997 by Calvo et al. [13]. The basic concept is that a polycationic polymer in aqueous solution passes, in appropriate conditions, from sol to dispersed gel following electrostatic crosslinking with an adequate anionic substance. This technique has been used with several quaternized chitosans carrying fixed, pH-independent positive charges, the most known of which is *N*-trimethyl chitosan (TMC). Sodium tripolyphosphate (TPP) has widely been employed as the ionotropic crosslinker [14-19].

The nanoparticles prepared by ionotropic gelation of quaternized chitosans with TPP were generally 200-300 nm in size, i.e., smaller than those obtained by the same method starting from plain chitosan which, by the way, showed lesser stability and tended to re-dissolve after some time from formation. The zeta potential was always positive, in the 10-20 mV range. The solution of the chitosan derivative into which the TPP solution was dripped would often contain a surfactant, usually Tween 80, to hinder nanoparticle aggregation and facilitate their re-dispersion after centrifugation. In fact, centrifugation was necessary to clear the particles of non-encapsulated drug.

The technique under discussion has also been used to prepare nanoparticles from thiolated derivatives of chitosan [20-23].

These polymers have shown mucoadhesive properties due to the ability of their thiol groups to form covalent disulfide bridges by reacting with the thiol residues present on the glycoproteins of mucus. For this reason the nanoparticles derived from these chitosan derivatives were themselves endowed with mucoadhesivity. The thiolated nanoparticles formed by ionotropic gelation with TPP were stabilized via oxidation of thiols with H_2O_2 which formed interchain disulfide bonds. These would bestow gastroresistance on the particles, which would be particularly appropriate in case of oral administration of the nanoparticle formulation. However the presence of some non-oxidized thiols on the nanoparticle surface was needed to confer enhanced mucoadhesivity on such a surface. This goal was actually achieved by Bernkop-Schnurch et al. [21]. These authors also studied the crosslinker effect on nanoparticle size. Under similar preparation conditions, sizes in the 200-300 nm range were obtained with TPP as the crosslinker, whereas sizes beyond the micron resulted using Na_2SO_4 .

2.2. Gelation from polyelectrolyte complex (PEC) formation

This method involved ionotropic gelation, just as described in the preceding section, only in the present case the crosslinker was a polyanionic polymer with charges opposite to those of the chitosan derivative, with which it formed a PEC. To this purpose *N*-carboxymethyl chitosan, poly(γ -glutamic acid), poly(aspartic acid) and hyaluronic acid were used as polyanions, while TMC and glycidyl trimethyl ammonium chitosan were the polycations [24-27]. In a case both the polyanion (hyaluronic acid) and the polycation (TMC) were thiolated and the nanoparticles were stabilized by the formation of interchain disulfide bonds [28].

2.3. Polymer-drug complexes

Some negatively charged active principles, such as insulin or gene drugs, when mixed with cationic chitosan derivatives in adequate proportions spontaneously formed nanoparticulate dispersions of insoluble complexes [29-34]. TMC nanoparticles obtained by ionotropic gelation with TPP in the presence of insulin were compared with nanoparticles obtained by PEC formation between TMC and insulin. In the latter instance higher encapsulation efficiency and zeta potential (positive), and smaller particle size were observed, which is particularly appropriate for particle internalization into cells. In addition, a higher stability in simulated intestinal fluid (pH 6.8) of the nanocomplex compared to the nanoparticles prepared with TPP resulted [31,32].

2.4. Self-assembly

Amphiphilic derivatives of chitosan in aqueous solution were found, at a critical aggregation concentration (CAC), to spontaneously arrange into nanoparticles of sizes in between 100-400 nm. Such derivatives were prepared by connecting hydrophobic structures to the chitosan or glycol chitosan backbone via the amino group of the chitosan repeating unit. Examples of the above amphiphilic derivatives are the following: glycol chitosan-5 β -cholanic acid conjugate [35-40]; palmitoyl chitosan [41]; palmitoyl glycol chitosan [42]; oleoyl chitosan [43]. Other amphiphiles were prepared from chitosans bearing fixed positive charges, in the case of quaternary ammonium palmitoyl glycol chitosan [42], or negative fixed charges, as in the case of linoleic acid-modified *O*-carboxymethyl chitosan [44], or deoxycholic acid-modified *N,O*-carboxymethyl chitosan [45]. Usually, after suspending the polymer in an aqueous medium, probe sonication was applied to limit particle size. The formation of nanoparticles was monitored and the CAC determined fluorometrically, or through UV absorption spectra, or measuring the enthalpy change by a microcalorimeter [42,44,45].

