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Chitosan-based nano-objects for drug delivery: a review of their chemical modifications, supramolecular organization and biological fate

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Abstract

Chitosan is a widely applied polysaccharide in different fields due to its versatility, biocompatibility and low toxicity. Its structure has reactive functional groups that can be modified without involving the chain backbone, which improves its physicochemical and biochemical properties. Several chemical modifications as alkylation, acylation, thiolation, grafting with polymers and active molecules, can be combined with various supramolecular chemistry approaches as crosslink, self-assembly, polyelectrolyte complexes, ionic gelation, polymerization to formulate chitosan-based nano-objects able to encapsulate many active pharmaceutical ingredients, eventually providing to chitosan new possibilities in the pharmaceutical, biomedical and biotechnological fields. This review summarizes the knowledge developed in some recent works published in the last years regarding chemical modifications of chitosan, the design of chitosan-based nano-objects for the encapsulation and controlled delivery of active pharmaceutical ingredients, their biodistribution, biodegradation and toxicology.

Keywords: Chitosan, Chitosan modification, Supramolecular chemistry, Nanogels, Nanoparticles, Self-assembly, Drug delivery, Biodistribution, Biodegradation, Toxicity.

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1. Introduction

Chitosan (CS) is a pseudo-natural polysaccharide, obtained by deacetylation of chitin in alkaline medium resulting in a random arrangement of β-*D*-glucosamine (GlcN) and N-acetyl-β-*D*-glucosamine (GlcNAc) units linked together by (1-4) glycosidic bonds (**Fig. 1**). Chitosan is one of the few cationic polysaccharide known to date, giving it many advantages for application in different fields such as food [1–3], crops [4,5], cosmetics [6,7], and in particular drug delivery [8–10]. The control of the physicochemical properties of chitosan, such as its degree of deacetylation and molar mass, are crucial for its biomedical applications [11]. In particular, chitosan is characterized by its mole fraction of N-acetyl groups, called degree of acetylation (DA), or by its mole fraction of N-acetyl groups removed from the chitin macromolecule during deacetylation, called degree of deacetylation (DD, with DD = 1 – DA) [12,13]. The term 'chitosan' is used when the DA of the macromolecule is less than 50% [11].

The behavior of chitosan in solution is directly linked to its acid-base properties. Indeed, chitosan is a cationic polyelectrolyte in acidic medium, whose state of ionization is described by the equilibrium in **Eq. 1**, with a pK_a generally found in the 6-6.5 range [14–16]. However, this pK_a is not constant and varies according to the degree of dissociation (α) of chitosan according to Katchalsky's relation [17] (**Eq. 2**).

$$CS\text{-}NH_2\ (aq) + H_3O^+\ (aq) \rightleftarrows CS\text{-}NH_3^+\ (aq) + H_2O\ (l) \qquad \textbf{(Eq. 1)}$$

$$pK_a = pH + \log\left(\frac{(1-\alpha)}{\alpha}\right) = pK_0 - \frac{\varepsilon \Delta\Psi\ (\alpha)}{kT} \qquad \textbf{(Eq. 2)}$$

where pK_0 is the intrinsic pK_a of an isolated and non-protonated amine function; ε the dielectric constant of the medium; $\Delta\Psi\ (\alpha)$ the potential difference between an ion placed on the surface of the polyelectrolyte and at an infinite distance; k the Boltzmann constant, and T the temperature.

The pK_a of chitosan also depends on its DA. Sorlier et al. demonstrated that for a wide range of DA (5.2% to 89.0%) and from a chitosan colloidal solution concentration and ionic

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strength, is possible to obtain a polynomial equation able to deduce the pKa, based only in the DA and the medium ionic strength [18].

Chitosan is a weak base, insoluble in alkaline solutions and organic solvents, but soluble in acidic aqueous media when its DA is less than 50% [19–21]. It is commonly solubilized in aqueous acetic acid solution (0.1 $mol \cdot L^{-1}$ or 1% $w \cdot v^{-1}$) [22] or strong acid solutions such as hydrochloric acid [23]. The solubility of chitosan is related at the same time to the DA, ionic strength, pH, the nature of the acid used as well as to the distribution of the acetyl groups on the polymer chain. The protonation of amino groups generates electrostatic repulsions between the polymer chains segments, allowing the solvation of the chains and their subsequent solubilization and expansion. Dissolution at neutral or basic pH can only be accessed with a pre-step of dissolution in an acid medium to protonate the amine groups; the pH can then be increased by adding a base without causing precipitation up to its amine group pK_a.

Chitosan is generally considered soluble up to a pH of 6 [14,22,24] to 6.5 [25–27]. Rinaudo et al. studied the role of amino groups protonation on the solubility of chitosan in the presence of a weak acid (acetic acid, AcOH), or a strong acid (HCl), at different acid concentrations. Independently on the polymer concentration ([N] = $CS\text{-}NH_2$ + $CS\text{-}NH_3^+$) or the type of acid, the complete solubilization of the chitosan was obtained for $\alpha \geq 0.5$ corresponding to a stoichiometric ratio $[AcOH]/[CS\text{-}NH_2] = 0.6$ or $[HCl]/[CS\text{-}NH_2] = 0.5\text{-}0.6$. The ion concentration required for complete solubilization of chitosan is proportional to the number of amine groups of chitosan ($CS\text{-}NH_2$) [22,23]. The solubility of chitosan is an obstacle to be circumvented to modulate or give it new physicochemical properties, especially for encapsulation of active substances. Chemical modifications of chitosan have been carried out at the deacetylated units, on

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the C6 hydroxyl group, and/or on the C2 primary amine group, usually without involving the glycosidic bonds and depolymerization.

Chitosan, in its native or a chemically modified form, has been widely used to produce nano-objects able to encapsulate drug molecules in order to improve their delivery and targeting. This review provides an overview of the most important chemical modifications of chitosan, the different supramolecular chemistry approaches to produce nano-objects and encapsulate drug molecules, their resulting biological fate, relevance for drug delivery and potential toxicity, and especially how these aspects are connected. Other recent reviews also cover the chemical modifications [28], their formulation at a larger scale [29] and other biomedical applications (wound dressing, dental material, ...) [30–32].

2. Chemical modifications of chitosan

Chitosan has been modified in various ways to introduce numerous hydrophilic or hydrophobic moieties, thanks to the reactivity of the primary amine, and to a lesser extent to hydroxyl groups. The main chemical modifications described in the literature are summarized in **Fig. 1** and discussed below with a focus on their relevance to drug delivery (**Table 1**).

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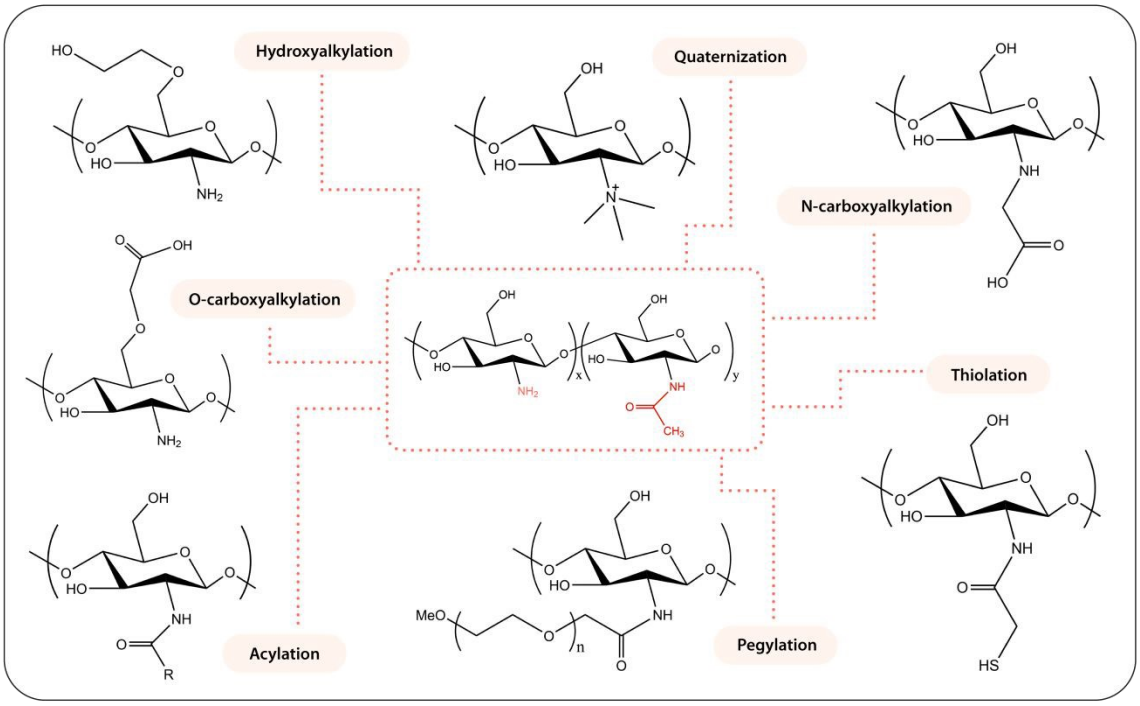

Figure 1. Overview of the main chemical modifications in chitosan structure described for its use in drug delivery, as reviewed below.

Table 1. Summary of major chitosan derivatives used in drug delivery and the encapsulated drugs.

