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

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1. Introduction

Chitosan (CS) is a pseudo-natural polysaccharide, obtained *via* the deacetylation of chitin in an alkaline medium, resulting in a random arrangement of β-D-glucosamine (GlcN) and *N*-acetyl-β-D-glucosamine (GlcNAc) units linked by (1-4) glycosidic bonds (Fig. 1). Chitosan is one of the few cationic polysaccharides known to date, possessing 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, is crucial for its biomedical applications.[11] In particular, chitosan is characterized by the mole fraction of its *N*-acetyl groups, called degree of acetylation (DA), or by the mole fraction of its *N*-acetyl groups removed from the chitin macromolecule during deacetylation, which is called the 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 an acidic medium, whose state of ionization is described by the equilibrium given in eqn (1), with a pK_a value generally in the range of 6–6.5.[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] (eqn (2)):

$$\text{CS-NH}_2\ (\text{aq}) + \text{H}_3\text{O}^+\ (\text{aq}) \rightleftarrows \text{CS-NH}_3^+\ (\text{aq}) + \text{H}_2\text{O}\ (\text{l}), \quad (1)$$

$$pK_a = \text{pH} + \log\left(\frac{(1-\alpha)}{\alpha}\right) = pK_0 - \frac{\varepsilon \Delta\Psi(\alpha)}{kT}, \quad (2)$$

where pK_0 is the intrinsic pK_a of an isolated and non-protonated amine function, ε is the dielectric constant of the medium, $\Delta\Psi(\alpha)$ is the potential difference between an ion placed on the surface of the polyelectrolyte and at an infinite distance, k is the Boltzmann constant, and T is 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%–89.0%) and from a chitosan colloidal solution concentration and ionic strength, it is possible to obtain a polynomial equation to deduce pK_a based only on the DA and medium ionic strength.[18]

Chitosan is a weak base and 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

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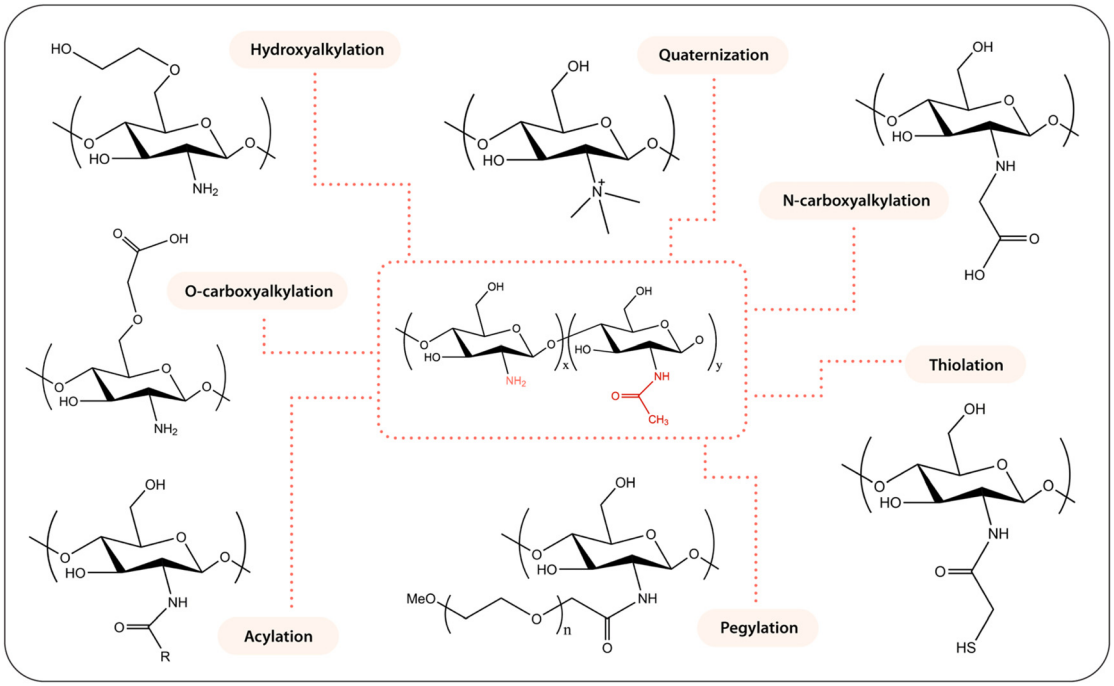

Fig. 1 Overview of the main chemical modifications in the structure of chitosan described for its use in drug delivery, as reviewed below.