The CAC for the hydrophobically modified chitosan derivatives is usually in the μ M range, whereas the CMC of small-molecular weight surfactants is in the mM range. This is one of the most important characteristics of amphiphilic polymers, pointing to stability of the self-aggregates in dilute conditions, such as those the nanoparticles are supposed to encounter after administration to the organism. The CAC values of these polymers have been found to decrease with increasing hydrophobic content of derivatives [44]. In fact, the nanoparticles formed from these chitosan derivatives are characterized by a core-shell structure, i.e., a hydrophobic core in a hydrophilic shell. The drug encapsulation method was chosen on the basis of the hydrophilic or hydrophobic nature of the drug. With hydrophobic drugs the solution of polymer and drug in a water-miscible organic solvent was mixed with an aqueous medium and the organic solvent was cleared away by dialysis or evaporation [36,39,40]. Hydrophobic drugs having a fair water solubility and polar drugs have been loaded into nanoparticles via direct addition to the aqueous polymer dispersion [41,42,44,45]. The non-encapsulated drug has been separated by ultracentrifugation, filtration or dialysis.

3. Interactions with cells and tissues

3.1. Quaternized derivatives

Chitosan has been found to open the tight junctions connecting epithelial cells, through an interaction of its positively charged amino groups with negatively charged sites in the tight junctions, thereby promoting paracellular transepithelial absorption of drugs, peptides and proteins [10,46-57]. The major drawback of unmodified chitosan as an absorption promoter is its insolubility at physiological neutral pH. Therefore the primary amino groups of its repeating units have been quaternized to bestow fixed, pH-independent positive charges on the polymer, thus making it soluble and active as an absorption promoter at physiological pH. In fact, TMC was found to act as an enhancer of drug, peptide and protein permeability across intestinal, nasal, buccal, ocular epithelium [47, 58-66]. TMC was shown to promote not only paracellular but also transcellular drug absorption [66]. Other quaternized chitosan derivatives, namely, *N*-triethylchitosan (TEC) [68], *N,N*-dimethyl *N*-ethylchitosan (DMEC) [69] and *N,N*-diethyl *N*-methylchitosan (DEMC) [70] have been synthesized. Positively charged chitosan and its quaternized derivatives have also exhibited mucoadhesive properties, determined by ionic interactions with the negatively charged sialic acid residues of mucins at neutral or slightly alkaline pH [71].

Particles in the nanosize range have resulted from the interaction of quaternized chitosans with polyanions. Proteins or macromolecular drug models have been encapsulated in these nanoparticles. Ovalbumin (OVA) was encapsulated in nanoparticles, obtained by ionotropic gelation of TMC with TPP, and studied as a nasal delivery system for proteins [19]. No cytotoxicity of nanoparticles on Calu-3 cells, a model of human respiratory function, was evidenced, whereas a partially reversible cilio-inhibiting effect on the ciliary beat frequency of chicken trachea was observed. Confocal laser scanning microscopy (CLSM) of nasal epithelia and nasal associated lymphoid tissue (NALT), incubated with nanoparticles loaded with fluorescein-labelled albumin, showed the presence of fluorescent nanoparticles throughout the cytoplasm of these cells, indicating the transport of albumin-associated TMC/TPP nanoparticles across the nasal mucosa. These findings led the authors to point to these nanoparticles as a potential delivery system for transport of proteins through the nasal mucosa.

Other authors studied similar TMC/TPP nanoparticles, loaded with fluorescein isothiocyanate dextran, molecular weight 4400 Da (FD4), as a model of macromolecular drugs [17]. In analogy with the free TMC, the TMC/TPP nanoparticles exhibited the property of opening the tight junctions between cells in the Caco-2 monolayer *in vitro* and the rat intestinal epithelium *ex vivo*, thus promoting the permeation of FD4 across the two epithelium models. The nanoparticles also shared, with the free TMC, the property of adhering to the intestinal mucosa. Using CLSM, Sandri et al. [17] showed internalization of their nanoparticles into Caco-2 cells and excised rat jejunum tissue.