Chitosan derivative	Encapsulated drug	Reference
Trimethyl chitosan	Camptothecin	[33,34]
	Candesartan-cilexetil	[35]
	Insulin	[36]
	Resveratrol	[37]
	Vancomycin	[38]
	Vitamins	[39]
N-octyl-O-sulfate chitosan	Docetaxel	[40]
	Paclitaxel	[41,42]
Glycol-chitosan	Camptothecin	[43]
	Cisplatin	[44]
	Dexamethasone	[45]
	Docetaxel	[46]
	Doxorubicin	[47,48]
	Paclitaxel	[49]
N-carboxymethyl chitosan	Idebenone	[50]
	Ofloxacin	[51]
O-carboxymethyl chitosan	Camptothecin	[52]
	Curcumin	[53]
	Gatifloxacin	[54]
	Metformin	[55]
	Methotrexate	[56]

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	Tetracycline	[57]
N,O-carboxymethyl chitosan	Dopamine	[58]
Oleoyl-chitosan	Doxorubicin	[59]
N-succinyl-chitosan	5-aminosalicylic acid	[60]
	5-fluorouacil	[61]
	Hydroxycamptothecin	[62]
Chitosan-*g*-stearic acid	Acyclovir	[63]
	Doxorubicin	[64]
	Tamoxifen	[65]
Chitosan-thioglycolic acid	Cyclobenzaprine	[66]
	Gemcitabine	[67]
	Leuprolide	[68]
	Theophylline	[69]
	Tizanidine	[70]
Chitosan-6-mercaptonicotinic acid	Insulin	[71]
Chitosan-2-iminothiolane	Insulin	[72]
Chitosan-N-acetyl-L-cysteine	Insulin	[73]
Chitosan-N-(4-hydroxyphenyl)-methacrylamide	Curcumin Gene transfection	[74]
PEGylated chitosan	Insulin	[75]
	Methotrexate, mitomycin C	[76]
	Ormeloxifene	[77]
	Resveratrol	[78]
	Rosuvastatin	[79]
	Indole-3-carbinol	[80]
Chitosan-*g*-β-cyclodextrin	Etoposide	[81]
	Ketoprofen	[82]
	Ovalbumin	[83]
Chitosan-*g*-polycaprolactone	Paclitaxel	[84]
Chitosan-*g*-poly(lactic-*co*-glycolic acid)	Paclitaxel	[85,86]
	Tamoxifen	[87]
Quaternized chitosan	Ketoconazole	[88]

2.1. Alkylation of chitosan

2.1.1. Quaternization

The alkylation was obtained by bonding carbon chains to the amino or hydroxyl groups of the chitosan. Rinaudo's group [89,90] described the quaternization of chitosan by alkylation of amine groups with methyl iodide under alkaline conditions (NaOH). The iodide ion was subsequently replaced by a chloride ion using an ion exchange process, to obtain a more stable chitosan salt: N-trimethyl chitosan chloride (TMC). This quaternization allows the introduction of

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permanent positive charges, enabling the polymer to be a cationic polyelectrolyte regardless of the pH. Its solubility depends directly on its degree of methylation [91]. Pardeshi et al. synthesized a TMC by the same technique and evaluated the mucoadhesive strength and bioadhesive potential of TMC. Due to the greater cationic nature of TMC, the mucoadhesive strength increased by 3.4-fold compared to unmodified chitosan [92].

Du Plessis et al. compared different degrees of quaternization of chitosan for nasal and rectal administration of insulin to rats. Highly quaternized TMC (61.2 %) increased insulin absorption at neutral pH, while chitosan hydrochloride and low quaternized TMC (12.3 %) were ineffective [93]. This effect was correlated to a more efficient interaction of TMC with anionic components of the cell membrane, potentiating the absorption in different values of pH. TMC was also applied as drug delivery for DNA [94] and used in wound dressing [95], as antibacterial [96], and antioxidant [97].

2.1.2. N-alkylation

The N-alkylation of chitosan has been achieved by grafting alkyl chains of different lengths [98]. The main parameters influencing the hydrophobic interactions by Van der Waals forces are the polymer concentration, the number of carbons grafted, temperature, and the ionic strength of the medium. Karam et al. described N-alkyl chitosan derivatives through reductive amination reactions using 1-dodecanal (C12) or 1-tetradecanal (C14). The N-dodecyl derivatives exhibit lower viscosities when compared to N-tetradecyl derivatives, both measured in 0.3 mol L^{-1} acetate buffer. The viscosity affects the hydrophobic interactions between the alkyl chains. N-tetradecyl products result in crosslink networks, whilst for N-dodecyl derivatives, the hydrophobic

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interactions facilitate the self-aggregation process to self-assemble into a nano-object (**see section 3.3**) [99].

Dang et al. modified chitosan with decanoic acid with different substitution degrees of amino groups. The derivatives with a higher degree of substitution showed low toxicity to L929 cells, mainly due to the insertion of the alkyl chains into the lipid bilayer without destroying the cell membrane, promoting cell adhesion. Increasing the content of alkyl chains also reduced the hemolysis rates of these conjugates in cells and promoted faster wound healing when compared to chitosan grafted with a low degree of substitution [100]. Liu et al. demonstrated an increase of transfection efficiency of plasmid-mediated by N-alkylation of chitosan dependent on the number of the carbons in the alkyl side chains, directly related to the hydrophobicity of N-alkyl chitosan [101].

2.1.3. Hydroxyalkylation

The hydroxyalkylation of chitosan was obtained by reaction of epoxides (ethylene oxide, propylene oxide, butylene oxide, etc.) with its primary amine and/or hydroxyl group, depending on the pH, solvent, and reaction temperature [102]. Due to its water solubility, chitosan glycol, a chitosan derivative conjugated with hydrophilic ethylene glycol branches, has been extensively studied for additional chemical modifications, particularly for the addition of hydrophobic groups to obtain amphiphilic derivatives (**see section 2.2**).

Mallick et al. described glycol chitosan-dequalinium, a chitosan glycol branched with a quaternary ammonium cation that contains two quaternary quinolinium units linked by an N-decylene chain, able to form micelles exhibiting a low toxicity towards HeLa and HDF cells [103]. Glycol chitosan was also studied by Yu et al., where the dexamethasone-loaded glycol chitosan

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nanoparticles showed an increase in the precorneal duration of action due to the ionic interactions with the cornea's surface when compared to free dexamethasone [45]. Other amphiphilic derivatives of chitosan glycol have also been developed, in particular with tocopherol [104], palmitoyl [105–107], hexadecyl [108,109], cholesterol [110], and N-acetyl-histidine [111].

2.1.4. Carboxyalkylation

Carboxyalkylation allows the introduction of carboxyalkyl groups on chitosan, giving it zwitterionic properties. By electrostatic repulsion, the carboxylic groups prevent potential intramolecular interactions between the polymer chains and improve their solubilization in water [112]. By varying the degree of carboxyalkylation, various charge densities on the polymer chains can be obtained and therefore control the solubility of chitosan at various pH values. N- and O-carboxyalkyl chitosan are obtained by reaction with monohalocarboxylic acids under different conditions to control the selectivity of the reaction in the C2 or C6 position [113,114].

The most common reaction to form O-carboxyalkyl chitosan is with monochloroacetic acid and sodium hydroxide. Glyoxylic acid can also be used to selectively produce N-carboxyalkyl chitosan derivatives by reductive amination [115,116]. Adnan et al. synthetized different O-carboxymethylated chitosan by using different ratios of monochloroacetic acid in isopropanol. The samples were tested to evaluate analgesic and anti-inflammatory activity *in vivo*. An increase in analgesia was noted and the paw volume did not present significant difference after treatment with the polymers. The O-CMC inhibited the release of pro-inflammatory mediators and did not present significant difference with the Paracetamol treated group, suggesting a strong analgesic effect [117].

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Carboxymethyl chitosan is mainly used for the delivery of gatifloxacin [54], camptothecin [52], methotrexate [56], tetracycline [57], curcumin [53,118], idebenone [50], and metformin [55].

2.2. Acylation of chitosan

The acylation of chitosan introduces hydrophobic groups at the C2 and/or C6 position of the chitosan via an ester bond by reaction with chlorides or acyl anhydrates. The hydrophobic groups are generally chains of fatty acids (C6-C18) such as oleic acid [59], linoleic acid [119], lauric acid [120], palmitoyl acid [121], stearic acid [64], and 5β-cholic acid [122,123], which increases the hydrophobic nature of chitosan and therefore allows the polymer, under certain conditions, to self-assemble into nano-objects in an aqueous medium (**see section 3.3**). This type of nanoparticle has also been developed for the encapsulation of hydrophobic active substances. Due to their amphoteric properties, short-chain acylated chitosans have also been developed, soluble in acidic and basic media [124]. One of them is N-succinyl-chitosan, a carboxyacylated derivative obtained by introducing a succinyl group [27] which has been investigated for the formulation of many nano-objects [125–127] and the encapsulation of anti-cancer drugs such as mitomycin C [128], doxorubicin [129], camptothecin [62], and paclitaxel [130].

Cho et al. investigated the impact of acyl chain length on drug encapsulation and delivery. Chitosan grafted with acyl chains of 1 to 16 carbons were used to formulate nanoparticles by self-assembly to encapsulate vitamin C, with a drug loading efficiency increased from C3 to C12. With longer N-acyl side chains, the resistance in acidic media also becomes stronger, leading to a slower drug release attributed to stronger hydrophobic interaction within N-acyl chitosan-based nanoparticles [131]. Echazú et al. modified chitosan with dodecenylsuccinic anhydride to develop

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hydrogels for buccal delivery of thymol. Rheological measurements showed that the elastic behaviour was predominant, and the acylation modified both the storage and loss modulus of the chitosan hydrogel. Moreover, the swelling test indicated an increase in the hydrophobicity of the modified chitosan compared to the unmodified one, facilitating the affinity of poorly water soluble compounds, such as the thymol [132].

2.3. Chitosan thiolation

The Bernkop-Schnürch group described the synthesis of various thiolated chitosans (thiomers). The first was a chitosan - thioglycolic acid (TGA) conjugate obtained by forming an amide bond between the primary amine group of chitosan and the carboxylic acid group of TGA activated by a carbodiimide [133–135]. Subsequently, other thiolated chitosan were synthesized by modifying the amine group with 2-iminothiolane [136,137] or glutathione [138]. Thiolated chitosan conjugates have *in situ* gelation properties, as the thiol groups oxidize between pH = 5 and 6.8, forming inter- and intramolecular disulfide bonds [136]. The gelling behavior of thiolated chitosan depend on the polymer chains' entanglement and the rearrangement of disulfide bonds [139]. These properties of mucoadhesion and *in situ* gelation have been exploited for the formulation of liquid and semi-liquid forms as well as for the formulation of nanocarriers.

A correlation was found between the degree of thiolation and the adhesion properties of the polymer. For example, chitosan-thioglycolic acid demonstrates a 5- to 10-times greater mucoadhesive effect than unmodified chitosan [136,137]. The mucoadhesion was also found to be influenced by the formation of disulfide bonds between its thiol groups and cysteine residues of the glycoproteins [69,140], as illustrated by Krauland et al in the case of nasal absorption of insulin

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by chitosan-4-thiobutylamidine conjugates [141] and Lee et al. in the case of drug delivery to the bronchial epithelium [69].