in aqueous acetic acid solution (0.1 mol L^{-1} or 1% w v^{-1})[22] or strong acid solutions such as hydrochloric acid.[23] The solubility of chitosan is related at the same time to its DA, ionic strength, pH, the nature of the acid used and 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 group protonation on the solubility of chitosan in the presence of a weak acid (acetic acid, AcOH) or strong acid (HCl) at different acid concentrations. Independent of the polymer concentration ([N] = CS-NH_2 + CS-NH_3^+) or the type of acid, the complete solubilization of chitosan was obtained for $\alpha \geq 0.5$, corresponding to a stoichiometric ratio [AcOH]/[CS-NH_2] = 0.6 or [HCl]/[CS-NH_2] = 0.5–0.6. The ion concentration required for complete solubilization of chitosan is proportional to its number of amine groups (CS-NH_2).[22,23] The solubility of chitosan is an obstacle that needs to be circumvented to modulate or give it new physicochemical properties, especially for the encapsulation of active substances. Chemical modification of chitosan has been carried out at its deacetylated units, on its C6 hydroxyl group, and/or its 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 that can encapsulate drug molecules to improve their delivery and targeting. This review provides an overview of the most important chemical modification 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 its chemical modification,[28] formulation on a larger scale[29] and other biomedical applications (wound dressings, dental materials, *etc.*).[30–32]

2. Chemical modification of chitosan

Chitosan has been modified in various ways to introduce numerous hydrophilic or hydrophobic moieties, owing to the reactivity of its 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).

2.1. Alkylation of chitosan

2.1.1. Quaternization. Alkylation was achieved by bonding carbon chains to the amino or hydroxyl groups of chitosan. Rinaudo's group[89,90] described the quaternization of chitosan *via* the alkylation of its amine groups with methyl iodide under alkaline conditions (NaOH). The iodide ion was subsequently replaced by a chloride ion using an ion exchange

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

Chitosan derivative	Encapsulated drug	Ref.
Trimethyl chitosan	Camptothecin	
	Candesartan-cilexetil	33–35
	Insulin	36
	Resveratrol	37
	Vancomycin	38
	Vitamins	39
N-Octyl-*O*-sulfate chitosan	Docetaxel	40
	Paclitaxel	41 and 42
Glycol-chitosan	Camptothecin	43
	Cisplatin	44
	Dexamethasone	45
	Docetaxel	46
	Doxorubicin	47 and 48
	Paclitaxel	49
N-Carboxymethyl chitosan	Idebenone	50
	Ofloxacin	51
O-Carboxymethyl chitosan	Camptothecin	52
	Curcumin	53
	Gatifloxacin	54
	Metformin	55
	Methotrexate	56
	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 and 86
	Tamoxifen	87
Quaternized chitosan	Ketoconazole	88

process to obtain a more stable chitosan salt, *N*-trimethyl chitosan chloride (TMC). This quaternization allows the introduction of permanent positive charges, enabling the polymer to be a cationic polyelectrolyte regardless of the pH. Its solubility directly depends on its degree of methylation.[91] Pardeshi *et al.* synthesized a TMC using the same technique and evaluated its mucoadhesive strength and bioadhesive potential. 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 chitosan with different degrees of quaternization for the 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 with the more efficient interaction of TMC with the anionic components of the cell membrane, potentiating the absorption at different values of pH. TMC was also applied as a drug delivery agent 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 it with 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 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 in the case of *N*-dodecyl derivatives, the hydrophobic 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 degrees of substitution with 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 compared to chitosan grafted with a low degree of substitution.[100] Liu *et al.* demonstrated an increase in the transfection efficiency of plasmids mediated by the *N*-alkylation of chitosan depending on the number of carbons in the alkyl side chains, which is directly related to the hydrophobicity of *N*-alkyl chitosan.[101]

2.1.3. Hydroxyalkylation. The hydroxyalkylation of chitosan was achieved *via* the 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 branched chitosan glycol with a quaternary ammonium cation that contains two quaternary quinolinium units linked by an *N*-decylene chain, which can form micelles exhibiting a low toxicity towards HeLa and HDF cells.[103] Glycol chitosan was also studied by Yu *et al.*, where dexamethasone-loaded glycol chitosan nanoparticles showed an increase in the precorneal duration of action due to their ionic interactions with the surface of the cornea 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 using different ratios of monochloroacetic acid in isopropanol. The samples were tested to evaluate their 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. *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]