Nanoparticles encapsulating fluorescein-labelled bovine serum albumin (BSA) were obtained by ionotropic gelation of alginate-modified TMC with TPP [16]. According to the authors the transport of alginate-modified TMC nanoparticles across the Caco-2 cell *in vitro* model of

gastrointestinal (GI) epithelium was more efficient than that produced by non-modified TMC nanoparticles. However, alginate modification barely had any effect on the trans-epithelial electrical resistance or on paracellular protein transport. Then the hypothesis was made that alginate modification facilitated nanoparticle transport across the Caco-2 monolayer by the transcellular route (transcytosis) by virtue of a reduction of particle size to 100-200 nm [16]. The supposedly permeated nanoparticles were assayed by measuring the fluorescence of fluorescein-labelled BSA, which was assumed to be completely associated with the particles. Similar nanoparticles as the above were loaded with urease, a vaccine protein against *Helicobacter pylori* infection. Immunization studies in mice showed that oral administration of urease-loaded TMC nanoparticles generated high titers of both IgG and S-IgA antibodies. The immunostimulating effect was caused by nanoparticle mucoadhesivity and transcytosis by M cells in gut associated lymphoid tissue [16].

OVA-loaded nanoparticles have been prepared from TMC using unmethylated CpG DNA as adjuvant and crosslinker, in place of TPP, for nasal vaccination in mice [15]. TMC/CpG/OVA showed similar physical properties as TMC/TPP/OVA in terms of particle size, zeta-potential and antigen release characteristics, but TMC/CpG/OVA induced a 10-fold higher IgG2a response than TMC/TPP/OVA, and a strong humoral and Th1 type cellular immune responses after nasal vaccination [15].

Nanoparticles derived from the polyelectrolytic complexation of TMC by the polyanionic mono-*N*-carboxymethyl chitosan (MCC), and loaded with fluorescein-labelled BSA were taken up into mouse Balb/c monocyte macrophages. Mice were nasally immunized with tetanus toxoid-loaded TMC/MCC complex nanoparticles. These were shown to induce both mucosal and systemic immune response [24].

Insulin was formulated into nanoparticles formed from quaternized chitosans such as TMC or DEMC via either ionotropic gelation with TPP, or polyelectrolyte complexation by the polyanionic insulin. The PEC method resulted in higher insulin loading efficiency and nanoparticle zeta-potential [31].

Similar nanoparticulate systems loaded with insulin were prepared from other quaternized chitosans, namely, *N*-triethyl chitosan (TEC) and *N*-dimethylethyl chitosan (DMEC), by the PEC method [30]. Insulin was transported ex vivo across the colon membrane of rats when it was formulated into nanoparticles made of quaternized derivatives, better than into those made of plain chitosan. In vivo colon absorption of insulin was enhanced by using insulin-loaded nanoparticles compared to free insulin. Insulin absorption from rat colon was evaluated by its hypoglycemic effect [30].

Poly(γ -glutamic acid) was used by Mi et al. [25] as the anionic polyelectrolyte complexing agent to prepare nanoparticles from TMC by the PEC method, for the oral delivery of insulin. According to the authors insulin was transported across the Caco-2 cell in vitro model of GI epithelium via the paracellular route. In fact, CLSM confirmed the opening of the tight junctions between cells caused by the nanoparticles. The authors propose a mechanism whereby the orally administered nanoparticles with mucoadhesive TMC on their surfaces may adhere and infiltrate into the intestinal mucus, mediate the opening of tight junctions between

enterocytes, undergo disintegration, and release insulin, which would permeate through the paracellular pathway to the bloodstream. This hypothesis is contrasting with that, proposed by Chen et al. [16], of protein being carried by TMC/alginate/TPP nanoparticles across the Caco-2 monolayer by transcytosis.