Other applications covered the P-glycoprotein (P-gp) inhibitory properties of these conjugates. Sakloetsakun et al. showed that the permeation enhancement of thiolated chitosan was achieved due to its capacity to form a disulfide bond between the cysteine of protein tyrosine phosphatase (PTPase), accelerating the reduction of GSH. Furthermore, the absorptive apparent permeability across the rat intestine was directly dependent on the pKa of the ligands and the amount of thiol groups conjugates on chitosan, impacting the P-gp inhibitory properties of the derivative [142]. Usually, thiolated chitosan nanoparticles were prepared by ionic gelation method [70,143–145], and this polymer was employed in the delivery of tizanidine [70], curcumin [145], cyclobenzaprine [66], leuprolide [146], and insulin [71,73].

2.4. Other chemical functionalization of chitosan

In addition to the chemical modifications of chitosan described above, the functionalization of chitosan by other polymers or by active substances has a great interest to improve the physicochemical properties of chitosan or the active substance.

2.4.1 Polyethylene glycol (PEG)

PEG is a nonionic hydrophilic polymer approved by several drug safety agencies (FDA, EMA) that can be synthesized with different molar masses and different functionalizable groups in the terminal position. For the functionalization of chitosan, PEG must be first chemically modified by adding a terminal group such as an aldehyde, a carboxylic acid, a carbonate, or an acrylate, which can react with the amine or hydroxyl groups of chitosan. Methoxy-PEG (mPEG)

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is predominantly used instead of PEG to prevent crosslinking between polymers [147–149]. PEGylation of chitosan generally takes place at the level of the amine group [150]. However, some studies described PEG grafting at the level of the hydroxyl group at the C6 position of chitosan [151–153]. Different methods have also been developed, such as radical polymerization [154], click chemistry [155], or crosslinking [156,157].

Through its hydrophilic properties, PEG improves the solubility of chitosan depending on the degree of substitution and the chain length of PEG [147,150,158]. Depending on the degree of PEGylation, PEGylated chitosan can form complexes with polyanions [159–164], or self-assemble in aqueous medium thanks to the formation of intermolecular hydrogen bonds (see sections 3.2. and 3.3. for the mechanisms of assembly, respectively) [157]. In particular, high molar mass of PEG improves the self-assembly of nanoparticles with smaller sizes.

The molar mass of PEG modulates the rheological properties of PEGylated chitosan hydrogels. Del Olmo et al. developed several hydrogels based on 400 and 1000 $g \cdot mol^{-1}$ PEG. The decrease in the molar mass of PEG and the increase in CS:PEG weight ratio (from 1:0.4 up to 1:1) directly interfered with the increase in viscosity and both storage and loss modules, and also increased the stability of the hydrogels [165]. Other effects of molecular weight were reported by Anraku et al., who showed that low M_w chitosan had more pronounced scavenging activities for DPPH radicals, with corresponding nanoparticle with a low particle size presenting a better activity in DPPH test than the larger. Furthermore, low M_w PEGylated chitosan was able to protect the liver against damages [166].

The PEGylated chitosan nanostructures were developed to reduce toxicity and enhance drug delivery (see sections 6.1. for the detailed toxicology). In cell culture, non-modified chitosan was observed in a large amount on the cell membrane, while chitosan grafted with 5 to 10 kg mol-

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[1] PEG and complexed to siRNA was observed within cells, increasing their enhanced transfection efficiency [167]. Bae et al. produced PEG-grafted chitosan micelles with heparin, which showed good cytotoxic activity against B16F10 cells and increased the treatment's intracellular transport across the cell membrane [160]. *In vivo,* Malhotra et al. found that the absorption of nanoparticles based on chitosan-PEG derivatives occurred across the nasal epithelial tissue 4h after administration to mice and was cleared after 16h, allowing a measurable biodistribution in the cerebral cortex which disappeared before 16h [168]. The PEGylated chitosan nanostructures were also applied to the delivery of doxorubicin [169], insulin [163], methotrexate, mitomycin C [76], puerarin [170], and ibuprofen [171].

2.4.2 Other polymers

Other less studied polymers have been grafted to chitosan such as dextran [172,173], poly (vinylpyrrolidone) (PVP) [174], poly (caprolactone) (PCL) [175–178], or even poly (ethylenimine) (PEI) [179–183], a cationic polymer used as a non-viral vector in gene therapy. Chitosan copolymers can also form complexes with polyanions (**see section 3.2.**) or self-assemble in an aqueous medium (**see 3.3.**). Different groups have also been interested in synthesizing copolymers of CS-PEI-PEG [184] and CS-PCL-PEG [185–187], allowing the combination of the physicochemical properties of different polymers.

2.4.3 Cyclodextrins

With their hydrophobic cavity, cyclodextrins can non-covalently bind aromatic molecules and other small organic molecules and form inclusion complexes. Grafting cyclodextrins with chitosan allows obtaining a molecular transporter system that protects against degradation,

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increases the apparent solubility, reduces the undesirable effects, and controls the release of the substance active. Furusaki et al. described the grafting of β-cyclodextrin on chitosan by coupling a carboxymethyl-β-cyclodextrin in the presence of carbodiimide [188]. To a lesser extent, cyclodextrin grafting can be achieved by nucleophilic substitution, reductive amination, or in the presence of other chemical groups such as tosyl chloride or hexamethylene diisocyanate [114].

Many systems have been developed for the encapsulation of active substances or macromolecules: heparin [189], glutathione [190], ketoprofen [82], insulin [191], doxorubicin [192,193], BSA [194], triclosan and furosemide [195].

2.4.4 Metal ions

Through its chelating properties, chitosan can form organometallic complexes with different metal ions. Chitosan forms coordination complexes with metals on the free electron pairs present on the oxygen and nitrogen atoms of chitosan [196]. Reynaud et al. have developed organometallic complexes of chitosan with different metals (Fe^{2+}, Fe^{3+} and Zn^{2+}) formulated in the form of microparticles to eliminate residual medicinal active substances in the digestive tract [197]. Other groups have used chitosan-iron complexes in water treatment to remove chromium [198] or chitosan-zinc complexes to enhance the antimicrobial activity of chitosan [199]. Giacalone et al. used organometallic complexes of chitosan-iron in order to stabilize nano-objects based on chitosan and polyphosphate molecules (**see 3.2.1**) [200].

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2.4.5 Targeting ligands

Chitosan can also be functionalized with a variety of ligands to enhance cell-specific targeting and internalization of nano-objects by receptor-mediated endocytosis pathways. Conjugation can take place on native chitosan or on chitosan already chemically modified, before or after formation of chitosan nanoparticles. The different types of ligands studied as well as their targets of interest have been summarized in **Table 2**.

Table 2. Chitosan-grafted ligands and their applications.

Ligands	Targets of interest	Molecules delivered
Galactose	Liver cells expressing asialoglycoprotein receptors	Oridonin [201], Norcantharidin [202], Glycyrrhizin [203], Curcumin [178] , DNA [173,174,204–209]
Mannose	Antigen-presenting cells, e.g., macrophages and dendritic cells	Rifampicin [210], DNA [211,212], Oligonucleotide [213]
Transferrin Protein KNOB	Targeting cancer cells e.g., HEK293 and HeLa cells Increased transfection and transcytosis	Doxorubicin [214], Methotrexate [215], DNA [216,217]
CPP	Increased transfection	DNA [218–222], siRNA [223–227]
Peptide RGD	Targeting cells expressing integrin receptors αvβ3 and αvβ5	Doxorubicin [228,229], siRNA [230]
Folic acid	Membrane targeting of overexpressed folate receptors on cancer cells (KB, OV2008, MCR-7)	Paclitaxel [231], 5-ALA [232], Doxorubicin [233], DNA [234,235]
Glycyrrhizin	Liver cells (asialoglycoprotein receptor)	Doxorubicin [236], Paclitaxel [237], Atorvastatin [238], Lamivudine [239], Adryamicin [240]
Urocanic acid	Proton sponge enhancing cytoplasmic release	DNA [241,242], siRNA [243], p53 gene [244]
Active substances		Doxorubicine, Mitomycine C, Paclitaxel

CPP: cell penetrating cells; RGD: arginine-glycine-aspartate tripeptide; 5-ALA: 5-aminolevulinic acid; PTEN: phosphatase and tensin homolog.

2.4.5.1 Saccharides

Saccharides play an important role in biological recognition at the cell surface level. They can interact specifically with membrane receptors allowing active targeting. For example, galactose conjugation has been studied to improve hepatic targeting, due to the high affinity for

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the asialoglycoprotein receptors (ASGR) mainly present on the surface of hepatocytes. These receptors bind and internalize glycoproteins with a terminal galactose group [245]. Galactosylated chitosan was obtained by coupling with lactobionic acid carrying galactose groups via a carbodiimide or directly with D-galactose [204]. Lactose conjugation has also been reported in the literature [246,247].

Another saccharide of interest for active targeting is mannose. Mannose receptors are mainly present at the level of antigen presenting cells such as dendritic cells and macrophages, playing an important role in the immune system. These cells are overexpressed at pathological sites such as tumors, atherosclerotic plaques, arthritic joints and infection sites [248]. Mannosylated chitosan is mainly obtained by coupling mannopyranosylphenyl-isothiocyanate with the amine groups of chitosan [211–213,249].

2.4.5.2 Proteins and peptides

Transferrin is a protein involved in the cellular transport of iron in the body, by the interaction with cell surface receptors to facilitate iron absorption. Transferrin receptors have been shown to be overexpressed in cancer cells of various malignancies [250,251]. Mao et al. developed two methods of post-formation transferrin conjugation to chitosan nanoparticles. The first consisted in introducing aldehyde groups by periodate oxidation, thus the modified transferrin can then react with the amine groups on the surface of the nanoparticles. This method minimizes steric hindrances and loss of protein activity. The second method is based on a reversible conjugation, the transferrin has been linked to the nanoparticles by disulphide bonds. These bonds allow the cleavage of transferrin after cell capture as well as the degradation of nanoparticles and the release of DNA. However, *in vitro*, nanoparticles conjugated with transferrin did not show an

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enhancement of DNA transfection. Mao et al. therefore developed nanoparticles with another protein: the KNOB protein, globular domain C-terminus of adenovirus capsid fibrous protein which was conjugated to chitosan in the same way by disulfide bond. These conjugated nanoparticles improved transfection [216].