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 its C2 and/or C6 position *via* an ester bond by reaction with chlorides or acyl anhydrates. The hydrophobic groups are generally fatty acid (C6–C18) chains such as oleic acid,[59] linoleic acid,[119] lauric acid,[120] palmitoyl acid,[121] stearic acid,[64] and 5β-cholic acid,[122,123] which increase the hydrophobic nature of chitosan, and therefore allow the polymer, under certain conditions, to self-assemble into nano-objects in 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 chitosan has also been developed, which is soluble in both 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 was used to formulate nanoparticles by self-assembly to encapsulate vitamin C, with the drug loading efficiency increasing from C3 to C12. With longer *N*-acyl side chains, the resistance in acidic media also becomes stronger, leading to slower drug release, which is attributed to the stronger hydrophobic interaction within *N*-acyl chitosan-based nanoparticles.[131] Echazú *et al.* modified chitosan with dodecenylsuccinic anhydride to develop hydrogels for the buccal delivery of thymol. Rheological measurements showed that the elastic behaviour was predominant, and 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 chitosans were synthesized by modifying the amine group with 2-iminothiolane[136,137] or glutathione.[138] Thiolated chitosan conjugates have *in situ* gelation properties, given that the thiol groups oxidize between pH = 5 and 6.8, forming inter- and intramolecular disulfide bonds.[136] The gelling behavior of thiolated chitosan depends on the polymer chain 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 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), acceler-

ating the reduction of GSH. Furthermore, the absorptive apparent permeability across the rat intestine was directly dependent on the pK_a of the ligands and the amount of thiol group conjugates on chitosan, impacting the P-gp inhibitory properties of the derivative.[142] Usually, thiolated chitosan nanoparticles were prepared by the 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 modification of chitosan described above, the functionalization of chitosan by other polymers or by active substances has 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 its 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) 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] and 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, the 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 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 the storage and loss moduli, 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 the corresponding nanoparticles with a low particle size presenting better activity in the DPPH test than that with a larger particle size. Furthermore, low M_w PEGylated chitosan was able to protect the liver against damage.[166]

PEGylated chitosan nanostructures were developed to reduce toxicity and enhance drug delivery (see section 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^{-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 intracellular transport of the treatment 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 4 h after administration to mice and cleared after 16 h, allowing a measurable biodistribution in the cerebral cortex, which disappeared before 16 h.[168] PEGylated chitosan nanostructures were also applied for 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 section 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, 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 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 such as 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 its oxygen and nitrogen atoms.[196] Reynaud *et al.* 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 to stabilize nanoobjects based on chitosan and polyphosphate molecules (see section 3.2.1).[200]

2.4.5 Targeting ligands. Chitosan can also be functionalized with a variety of ligands to enhance the 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 the formation of chitosan nanoparticles. The different types of ligands studied as well as their targets of interest are summarized in Table 2.

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 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 mannopyranosyl phenyl-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 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 for the post-formation conjugation of transferrin to chitosan nanoparticles. The first involved introducing aldehyde groups by periodate oxidation, and thus the modified transferrin can react with the amine groups on the surface of the nanoparticles. This method minimizes steric hindrance and loss of protein activity. The second method is based on reversible conjugation, where transferrin is 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*, the nanoparticles conjugated with transferrin did not show an enhancement in DNA transfection. Therefore, Mao *et al.* 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 the 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 and 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] In the case of 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

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	Targeting cancer cells *e.g.*, HEK293 and HeLa cells. Increased transfection and transcytosis	Doxorubicin,[214] methotrexate,[215] DNA[216,217]
KNOB		
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,[236,240] Paclitaxel,[237] Atorvastatin,[238] Lamivudine[239]
Urocanic acid	Proton sponge enhancing cytoplasmic release	DNA,[241,242] siRNA,[243] p53 gene[244]
Active substances		Doxorubicin, mitomycin C, paclitaxel

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

hepatocytes.[261] 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 quantity of 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, 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, which is stable in physiological medium but cleavable at the site of action by hydrolysis or enzymatic degradation. For example, a 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.* 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] The 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 a glycol chitosan derivative stimulates cell proliferation, metabolism, and differentiation due to the 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 has been widely used to formulate nano-objects encapsulating active substances for multiple routes of administration. These nano-objects can be obtained in different ways including chemical crosslinking, ionic crosslinking (ionic gelation or polyelectrolyte complexes), and even self-assembly (Fig. 2).