TMC was modified with the specific ligand CSKSSDYQC peptide (CSK) to prepare ionotropically crosslinked TMC-CSK/TPP nanoparticles, loaded with fluorescein isothiocyanate (FITC)-labelled insulin, targeted to the mucus-producing goblet cells [45]. In transport studies across Caco-2/HT29-MTX co-cultured cell monolayer, simulating mucus-producing intestinal epithelium, the CSK modification showed enhanced drug transport ability, even if the target recognition was partially affected by mucus. In pharmacological and pharmacokinetic studies in diabetic rats, the orally administered CSK-modified nanoparticles produced a stronger hypoglycemic effect than the unmodified ones, prompting the authors to state that the former were sufficiently effective as goblet cell-targeting nanocarriers for oral delivery of insulin.

An oral delivery system for paclitaxel, a mitotic inhibitor used in cancer chemotherapy, was devised by encapsulating the drug in *N*-(2-hydroxy-3-trimethylammonium) propyl chitosan chloride (HTCC) nanoparticles prepared by the O/W/O double emulsion temperature-programmed solidification method [72]. CLSM studies suggested that the HTCC nanoparticles could be transported across Caco-2 monolayers via the opening of tight junctions between cells. Also the *in vivo* absorption of these nanoparticles by the small intestine of rats was shown. These transport properties of nanoparticles were ascribed to their positive surface charge, which was also considered responsible for an enhanced nanoparticle uptake by carcinoma cells. Biodistribution studies after oral administration in subcutaneous LLC tumor-bearing mice showed accumulation of paclitaxel-loaded HTCC nanoparticles in liver, spleen, lung, and kidney tissues, which was ascribed to the uptake of nanoparticles by the reticuloendothelial system, and in tumour tissue through the enhanced permeability and retention (EPR) effect. These results are particularly intriguing as they open the prospect of a targeted oral treatment of cancer by nanomedicine.

3.2. Thiolated derivatives

The thiol groups immobilized on these polymers are supposed to give exchange reactions with disulfide bonds within the mucus, or oxidation reactions with cysteine-rich subdomains of mucus glycoproteins [73, 74], both resulting in the formation of disulfide bonds between thiolated chitosan derivatives and the mucus, which improve the polymer mucoadhesivity. Nanoparticles prepared from this type of chitosan derivatives were supposed to be themselves mucoadhesive, and hence, apt to make nanocarriers for oral drug delivery. In fact, enhanced mucoadhesive properties of nanoparticles prepared by gelation of chitosan-*N*-acetyl cysteine conjugate (chitosan-NAC) with TPP, compared with unmodified chitosan nanoparticles, were found by Wang et al. [23]. Enhanced insulin *in vivo* absorption via nasal mucosa was found by these authors when insulin-loaded chitosan-NAC/TPP nanoparticles were administered intranasally to rats.

Another thiolated chitosan derivative, chitosan-4-thiobutylamidine (chitosan-TBA) was used by Bernkop-Schnürch et al. [22] to develop a mucoadhesive nanoparticulate delivery system.

Polymer	Gelling agent	Drug	Diameter (nm)	Zeta potential (mV)	Reference
TMC-CSK	TPP	insulin	200-350	3-10	[14]
HTCC	o/w/o double emulsion	paclitaxel	130	21	[72]
TMC	cisplatin-hyaluronate	cisplatin	450	45	[27]
TMC	TPP	OVA	300	20	[15]
TMC	MCC	tetanus toxoid, FITC-BSA	not reported	not reported	[24]
HTCC	poly(aspartic acid)	BSA	200-300	55	[26]
TMC	poly(γ -glutamic acid)	insulin	100	30	[25]
TEC, DMEC	insulin	insulin	200	25	[30]
TMC, DEMC	TPP, insulin	insulin	250	25	[31]
TMC	TPP	FITC-BSA	300	14	[16]
TMC	TPP	OVA	254-300	20-61	[19]
TMC	TPP	FD4	200	-	[17]

Table 1. Main characteristics of nanoparticles based on quaternized chitosans

The polymer was first crosslinked ionotropically by TPP, followed by stabilization of the resulting nanoparticles via formation of inter- and intrachain disulfide bonds by thiol oxidation with H₂O₂. Subsequently, TPP was removed by dialysis. The covalently crosslinked particles would not disintegrate in the acidic medium of the stomach. The adhesion to porcine intestinal mucosa was studied after incorporation of fluorescein diacetate into nanoparticles. The more thiol groups were oxidized, the lower was the nanoparticle mucoadhesivity, nevertheless, even when as much as 90% of all thiols were oxidized the mucoadhesivity of chitosan-TBA nanoparticles was twice as high as that of unmodified chitosan nanoparticles.