RGD peptides are peptides containing arginine-glycine-aspartate with the ability to target cells expressing integrin receptors at their cell membranes [252,253], such as cancer cells [254,255]. The RGD peptide is mainly conjugated to chitosan by reaction with carbodiimides [228–230] but it has also been physically encapsulated within chitosan-based nanoparticles [256,257].

To improve cellular uptake of different molecular structures (from small molecules to DNA fragments and nanoparticles), different groups have synthesized conjugates of chitosan with cell penetrating peptides (CPP). They are short sequence peptides (5 to 30 amino acids) that can interact and cross membranes [219]. Chitosan has been modified for nucleic acid (DNA and small interfering RNA, siRNA) delivery with various CPPs including TAT [218,221,223,224,258], penetratin [219], and oligoarginines (7–9 units) [220,226,227].

2.4.5.3 Small molecules

Different small molecules have been conjugated to chitosan mainly by reaction with carbodiimides to target certain cell types. For example, folic acid has been conjugated to chitosan for its ability to target overexpressed folate receptors particularly in cancer cells [259]. As for urocanic acid, a molecule bearing an imidazole group, it can act as a proton sponge and improve the release of active substances into the cytoplasm after endocytosis [260]. Another example, glycyrrhizin, the main compound extracted from the root of Glycyrrhiza glabra (liquorice), has shown specific affinity for ASGR receptors present on the surface of hepatocytes [261].

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Glycyrrhizin was grafted directly onto chitosan [236–238], or grafted post-formation of chitosan nanoparticles [239,240].

2.4.6 Active substances

To increase the quantities of the active substances delivered and their retention at the site of action, different groups investigated their conjugation to chitosan. As a result, numerous conjugates have been developed to deliver generally hydrophobic, mainly anti-cancer, active substances. The chitosan-active substance conjugates consisting of a hydrophilic part (the polymer) and a hydrophobic part (the active substance) can behave as amphiphilic polymers and self-assemble as nanoparticles in aqueous media (**see section 3.3**) [262].

The active substance can be conjugated to chitosan via a biodegradable linker, stable in physiological medium but cleavable at the site of action by hydrolysis or enzymatic degradation. For example, the chitosan glycol-doxorubicin conjugate (DOX-GC) has been obtained by a chemical reaction between N-*cis*-aconityl-doxorubicin and chitosan glycol by reaction with carbodiimides. The N-*cis*-aconityl linker is stable at physiological pH but is hydrolyzed at acidic pH (4.5-6.5), allowing the release of the active substance at the endosomes and lysosomes of tumor cells. Son et al. have described DOX-GC nanoparticles with a high yield (97%) and charge rate (38% m m^{-1}). These intravenously administered DOX-GC nanoparticles accumulated preferentially in the tumor with the release of the active substance (**see section 4**) [47].

2.5 Combinations of chemical modifications

The combination of several chemical modifications of chitosan has been reported on numerous occasions. For example, the synthesis of alkylated and thiolated chitosan has been developed to improve the solubility and mucoadhesion of chitosan [263,264]. For its part, the

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synthesis of chitosan alkylated with fatty acid chains or PEG has made it possible to obtain nano-objects by self-assembly for the encapsulation of active substances [159,265,266]. The conjugation of PEGylated chitosan with folic acid increases the solubility of chitosan while improving cell targeting [158].

The thiolation of carboxymethyl chitosan was synthesized in a self-assembled particle via a disulfide bond crosslinked to encapsulate methotrexate for a tumor-specific drug release [267], gene carriers [264], and insulin orally delivered [263]. The thiolation of glycol chitosan enhances the pulmonary absorption of calcitonin compared to a glycol chitosan nanoparticle [268]. The octanoylation of glycol chitosan derivative stimulates cell proliferation, metabolism, and differentiation, due to an increase in the duration of the G1 phase [269].

3 Formation of nano-objects based on chitosan

Due to its various physicochemical and biological properties, native and chemically modified chitosan have been widely used to formulate nano-objects encapsulating active substances for multiple routes of administration. These nano-objects can be obtained in different ways: by chemical crosslinking, ionic crosslinking (ionic gelation or polyelectrolyte complexes), or even self-assembly (**Fig. 2**).

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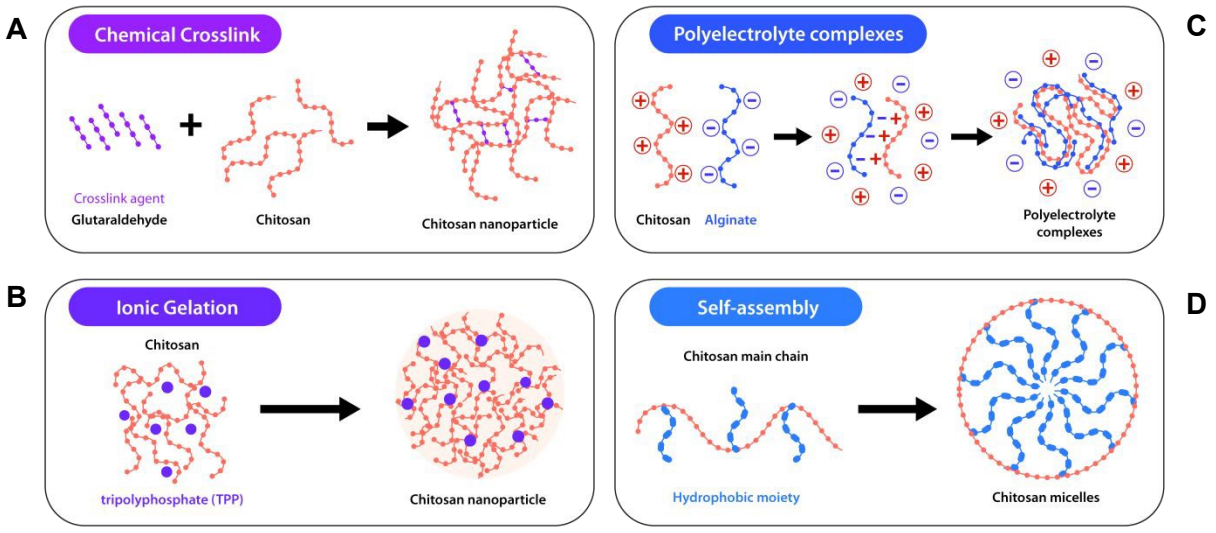

Figure 2. Mains strategies for the formulation of nano-objects based on native or modified chitosan chains: **(A)** chemical crosslinking by formation of covalent bounds between chitosan chains; **(B)** ionic gelation by formation of ionic bounds between chitosan chains and small polyions such as TPP; **(C)** formation of polyelectrolyte complexes by mixing chitosan with anionic polymer chains; **(D)** self-assembly of specific amphiphilic derivatives of chitosan.

3.1 Formation of nano-objects by chemical crosslinking

The first chitosan nanoparticles described were obtained by chemical crosslinking (Fig 2A). The most commonly crosslinking agent used is glutaraldehyde which binds covalently by its aldehyde groups to the primary amine groups of chitosan and, in some cases, to the primary amine groups the active substance (Schiff reaction), allowing the formation of nanoparticles at specific concentrations and ratios. Different manufacturing methods have been developed and described on numerous occasions in different reviews [270–274].

Paclitaxel has also been encapsulated by chemical crosslinking [275]. However, because of the cytotoxicity of glutaraldehyde, recent studies have focused on using other crosslinking agents such as genipin, an agent of natural origin isolated from the fruit of *Gardenia jasminoides* Ellis. Due to better stability (resistance to enzymatic degradation) and lower cytotoxicity than glutaraldehyde [276,277], genipin has been used for the encapsulation of various active ingredients

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for targeting enteropathogenic bacteria [278–280]. In addition, other naturally occurring crosslinking agents have been investigated with di- and tri-carboxylic acids (tartaric, malic, succinic, citric, PEG-COOH) [281–283].

Del Olmo et al., used chitosan crosslinked with genipin hydrogels for sustained release of several drugs. The rheological parameters of viscosity, storage and loss modulus were obtained when 1:0.2 CS:genipin weight ratio, due to the greater number of chemical linkages formed between them. Time reaction was also an important parameter since hydrogels showed a decrease in rheological parameters with the increase of time reaction. The hydrogels also presented and healing of ulcerated wounds and an improvement in metabolic activity and an increase in collagen and elastin levels compared to negative control [284].

The nanoparticles obtained by chemical crosslinking exhibit good encapsulation of the active substance and good stability in a physiological medium due to the intermolecular chemical bonds involved. However, these nanoparticles are obtained under aggressive experimental conditions, which may alter the active substance. Moreover, solvent and reagent removal problems may be encountered, prompting interest for manufacturing methods in milder conditions.

3.2 Formation of nano-objects by ionic crosslinking

Protonated chitosan chains can interact with the negative charges carried by an ionic crosslinking agent, an active substance, or a polymer. Two main approaches rely on this idea: ionic gelation (Fig. 2B) and complexation of polyelectrolytes (Fig. 2C).

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3.2.1 Ionic gelation

The group of Alonso et al. was the first to formulate chitosan-based nanoparticles by the ionic gelation process [285,286], US6649192B2 patent). This simple technique relies on the spontaneous formation of nano-objects by adding, dropwise, a solution of sodium polyanion triphosphate (TPP), used as an ionic crosslinking agent, to the (protonated) chitosan solution. A colloidal suspension is obtained by inter and intramolecular electrostatic interactions between the phosphate groups of the negatively charged TPP and the amine groups of the positively charged chitosan. The resulting nano-objects are referred to as ‘nanogels’ or ‘nanoparticles’ in the literature.

Due the nature of the reversible electrostatic interactions involved between chitosan chains and TPP, several parameters can affect the physicochemical properties of the nano-objects (size, surface charge, compactness), such as the concentrations of chitosan and TPP as well as the CS/TPP ratio [287–294], the molar mass and the DD of chitosan [289,290,293–296], the initial pH of the chitosan solution [287,289,296], the incorporation of another polymer such as PEG [288] and most importantly, ionic strength, which plays a crucial role in the colloidal stability of CS/TPP nanoparticles. The colloidal instability of these nanoparticles results in either the dissociation of the nanoparticles or their aggregation [297]. An important source of dissociation of CS/TPP nanoparticles prepared in a salt-free acetic acid solution is a mere dilution in isotonic (150 mmol L^{-1}) NaCl [298], which highlights the importance of competitive ionic interactions in a salt-rich environment. Huang et al. also demonstrated that, in NaCl (150 mmol L^{-1}, pH = 4), lowering DD leads to a decrease in the aggregation but also an increase in the dissociation of nanoparticles, reflecting weaker electrostatic interactions with the most deacetylated chitosan. Conversely, in phosphate buffered saline PBS (pH = 7.2), the most deacetylated chitosan-based nanoparticles do

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not dissociate but precipitate because of the low solubility of chitosan. The dissociation or aggregation of nanoparticles is therefore correlated to the strength of the interactions between chitosan and TPP and is dependent on ionic strength and pH [294].

Several strategies have addressed the potential instability of CS/TPP nanoparticles in physiological ionic media. Increasing the ionic strength of the nanoparticle formation medium by adding salt (150 mmol L^{-1} NaCl) results in the formation of more compact and stable nanoparticles that resist dilution in physiological media [292,299,300]. Giacalone et al. also successfully improved the colloidal stability of chitosan nanoparticles by incorporating Iron (III) ions during their preparation. Iron (III) forms coordination complexes with chitosan and phosphate groups and allows additional and overall stronger interactions (less sensitive to ionic strength) to be formed between chitosan and the active substance, improving colloidal stability [200].

These studies were performed on CS/TPP nanoparticles that do not encapsulate any active substance. However, depending on the nature of the active substance, the electrostatic interactions can be stronger. Exemplifying this, Kalam et al. developed tedizolid phosphate encapsulated chitosan nanoparticles via ionic gelation method for ocular delivery [301]. Particles formed by low weight ratio of CS/TPP obtained optimum-sized particles with high encapsulation (82%) and a good drug loading capacity (7%), indicating an ionic interaction between the anionic groups of TPP with the amine groups of CS in the weight ratio (CS was 3.12-fold higher than TPP). When the weight ratio was decreased (CS was 2.77-fold TPP), the ionic interaction between them it was not sufficient. Nanoparticles containing tedizolid did not showed symptoms of discomfort in the ocular irritation study in rabbits, and the transcorneal permeation of tedizolid by the particles was 1.6-fold increase in flux and the permeability coefficient, indicating its higher permeation compared to the free drug.

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CS/TPP nanoparticles prepared by the ionic gelation process have been used to encapsulate many active substances, showing their versatility as a drug delivery system [302,303]. The potential of an active substance itself to induce the ionic gelation of chitosan has been proposed by Giacalone et al. as a strategy to improve drug loading (**Fig. 3**). The nucleotide adenosine triphosphate (ATP) and the nucleotide analog azidothymidine triphosphate (AzT-TP), both of which have a triphosphate group similar to TPP, formed nanoparticles by mere mixing with chitosan in absence of TPP, resulting in a drug loading as high as 44% by weight [298]. Russo et al. have also demonstrated it with foscarnet, a molecule also having a triphosphate group [304].

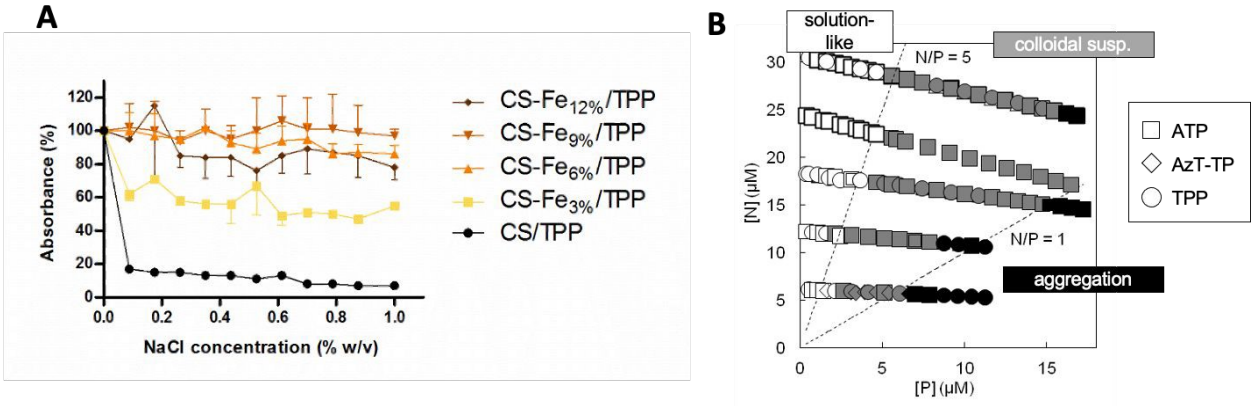

Figure 3. Illustration of drug delivery challenges and opportunities associated to the ionic gelation of chitosan. **(A)** The dilution of CS/TPP nanogels in electrolytes may cause their rapid disassembly, as shown by turbidity measurements following nanogel dilution in 0-150 mM (~0-0.9 % w/v) NaCl medium. This effect can be reversed by the introduction of Fe^{3+} coordination complexes within the nanogels (reproduced from [200] with permission from Elsevier, copyright 2014). **(B)** Active drug molecules containing a triphosphate group can induce chitosan gelation similarly to TPP, leading to drug loading values higher than typically obtained in nanocarrier formulation, as shown in the case of the nucleotide adenosine triphosphate (ATP) and the nucleotide analogue azidothymidine triphosphate (AzT-TP) (reproduced from [298] with permission from American Chemical Society, copyright 2013).

3.2.2 Polyelectrolyte complexes (PEC)

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Polyelectrolytes are macromolecules carrying numerous positive (polycation) or negative (polyanion) charges on their structure. By simply mixing two or more oppositely charged polyelectrolytes, PECs can be obtained spontaneously through the formation of electrostatic interactions as well as hydrogen and hydrophobic bonds [305,306]. In an acidic medium, chitosan behaves like a cationic polyelectrolyte and can therefore interact with anionic polyelectrolytes to form complexes. Many such complexes have reported with chitosan and various polyanions for the design of drug delivery systems: alginate [297,307–315], carrageenan [310,316,317], glucomannan [318,319], pectin [320,321], hyaluronic acid [322–325], carboxymethylcellulose [326], chondroitin sulfate [327,328], polyglutamic acid [329–331], heparins [322,332–334], dextran sulfate [333,335–339].

The formation of PECs depends on many parameters, particularly: (i) the nature of the polyelectrolytes, (ii) the degree of ionization of each polyelectrolyte, (iii) the density and distribution of charges on the chains of polyelectrolytes, (iv) the concentration of polyelectrolytes, (v) the ratio between the two polyelectrolytes [308,321,324,325,332,335]. Quiñones et al. showed the effect of the charge ratio of polyelectrolytes on the size and charge of the polyelectrolyte complexes formed. As the charge ratio moves away from neutrality, the nanoparticles formed are charged with excess polyelectrolyte. On the other hand, if the charge ratio is close to 1, uncharged particles are obtained, forming aggregates [305]. The formation also depends on the reaction parameters: temperature, ionic strength, and pH. PECs' colloidal stability heavily depend on ionic strength and is controlled by the dissociation or aggregation of the complexes [340]. The colloidal stability of PECs has been improved with the use of zinc [324,341]. Different groups have developed systems combining these two manufacturing processes, using a chemical or ionic crosslinking agent to formulate PECs [305].

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3.3 Formation of nano-objects by self-assembly

Native chitosan has difficulty to self-assemble into nano-objects in aqueous media, unlike some chitosan derivatives. The grafting of hydrophobic fragments such as fatty acids by acylation (**see section 2.2**) or certain polymers (**see section 2.4.**) allows the conjugates, by hydrophobic interactions, to self-assemble into nano-objects in an aqueous medium (Fig. 2D). By varying the type and degree of substitution of the hydrophobic moiety, the size and zeta potential of nano-objects and the release profile of the active substance can be modulated. This type of nanoparticle has been mainly used for the encapsulation of anti-cancer drugs: epirubicin [342], paclitaxel [41,42,49,84,111,343], doxorubicin [46–48,129,344], mitomycin C [345], camptothecin [43,346].

3.4 Formation of nano-objects by polymerization

The chitosan-based nanoparticles have also been obtained by grafting a polymerizable group onto the chitosan leading to the formation of polymer chains. The polymerization then generates inter and intra-molecular bonds between the carboxylic groups of the polymer and the amine groups of the chitosan forming nanoparticles. The most widely used copolymerization method is controlled or uncontrolled radical polymerization by free radicals with vinyl monomers. By this method, various copolymers have been synthesized with acrylates [347–351] or styrenes [352].

The chemical flexibility of chitosan, due to the numerous possible chemical modifications, is important to obtaining biomaterials suitable for different therapeutic applications. However, this diversity of chitosan leads to new chemical and particulate entities whose biodistribution, biodegradation and toxicology profiles are still poorly understood today.

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4 Biodistribution

The biodistribution of chitosan-based systems strongly depends on their route of administration, which originates in the diversity of the chemical modifications of the polymer and of the nano-formulation types based on them. The understanding of their *in vivo* fate after administration is crucial to evaluate their potential to improve drug delivery, but also to understand the potential toxicity of these drug delivery systems. In this respect, most studies have used the main parenteral routes.

4.1 Intravenous route

He et al. investigated the impact of the size and surface charge of nanoparticles on biodistribution in H-22 tumor bearing mice (**Fig. 4**). To do so, two types of chitosan conjugated with rhodamine, carboxymethyl-chitosan (RhB-CMC) and chitosan hydrochloride (RhB-CH), were associated to FITC-labeled protamine sulfate (FITC-PS) and camptothecin (CPT) to yield nanoparticles with variable physicochemical properties in terms of size (150-500 nm) and zeta potential (-40 to +35 mV) [353]. The study overall demonstrates that the surface charge influences the biodistribution of chitosan-based nanoparticles, with negatively charged nanoparticles accumulating more in the tumor compared to positively charged nanoparticles, which are cleared more quickly from the bloodstream and accumulate in the liver and spleen. The size of the nanoparticles also has an impact: the larger the nanoparticles, the more they are captured by the liver. In particular, small and negatively charged (150 nm, -15 mV) nanoparticles showed the greatest accumulation in the tumor due to an increased circulation time in the bloodstream. Higher surface charges and nanoparticle size caused a decrease in the circulation time in the blood and a

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higher accumulation in the liver and spleen. Renal elimination of nanoparticles was not found to be influenced by charge and size [353]. A higher hepatic accumulation of RhB-CH nanoparticles (+25 mV, 300 nm) compared to RhB-CH (+25 mV, 150 nm) was also observed by Hu et al., while the opposite trend was observed in the spleen. In the lungs, the accumulation of nanoparticles depends on the load, probably resulting from the formation of aggregates in the pulmonary capillaries.