Polymer	Gelling agent	Drug	Diameter (nm)	Zeta potential (mV)	Reference
chitosan-NAC	TPP	insulin	140-210	19-31	[23]
chitosan-TGA	DNA	DNA	75-120	2-20	[34]
chitosan-TBA	TPP	none	268	4-19	[22]
chitosan-TBA	TPP	none	240	5-11	[21]
thiolated TMC	thiolated HA	OVA	250-350	10-20	[28]
chitosan-TGA	none	pSEAP	212-113	4-8	[33]
glycol chitosan-TGA	TPP	calcitonin	230-330	21-27	[20]

Table 2. Main characteristics of nanoparticles based on thiolated chitosan derivatives

Glycol chitosan coupled with thioglycolic acid (TGA) was ionotropically gelled with TPP to yield nanoparticles, which showed a twofold increase in mucoadhesion to lung tissue after intra-tracheal administration to rats as compared to non-thiolated nanoparticles. Biocompatibility of nanoparticle formulations with lung tissue was demonstrated. Calcitonin-loaded glycol chitosan and glycol chitosan-TGA nanoparticles resulted in a pronounced hypocalcemic effect for at least 12 and 24 h and a bioavailability of 27 and 40%, respectively [20].

Verheul et al. [28] used the thiol groups of thiolated TMC to spontaneously form interchain disulfide crosslinks with the thiols of thiolated hyaluronic acid (HA), after ionic gelation. OVA-loaded stabilized TMC-S-S-HA nanoparticles demonstrated higher immunogenicity than not stabilized particles, indicated by higher IgG titers, in nasal and intradermal vaccination.

Besides showing enhanced mucoadhesivity and cell penetration properties, nanoparticles made of thiolated chitosans have appeared highly effective as gene delivery systems. Thiolated derivatives, prepared from 33-kDa chitosan by coupling with TGA, formed nanocomplexes with plasmid DNA encoding green fluorescent protein (GFP), that were able to bind and protect plasmid DNA from Dnase I digestion. Thiolated chitosan/DNA nanocomplexes induced higher GFP expression in HEK293, MDCK and Hep-2 cell lines than unmodified chitosan. Nanocomplexes of disulfide-crosslinked thiolated chitosan/DNA showed a sustained DNA release and continuous expression in cultured cells lasting up to 60 h post transfection. Intranasal administration of crosslinked thiolated chitosan/DNA nanocomplexes to mice yielded gene expression that lasted at least 14 days [34].

Nanoparticles containing the gene reporter pSEAP (recombinant Secreted Alkaline Phosphatase) were generated, based on a thiolated chitosan conjugate, chitosan-TGA, crosslinked by thiol oxidation with H_2O_2 to form disulfide crosslinks. Transfection of nanoparticles in Caco-2 cells led to increased protein expression compared to unmodified chitosan nanoparticles. Red blood cells lysis tests provided evidence for no cytotoxicity of nanoparticles. On the basis of their experimental results the authors stated that their crosslinked thiolated chitosan nanoparticles showed the potential for being used as a non-viral vector system for gene therapy [33].

3.3. Amphiphilic derivatives

Amphiphilic derivatives resulted when hydrophobic structures were attached to the hydrophilic chitosan backbone. In aqueous milieu these derivatives would self-assemble into nanoparticles to attain thermodynamic stability. Nanoparticles derived from the self-assembly of amphiphilic derivatives were often intended for cancer therapy. Glycol chitosan (hydrophilic)-cholanolic acid (hydrophobic) conjugates self-assembled to form nanoparticles, the in vivo tissue distribution, time-dependent excretion and tumor accumulation of which were monitored in tumor-bearing mice by Park et al. [37]. The particles exhibited prolonged blood circulation time, decreased time-dependent excretion from the body, and increased tumor accumulation with increasing polymer molecular weight. The enhanced tumor targeting by nanoparticles made of high molecular weight glycol chitosan-cholanolic acid was ascribed to a better in vivo stability, related to an improvement in blood circulation time [37].