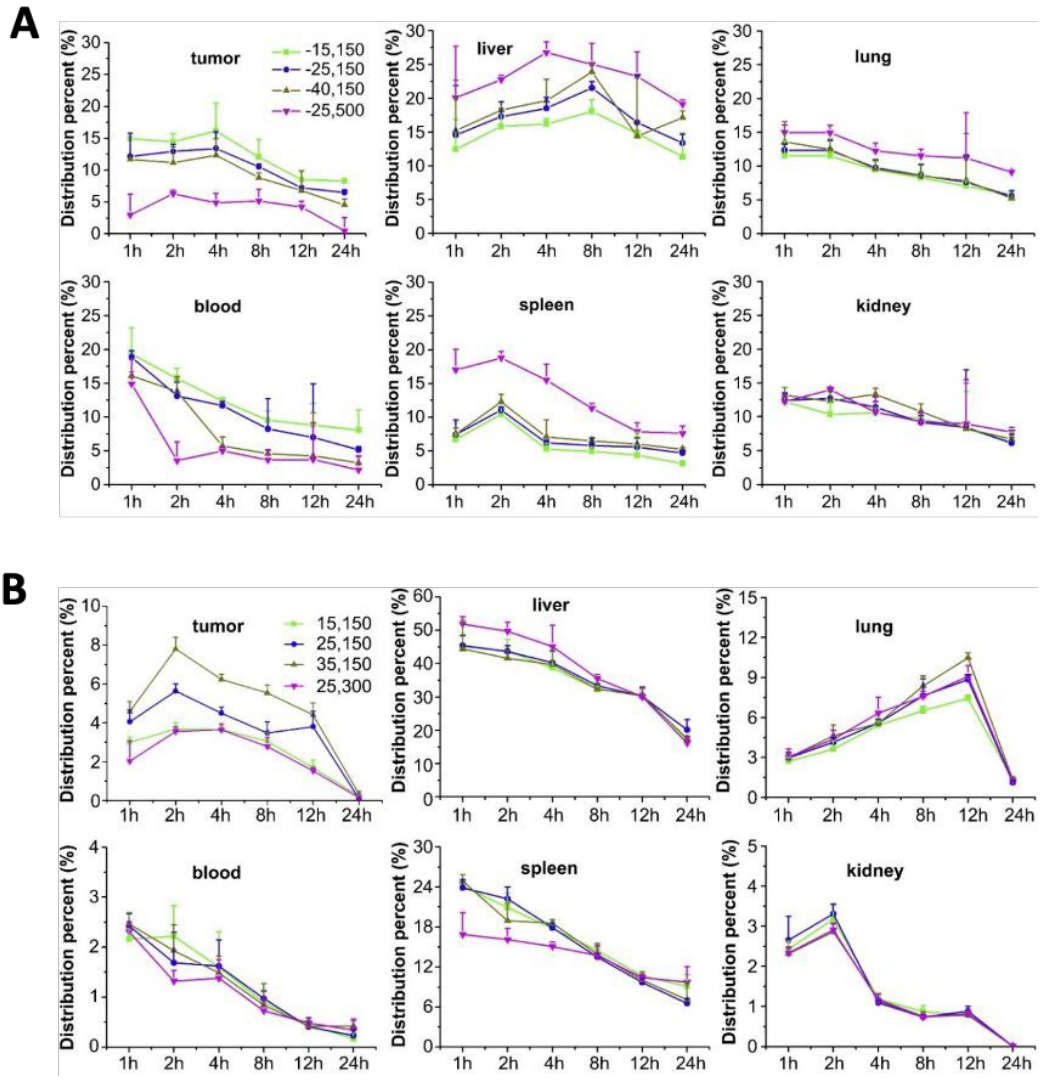

Figure 4. Biodistribution of chitosan-based nanoparticles displaying various size and surface charge (legend: zeta potential [mV], size [nm]) obtained from **(A)** carboxymethyl-chitosan and **(B)** chitosan hydrochloride, following intravenous administration to H-22 tumor-bearing mice (reproduced from [353] with permission from Elsevier, copyright 2010).

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The biodistribution of chitosan glycol-based nanoparticles with different anti-cancer agents (cisplatin [44], doxorubicin [48], docetaxel [46] has also been described in the literature. Again, an accumulation of nanoparticles in the tumor was observed as well as small amounts in the organs, decreasing over time. Anti-cancer drugs in nanoparticles exhibited lower toxicity and maintained or improved anti-cancer activity depending on the active substance. The impact of the physicochemical properties of nanoparticles of chitosan glycol-based nanoparticles has been investigated in terms of molar mass of the polymer (20-250 kg mol^{-1}), in mice carrying subcutaneous tumors (SCC7). Regardless of the molar mass, fluorescence was detected in liver, lung, kidneys, spleen, and heart, and gradually decreasing over time. All types of nanoparticles showed an accumulation at the tumor level dependent on the molar mass of chitosan glycol-cholic acid. With 20 kg mol^{-1} and 100 kg mol^{-1} chitosan-glycol, tumor accumulation was low and decreased after 6 h, while in the case of 250 kg mol^{-1}, higher and prolonged tumor fluorescence were recorded. The nanoparticles of lower molar masses were eliminated more quickly. According to Park et al., this difference is linked to the difference in the stability of nanoparticles in the bloodstream. However, this parameter has not been extensively studied [354].

4.2 Intra-peritoneal (IP) route

The group of Machida et al. [345] compared the biodistribution of nanoparticles of N-succinyl-chitosan labeled with FITC and conjugated with mitomycin C, injected by IV or IP routes in mice bearing subcutaneous tumors (Sarcoma 180). Depending on the route of administration, a difference in kinetics was observed whereas the biodistribution was kept unchanged. The nanoparticles have accumulated preferentially at the level of the tumor, and few have been found

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in the organs (kidney, spleen, liver, lungs). The amounts determined in the blood and the tumor were higher after IV administration: 15% of the dose reached the tumor after 24 h while after IP administration, the nanoparticles remained close to the point of injection, causing potential toxicity. The distribution and accumulation at the tumor level were similar to those obtained with N-succinyl-chitosan in solution with varying degrees of substitution [127].

4.3 Subcutaneous route

Lu et al. described chitosan-based nanoparticles for the encapsulation of mitoxantrone, an anti-cancer drug indicated against breast cancer [355]. After subcutaneous injection, the nanoparticles showed a slow absorption from the injection site and better accumulation in the lymph nodes than the solution's active substance. These nanoparticles have also made it possible to reduce the toxicity of the anti-cancer drug [355].

The majority of biodistribution studies reported in the literature were carried out in mice bearing subcutaneous tumors, impacting the biodistribution of chitosan nanoparticles. These studies have shown an accumulation of nanoparticles in the tumor due to increased circulation time in the blood. However, the biodistribution studies carried out do not compare the chitosan-based nanosystems and the polymer in solution. In addition, the impact of colloidal stability and physicochemical properties of nanoparticles (size, surface charge, molar mass) remains to be fully understood.

5 Biodegradation

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Once in the form of a polymer in solution, chitosan is degraded by hydrolysis of the β(1-4) glycosidic bonds, chemically in the stomach by acid catalysis [356]. The DA of chitosan indeed plays an essential role in the degradation. Zhang et al. studied the degradation of chitosan by isolated β-glucosidase and enzymes from the cecum and rat colon, whose activity is comparable to that of the human colon. Chitosan having a molar mass of 600 kg mol^{-1} and a low DA (77%) showed a faster degradation rate, and a correlation of *in vitro* and *ex-vivo* results has also been demonstrated [357,358].

From study to study, degradation rates vary depending on the type of chitosan, enzymes, enzyme concentration, study time, and degradation conditions [359]. The availability of amine groups also impacts the rate of degradation. Indeed, chemical modifications of chitosan can limit the accessibility of amino groups for hydrolysis of β 1-4 glycosidic bonds. For example, thiolated chitosan with thioglycolic acid (CS-TGA) is between 12.9-24.7% less degraded than native chitosan. This reduction is correlated with the degree of grafting of thiol groups on the chitosan. When CS-TGA is crosslinked (presence of a disulfide bridge), the degradation of chitosan is also reduced [360].

Oppositely, porous microparticles formulated with different acetylated chitosan (DA of 10-50%) have shown an increased degradation rate depending on the DA [361]. This was also observed when the chitosan was in the form of matrix [362] and fibers. Yang et al. have shown in vitro and in vivo that acetylated chitosan fibers degrade more than chitosan fibers [363]. Numerous studies of the degradation of acetylated chitosan have been carried out in films or fibers, which have great prospects in tissue engineering [364].

The presence of a crosslinking agent also significantly impacts the degradation of chitosan by preventing the access of enzymes to the β(1-4) glycosidic bonds. McConnell et al.

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evaluated it with different chitosan films prepared in the presence or not of different concentrations of chemical or ionic crosslinking agent, glutaraldehyde, or TPP, respectively. Non-crosslinked films were degraded by pancreatic and colonic enzymes from human colonic bacteria and porcine pancreatic enzymes in less than 4h, while films crosslinked with glutaraldehyde resisted any type of degradation. In contrast, crosslinked films with TPP only resisted degradation by pancreatic enzymes. This difference is linked to the nature of the crosslinking: glutaraldehyde forms covalent bonds, while TPP forms ionic and more 'flexible' bonds between chitosan chains [365].

The rate of degradation of chitosan therefore depends on the molar mass, the DA, and chemical modifications. So far, the *in vivo* degradation pathways of chitosan and its derivatives are not fully determined. Despite this, the modulation of the biodegradation of chitosan has many interests, more precisely in the field of the delivery of active substances for a controlled and/or prolonged release.

6 Toxicology of chitosan

6.1 *In vitro* toxicity

Many studies have investigated the effect of chitosan on cell viability, and its modulation by the polymer physicochemical properties and chemical modifications (**Table 3**). In general, it is difficult to conclude simply on the toxicity of chitosan. Not all cytotoxicity studies presented are comparable, each using different cell lines and incubation times. However, compared to cationic reference polymers such as PEI (IC_{50} <0.03 mg mL^{-1} [366]) or poly-L-lysine. Chitosan and its derivatives are not very toxic with an IC_{50} varying from 0.2 to 2 mg mL^{-1} in most cell models [302], with cytotoxicity depending on concentration and incubation time [266,367].

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The impact of molar mass and degree of substitution (deacetylation, trimethylation, PEGylation) must also be considered. According to Thanou et al., there is a threshold at which the molecule and the components of the cell have enough contact to have significant toxicity. This threshold would be between 40 and 60% degree of substitution. In terms of molar mass, chitosan with low molar masses (<10 kg mol^{-1}) would not exhibit significant toxicity [356], while the toxicity may increases with higher molar masses [266,366]. In particular, a study carried out on chitosan of 3 to 100 kg mol^{-1} exhibiting different degrees of trimethylation (DTM) showed that the toxicity of the polymer increases with trimethylation regardless of its molar mass and more significantly with polymers of higher molar masses. For an identical DTMs, the chitosan with the highest molar masses also showed the highest toxicities [366]. Mao et al. compared trimethylated (TMC) and PEGylated chitosan. The copolymers studied were synthesized with different molar masses and a DTM set at 40%. The cytotoxicity of TMC (400 kg mol^{-1}) was significantly decreased with an increasing degree of PEGylation (DP). The cytotoxicity is also dependent on the molar masses of the constituents of the copolymer TMC-PEG. For a fixed degree of PEGylation (DP = 6%), the 100 kg mol^{-1} TMC and 50 kg mol^{-1} TMC pegylated with PEG (5 kg mol^{-1}) showed 10 times less toxicity (IC_{50} > 0.5 mg mL^{-1}) than the TMC 400 kg mol^{-1}-PEG (5 kg mol^{-1}) (IC50 = 0.04 mg mL^{-1}). Conversely, for a molar mass of fixed TMC, lower cytotoxicity was obtained with PEG of higher molar mass (5 kg mol^{-1}) compared to PEG (550 g mol^{-1}), despite a higher degree of PEGylation: 36.7% for PEG (550 g mol^{-1}) against 6.4% for PEG (5 kg mol^{-1}). PEG grafting makes it possible to reduce the cationic surface charge of chitosan, leading to a reduction in interactions with cells and, therefore, in its cytotoxicity [266].

Table 3. Cytotoxicity of chitosan and its derivatives, measured by MTT test. Adapted from [356].

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Chitosan (DD, M_w)	IC_{50} (cells, incubation time)	Reference
Trimethylated chitosan, PEGylated	L929 cells, 24h	
84.7% DD, 40% DTM		
400 kg mol^{-1} PEG 5000 g mol^{-1}		
0% DP	0.015 mg mL^{-1}	[266]
12% DP	0.04 mg mL^{-1}	
25.7% DP	> 0.5 mg mL^{-1}	
27.4% DP	> 0.5 mg mL^{-1}	
Chitosan salts	Caco-2 cells, 2h	
87% DD, 20-460 kg mol^{-1}		
Aspartate	0.67-0.72 mg mL^{-1}	[367]
Glutamate	0.35 – 0.46 mg mL^{-1}	
Lactate	0.31 – 0.38 mg mL^{-1}	
Hydrochloride	0.22 – 0.27 mg mL^{-1}	
Chitosan lactate	B16F10 cells, 72h	
78% DD, < 50 kg mol^{-1}	2.50 mg mL^{-1}	[368]
82% DD, 150-170 kg mol^{-1}	2.00 ± 0.18 mg mL^{-1}	
Chitosan glutamate		
> 80% DD, 60-90 kg mol^{-1}	2.47 ± 0.14 mg mL^{-1}	
77% DD, 180-230 kg mol^{-1}	1.73 ± 1.39 mg mL^{-1}	
Chitosan hydrochloride	B16F10 cells, 72h	
85% DD, 60-90 kg mol^{-1}	2.24 ± 0.16 mg mL^{-1}	
81% DD, 100-130 kg mol^{-1}	0.21 ± 0.04 mg mL^{-1}	[368]
Chitosan glycol		
100% DD, 152 kg mol^{-1}	2.47 ± 0.15 mg mL^{-1}	
Trimethylated chitosan	MCF-7 cells, 24h	
3-6 kg mol^{-1} (oligomer)		
20% DTM	> 10 mg mL^{-1}	
44% DTM	> 10 mg mL^{-1}	
55% DTM	5.959 mg mL^{-1}	
94% DTM	0.417 mg mL^{-1}	[366]
100 kg mol^{-1}		
36% DTM	0.285 ± 0.1 mg mL^{-1}	
57% DTM	0.265 ± 0.05 mg mL^{-1}	
76% DTM	0.059 ± 0.03 mg mL^{-1}	
93% DTM	0.118 ± 0.28 mg mL^{-11}	

DD: degree of deacetylation; DP: degree of PEGylation, DTM: degree of trimethylation

All the *in vitro* studies presented show that the toxicity of chitosan is directly related to its charge density. Chemical modifications that do not induce an increase in charges, such as PEGylation, may reduce the toxicity of chitosan. Counterions present in the chitosan salts may increase the cationic charge of the chitosan and cause an increase in toxicity. Opanasopit et al. showed that the cytotoxicity of chitosan varied according to the type of chitosan salts

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(hydrochloride > lactate > glutamate > aspartate), with hydrochloride chitosan as the most toxic with an IC_{50} of 0.22-0.27 mg mL^{-1} [367].

A few more studies have focused on the impact of the physical-chemical properties of nano-objects (size, zeta potential, composition) based on chitosan on the cytotoxicity, knowing that such nano-formulations have already demonstrated their significant role in the cellular capture [353]. Huang et al. showed that chitosan and chitosan nanoparticles crosslinked with TPP exhibited comparable cytotoxicity with similar IC_{50} values. However, the cytotoxicity of chitosan and nanoparticles was reduced with the polymer DD decrease, from 88% to 61% [369].

Qi et al. have studied the impact of the surface charge and the size of CS/TPP nanoparticles, loaded or not with Cu (II). CS/TPP nanoparticles (40 nm, +51 mV) and CS/TPP/Cu nanoparticles (257 nm, +96 mV) showed more significant toxicity than that of chitosan in solution with respectively an IC_{50} of 15 and 6 µg mL^{-1}. The increased surface charge of nanoparticles increased electrostatic interactions with negatively charged membrane components of cells resulting in increased cytotoxicity, which was also impacted by the size of the nanoparticles. A variation in cytotoxicity was also observed depending on the cell line studied. The nanoparticles showed high cytotoxicity towards tumor cells and lower towards hepatic cells [370] (**Fig. 5D,E**).

Nasti et al. also studied the cytotoxicity profiles of CS/TPP nanoparticles on macrophages and fibroblasts. Two types of nanoparticles were analyzed in terms of size: nanoparticles of about 240 nm, coated or not of hyaluronic acid (HA), and nanoparticles around 360 nm. The HA coating allows the surface charge of the smaller nanoparticles to be changed, however, the surface charge value of CS/TPP/HA nanoparticles has not been reported [371]. Higher nanoparticle cytotoxicity was obtained for all types of nanoparticles on macrophages compared to fibroblasts (IC_{50} > 2 mg mL^{-1}). At the level of macrophages, a difference in cytotoxicity was also observed depending on

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the surface charge of the nanoparticles, with CS/TPP/HA nanoparticles showing less cytotoxicity compared to CS/TPP nanoparticles. However, depending on the size of the nanoparticles, the variation in cytotoxicity observed may not be statistically significant. This study demonstrated that the observed cytotoxicity is mainly linked to the internalization of nanoparticles, directly related to the surface charge of the nanoparticles [371].

Other studies have also shown the impact of the size and surface charge of nanoparticles on different cell lines: L929 cells [266] (**Fig. 5B,C**), COS-7 and MCF-7 cells [366], and hematopoietic stem cells [372]. All the studies show that the cytotoxicity of chitosan nanoparticles is directly related to the surface charge density resulting from amine groups, to the size of the nanoparticles and to the three-dimensional arrangement of the polymer. Depending on the conformation and the cationic charge of the nanoparticles, a decrease, or an increase in interactions with the anionic components of the glycoproteins present at the level of the cell membranes can be observed, impacting the cytotoxicity of the chitosan-based nanoparticles.

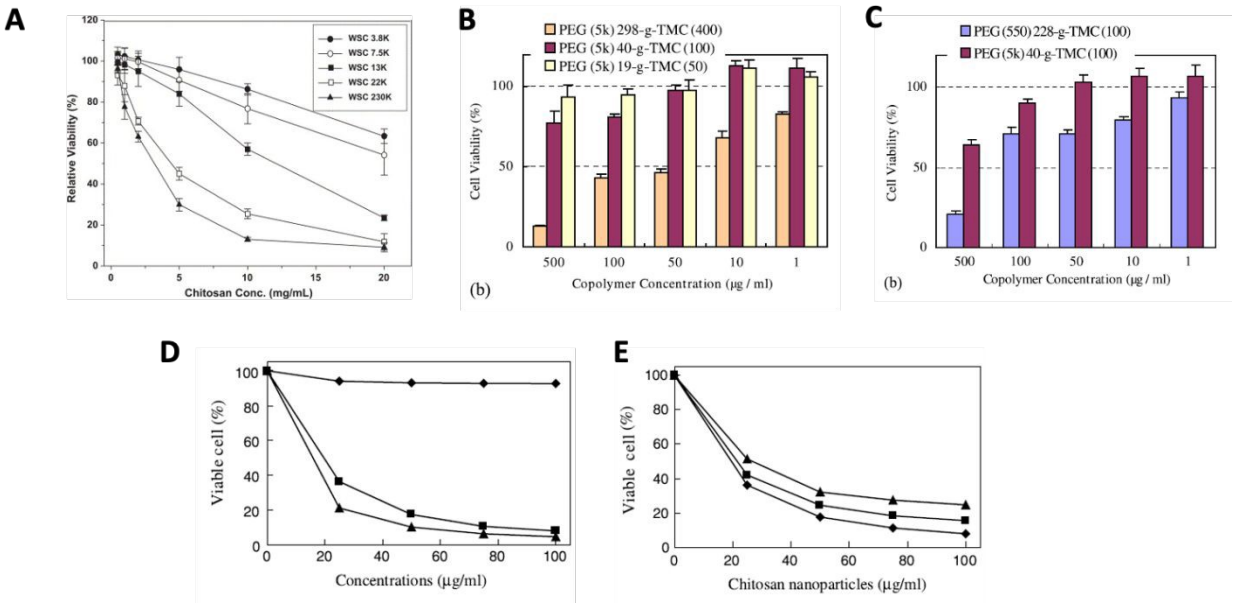

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Figure 5. Impact of chitosan chemistry and supramolecular organization on *in vitro* toxicity. **(A)** Cytotoxicity of soluble chitosan of various M_w: ● 3,8 kg mol^{-1}; ○ 7,5 kg mol^{-1}; ■ 13 kg mol^{-1}; □ 22 kg $mol^{-1}$and ▲ 230 kg mol^{-1} (reproduced from [373] with permission from Elsevier, copyright 2005). **(B, C)** Cytotoxicity of trimethyl chitosan-poly(ethylene glycol) (TMC-PEG) conjugates displaying various M_w of the TMC and PEG segments (reproduced from [266] with permission from Elsevier, copyright 2005). **(D)** Cytotoxicity of ◆ free CS, ■ CS/TPP nanoparticles and ▲CS/TPP/Cu nanoparticles, and **(E)** influence of CS/TPP nanoparticle size: ▲ 100 nm, ■ 70 nm and ◆ 40 nm (reproduced from [370] with permission from Elsevier, 2005).