Similar nanoparticles as the above, formed from glycol chitosan-cholanic acid conjugate, loaded with the anticancer drug camptothecin, exhibited significant antitumor effects and high tumor targeting ability towards MDA-MB231 human breast cancer xenografts subcutaneously implanted in nude mice. The significant antitumor efficacy of nanoparticles was ascribed to both their prolonged blood circulation and high accumulation in tumors through the EPR effect [39].

The cellular uptake mechanism and the intracellular fate of nanoparticles formed from glycol chitosan hydrophobically modified with cholanic acid have been reported [40]. These particles showed an enhanced distribution in the whole cells, compared to the parent hydrophilic glycol chitosan polymer. In vitro experiments with endocytic inhibitors suggested that the cellular uptake of these nanoparticles involved several distinct pathways, e.g., clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis. Such a property, along with low toxicity and biocompatibility suggested these hydrophobically modified glycol chitosan nanoparticles as a versatile carrier for the intracellular delivery of therapeutic agents [40].

A further hydrophobically modified chitosan derivative from which self-assembled nanoparticles were obtained was oleoyl chitosan. The toxicity profile of the relevant nanoparticles, evaluated in vitro via hemolysis test and MTT assay, was within acceptable limits. When loaded with the antitumor drug doxorubicin, oleoyl chitosan nanoparticles exhibited inhibitory rates on different human cancer cells (A549, Bel-7402, HeLa, and SGC-7901) significantly higher than the drug solution [43].

Folic acid was conjugated with *O*-carboxymethyl chitosan via the bifunctional 2,2'-(ethylenedioxy)-*bis*-(ethylamine) to obtain an amphiphilic chitosan derivative that would self-assemble into nanoparticles. Folate-mediated endocytosis significantly enhanced the cellular targeting of nanoparticles, thus facilitating apoptosis of cancer cells (HeLa, B16F1). Doxorubicin could be loaded into the nanoparticles. It was observed that survival in cancer cells treated with doxorubicin-loaded nanoparticles was lower than that of normal cells in similar concentrations [75].

The ability of nanoparticles prepared by self-assembly of chitosan amphiphiles to promote oral absorption of hydrophobic and hydrophilic drugs in rats was recently investigated by Siew et al. [42], using quaternary ammonium palmitoyl glycol chitosan as the basic material. The nanoparticles were found to enhance the oral absorption (C_{max}) of griseofulvin and cyclosporine A (hydrophobic) and, to a lesser extent, of ranitidine (hydrophilic). Hydrophobic drug absorption was facilitated by the nanomedicine by: (a) increasing the drug dissolution rate, (b) adhering to and penetrating the mucus layer, thus allowing intimate contact between the drug and the GI epithelium absorptive cells, and (c) enhancing transcellular drug transport. As for the absorption of the hydrophilic ranitidine, despite an 80% increase of C_{max} there was no appreciable opening of tight junctions by the nanoparticles. No uptake of this type of nanoparticles by epithelial cells is reported [42].

Polymer	Drug	Diameter (nm)	Zeta potential (mV)	Reference
griseofulvin cyclosporin A ranitidine	100-500	not reported	[42]
quaternary ammonium palmitoyl glycol chitosan
none	300-400	10	[40]
glycol chitosan-5β-cholanic acid
doxorubicin	150-200	10-20	[75]
O-carboxymethyl chitosan-2,2'-(ethylene dioxy)-bis-(ethylamine)-folic acid				
glycol chitosan-5β-cholanic acid	none	230-310	10-11	[37]
glycol chitosan-5β-cholanic acid	camptothecin	250-350	not reported	[39]

Polymer	Drug	Diameter (nm)	Zeta potential (mV)	Reference
oleoyl-chitosan	doxorubicin	250-350	not reported	[43]

Table 3. Main characteristics of nanoparticles based on amphiphilic chitosans

4. Concluding remarks

Three families of chitosan derivatives have been synthesized and used to prepare nanoparticles for pharmaceutical application, namely, polycations obtained by introducing quaternary ammonium groups on the polymer backbone; thiolated derivatives, and amphiphilic derivatives obtained by attaching hydrophobic structures to the chitosan or glycol chitosan backbone. The nanoparticles prepared from the quaternary ammonium-chitosan derivatives, especially via the PEC formation method, have shown improved stability and physical properties (smaller size, higher zeta potential) compared to nanoparticles from unmodified chitosan. The thiolated derivatives offered the opportunity to stabilize the nanoparticles by covalent crosslinks formed from interchain thiol oxidation to disulfide, which made the particles stable in the GI environment. The critical aggregation concentration of the amphiphilic hydrophobically modified chitosan derivatives is usually very low, which implies stability of the self aggregates in dilute conditions, such as those encountered by the nanoparticles in the organism. The nanoparticulate systems prepared from chitosan derivatives have generally shown acceptable cytotoxicity. In accord with the known behavior of particles of a size smaller than 500 nm, they have shown endocytic uptake by cells. Smaller particles with higher zeta potential have shown more aptitude to endocytosis. Iontropically crosslinked TMC nanoparticles are

a potential vehicle for transport of proteins across mucosal epithelia, as they have been found to open the tight junctions between epithelial cells. Indeed, nanoparticles based on quaternized chitosan are a promising vehicle for the oral administration of insulin, especially if the chitosan derivative is conjugated with the specific ligand CSKSSDYQC peptide. Also interesting is the nanosystem based on the quaternary ammonium-chitosan conjugate HTCC, which was orally absorbed by the rat small intestine and subsequently accumulated in carcinoma tissue by the EPR effect. These results are particularly intriguing as they open the prospect of a targeted oral treatment of cancer by nanomedicine. Nanoparticles prepared from thiolated chitosan derivatives have shown a particular mucoadhesivity implying a suitability for making nanocarriers for transmucosal protein delivery. Also this type of nanoparticles have appeared highly effective as gene delivery systems and have shown the potential for being used as a non-viral vector system for gene therapy. Nanoparticles derived from the self-assembly of amphiphilic chitosan derivatives were often intended for cancer therapy. Glycol chitosan hydrophobically modified with cholanic acid yielded nanoparticles with comparatively high *in vivo* stability, responsible for a prolonged blood circulation time, which led to high accumulation in tumors through the EPR effect. This type of nanoparticles can be taken up by cells through distinct pathways, which points to this system as a versatile carrier for the intracellular delivery of therapeutic agents. Folic acid, conjugated with *O*-carboxymethyl chitosan to obtain doxorubicin-loaded self-assembled nanoparticles, could mediate particle endocytosis by cancer cells with consequent cell apoptosis. In conclusion the present survey has endorsed the concept that chitosan derivatization can lead to new basic materials for nanosystems with unique pharmaceutical performances.

Abbreviations

BSA Bovine serum albumin

CAC Critical aggregation concentration

CLSM Confocal laser scanning microscopy

CSK CSKSSDYQC peptide

DEMC *N,N*-diethyl *N*-methyl chitosan

DMEC *N,N*-dimethyl *N*-ethyl chitosan

EPR Enhanced permeability and retention effect

FD4 Fluorescein isothiocyanate dextran, molecular weight 4400 Da

FITC Fluorescein isothiocyanate

GFP Green fluorescent protein

GI Gastrointestinal

HA Hyaluronic acid

HTCC *N*-(2-hydroxy-3-trimethylammonium) propyl chitosan chloride

LLC Lewis lung carcinoma

MCC Mono-*N*-carboxymethyl chitosan

NAC *N*-acetyl cysteine

NALT Nasal associated lymphoid tissue

OVA Ovalbumin

PEC Polyelectrolyte complex

pSEAP Recombinant secreted alkaline phosphatase

TBA Thiobutyl amidine

TEC *N*-triethyl chitosan

TGA Thioglycolic acid

TMC *N*-trimethyl chitosan

TPP Sodium tripolyphosphate

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