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6.2 *In vivo* toxicity

6.2.1 Parenteral route

Depending on its route of administration, chitosan does not present the same toxicity profile (**Table 4**). Intravenously, through the interaction of its cationic groups with plasma proteins or blood cells, chitosan can cause activation of complement [374,375].

Table 4. *In vivo* toxicity of chitosan and its derivatives after parenteral and oral administration. Adapted from [376].

Chitosan (DD, M_w)	Route	Study	Observations	Reference
Chitosan oligosaccharides 304 – 1.162 kg mol^{-1} Chitosan LMW 3 kg mol^{-1}	Parenteral route	Rabbit, IV injection 7.1-8.6 mg/kg during 5 days	Increased lysozyme activity after two or more injections	[377] [378]
Chitosan 80% DD, 30-40 kg mol^{-1}	Parenteral route	Mouse, IP or SC injection 1-5 mg Every 15 days for 84 days	Clinical signs and cellular abnormalities observed only after IP administration	[379]
N-succinyl chitosan (Suc-CS) nanoparticles Mitomycin C (MMC) 72% DS, 300 kg mol^{-1}	Parenteral route	Mouse, IP injection	Suc-CS: LD_{50} = 2000 mg/kg Suc-CS/MMC: LD_{50} = 25 mg/kg No clinical sign	[345]
N-octyl- nanoparticles Chitosan O-sulfate Paclitaxel	Parenteral route	Mouse, IV injection Rats, IV injection Rabbit, IV injection 6 mg/kg/day for 3 days Rabbit, IV injection 2mg/kg	92% DD, 65 kg mol^{-1} IV: LD_{50} = 72.16 mg/kg PI: LD_{50} = 81.28 mg/kg 97% DD, 65 kg mol^{-1} IV: LD_{50} = 102.59 mg/kg PI: LD_{50} = 130.53 mg/kg No pathological changes observed No hypersensitivity	[42,380]
Chitosan	Oral route	Rabbits, hens	No pathological changes	[378]

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3 kg mol^{-1}		700-800 mg/kg for 239 days		
Chitosan 80% DD, 30-40 kg mol^{-1}	Oral route	Mouse Food containing between 0.5-5% chitosan during 28 days	Weight loss and reduction of bacteria in the intestinal flora at the 5% dose	[379]
Chitosan oligomer 85% DD, 1.86 kg mol^{-1}	Oral route	Mouse 1000-10,000mg/kg Rats 750-3000 mg/kg for 30 days	LD_{50} > 10,000mg/kg No mutagenicity No clinical or pathological signs LD_{50} > 3000mg/kg No clinical, hematological or pathological signs	[381]
Chitosan n/a	Oral route	Rats 0-2545 mg/kg for 364 days 0-2545 mg/kg for 728 days	LD_{50} > 2323-2545mg/kg No toxic effects (clinical, hematological, biochemical, pathological) No carcinogenicity	[382]
Chitosan/TPP nanoparticles 85% DD, 80 kg mol^{-1}	Oral route	Mouse 100 mg/kg for 14 days	No clinical signs (diarrhea, fever, weight loss, etc.), pathological and inflammatory Non-significant hematological and biochemical abnormalities	[383]

DD: degree of deacetylation; Mw: molar mass; LMW: low molecular weight IV: intravenous; IP: intraperitoneal; LD_{50}: median lethal dose; DS: degree of N-succinylation

As for the toxicity of chitosan in the form of nano-objects, Hu et al. studied it with chitosan nanoparticles crosslinked with TPP in a zebrafish embryo model, commonly used for genotoxicity studies. High concentrations of nanoparticles (20-40 mg mL^{-1}) with a size of 200 or 340 nm were tested. A decrease in hatching and an increase in concentration-dependent mortality have been demonstrated. However, the malformations were only observed in embryos treated with the 200 nm nanoparticles, showing an impact on the size of the nanoparticles. At the cellular level, nanoparticles have caused physiological stress. These toxic effects were observed only for high

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concentrations of nanoparticles. Embryos exposed to lower concentrations (5 mg mL^{-1}) exhibited oxidative cellular stress but no tissue toxicity [384].

N-octyl-O-sulfate chitosan micelles encapsulating paclitaxel did not induce anaphylactic reactions, histopathological or hemolytic effects afterw intravenous injection with 6 mg kg^{-1} day in mice. These micelles have also made it possible to reduce the toxicity of the active molecule: the median lethal doses (LD_{50}) after intravenous or intraperitoneal administration were between 1.14 and 1.52 times higher than that of paclitaxel in the free form [42].

Overall, the use of chitosan-based nanosystems may be more challenging by parenteral route than oral or other local ones. In particular, subcutaneous administration of chitosan-based nanoparticles [385] faces less risks compared to the intravenous one, for which a lack of sufficient hemocompatibility and the formation of deadly emboli, despite the safe administration in several animal models, has been raised as a concern [31].

6.2.2 Oral route

Orally, chitosan is mainly used as an absorption promoter in the gastrointestinal tract due to its mucoadhesion properties and its ability to modulate permeability of active molecules due to the reversible opening of tight junctions between epithelial cells [386], which facilitates the paracellular transport of hydrophilic macromolecules [105,387–390].

The *in vivo* toxicity profile of chitosan after oral administration has been established in different species (mice, rats, rabbits, chickens) (**Table 4**). Absorption through the intestinal barrier is dependent on the molar mass of chitosan, and it is increased as its molar mass decreases and its water solubility increases [391]. Therefore, the oral route is not a preferred route of administration for the design of chitosan-based nano-objects for targeting tissues other than the gastrointestinal tract. Overall, chitosan exhibits no significant toxicity or minimal toxic effects regardless of the

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route of administration, which encourages its use as biomaterials for the administration of active drug substances.

Many examples can be found in relation to permeability enhancement of drugs. Yin et al. developed micelles of N-octyl-O, N-carboxymethyl chitosan to investigate the role in enhancing oral absorbance of silybin [392]. When incorporated into modified chitosan micelles, the drug in plasma concentrations was improved and remained detectable for more time than in free solution. Krauland et al. treated non-diabetic rats with thiolated chitosan tablets containing insulin orally, and the decrease of blood glucose level was higher when the insulin was administrated in the tablets, compared to free insulin and insulin in the control tablets [393]. Sudhakar et al. observed the difference in blood glucose level in rats between the free insulin administrated subcutaneously and insulin encapsulated in thiolated chitosan nanoparticles administrated orally. The free insulin decreased the blood glucose level in 30 minutes, while the encapsulated insulin showed a prolonged reduction of blood glucose. The presence of insulin in the plasma was also modified by the nanoparticles. After 1h injection of free insulin was reached the maximum, and the insulin-loaded nanoparticles, this maximum was observed at 2-3h after administration [394].

However, each *in vivo* toxicity study uses its own experimental conditions; standardization of procedures should be considered (Cf. section 7.). In addition, most toxicity studies described in the literature aim to show the effectiveness of the nanoparticles developed. This lack of toxicological knowledge is also due to the system himself. Chitosan forms nano-objects in the presence of other components such as a chemical or ionic crosslinking agent or an active substance, which may have their own toxicity.

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7 Conclusions and future perspectives

Nanoparticles composed of chitosan become an excellent option to compose biodegradable, biocompatible and versatile drug delivery systems with an overall low toxicity. Restrictions in chitosan solubility draws attention and chitosan derivatives were developed to become an excellent alternative to overcome this issue. The modification of chitosan may lead to a more suitable and less toxic delivery systems, improving the chitosan properties. In addition, numerous nanoscale architectures have been described, with various levels of complexity, drug encapsulation abilities and targeting properties. The application of chitosan derivatives in industry could be a promising perspective in the use of polymers in different fields, as food, agriculture and especially in health care.

Several challenges remain to be address for a full development of chitosan-based nanomedicine. As highlighted above, the variety of chitosan biopolymers used in the literature spans a wide range of molar masses, DAs, sources, etc., which complicates comparisons and consolidation of the published data (with occasional inconsistencies in the data and a limited understanding of the underlying mechanisms, as highlighted by some authors [30]). The translation from laboratory studies to industrial scale also faces several significant challenges, among which the scale-up and economic feasibility of chemical modifications and processes of nano-formulation in GMP conditions [29], in addition to the cost of the chitosan raw material depending on its source [395], and to the need to find more environmentally friendly extraction methods [30]. There is also an important need for standardization, as already identified in the nanomedicine field, in particular in the development of standard test methods and reference materials, and unified approaches among the community [396], as addressed e.g. by several European initiatives [397].

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Despite these hurdles, it should be noted that several commercial products have received approval for their use as wound dressings, hemostatic sealants and even as nerve conduit [28,30]. The scale up of chitosan-based nanoparticle fabrication has also been investigated to identify relevant formulation parameters [398]. Finally, another specificity of chitosan lies in its immunomodulatory effects [399]. It has shown potential immune-triggering capacity and potential to induce both cellular and humoral immunity [400–403], which holds promise for application as adjuvant in vaccination, but should be taken into account for other drug delivery purposes.

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Data availability statement

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.