3.1 Formation of nano-objects by chemical crosslinking

The first chitosan nanoparticles described were obtained by chemical crosslinking (Fig. 2A). The most common crosslinking agent used is glutaraldehyde, which binds covalently by its aldehyde groups to the primary amine groups of chito-

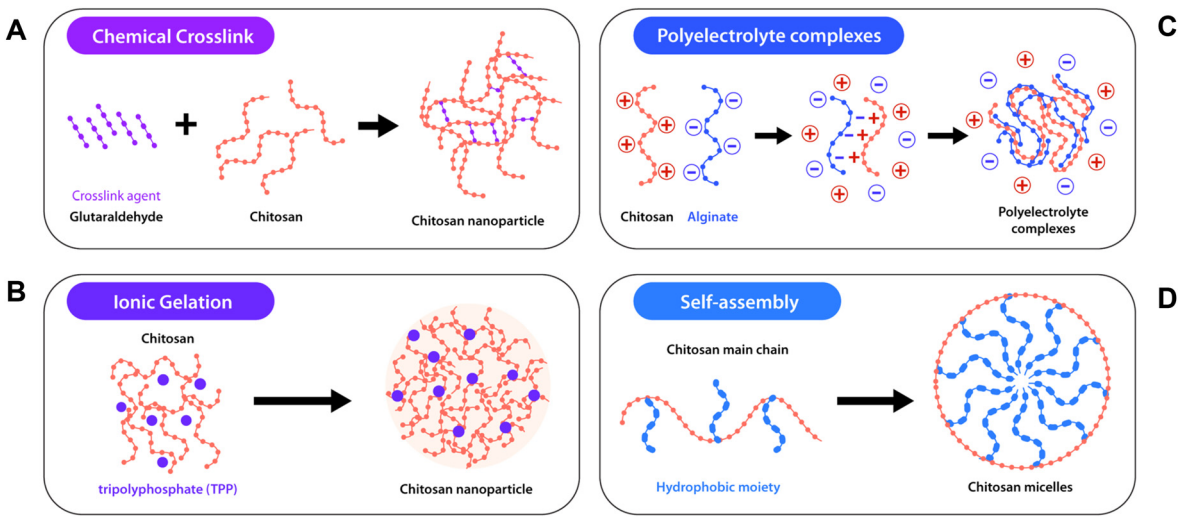

Fig. 2 Main strategies for the formulation of nano-objects based on native or modified chitosan chains: (A) chemical crosslinking by formation of covalent bounds between chitosan chains; (B) ionic gelation by formation of ionic bounds between chitosan chains and small polyions such as TPP; (C) formation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

san and, in some cases, to the primary amine groups of 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 its better stability (resistance to enzymatic degradation) and lower cytotoxicity than glutaraldehyde,[276,277] genipin has been used for the encapsulation of various active ingredients 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, and PEG-COOH).[281–283]

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

The nanoparticles obtained by chemical crosslinking exhibit good encapsulation of the active substance and good stability in 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 under 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).

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 a solution of sodium polyanion triphosphate (TPP), used as an ionic crosslinking agent, dropwise to a (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, and compactness), such as the concentration 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 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), a reduction in 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 not dissociate but precipitate because of the low solubility of chitosan. Therefore, the dissociation or aggregation of nanoparticles is correlated with the strength of the interactions between chitosan and TPP and dependent on the 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 the 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* the ionic gelation method for ocular delivery.[301] The particles formed using a low weight ratio of CS/TPP obtained optimum-sized particles with high encapsulation (82%) and a good drug loading capacity (7%), indicating 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 decreased (CS was 2.77-fold TPP), the ionic interaction between them was not sufficient. The nanoparticles containing tedizolid did not show symptoms of discomfort in the ocular irritation study in rabbits, and the transcorneal permeation of tedizolid by the particles resulted in a 1.6-fold increase in flux and the permeability coefficient, indicating their higher permeation compared to the free drug.

CS/TPP nanoparticles prepared *via* 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 the 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 the absence of TPP, resulting in a drug loading as high as 44% by weight.[298] Russo *et al.* also demonstrated this with foscarnet, a molecule also having a triphosphate group.[304]

3.2.2 Polyelectrolyte complexes (PEC). 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 therefore can interact with anionic polyelectrolytes to form complexes. Many of these complexes have reported with chitosan and various polyanions for the design of drug delivery systems including 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] heparin,[322,332–334] and 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, and (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 formed polyelectrolyte complexes. As the charge ratio moves away from neutrality, the nanoparticles formed are charged with excess polyelectrolyte. Alternatively, if the charge ratio is close to 1, uncharged particles are obtained, forming aggregates.[305] Their formation also depends on the reaction parameters including temperature, ionic strength, and pH. The colloidal stability of PECs heavily depends on their 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]

3.3 Formation of nano-objects by self-assembly

Native chitosan has difficulty in self-assembling 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 including epirubicin,[342] paclitaxel,[41,42,49,84,111,343] doxorubicin,[46–48,129,344] mitomycin C,[345] and camptothecin.[43,346]

3.4 Formation of nano-objects by polymerization

Chitosan-based nanoparticles have also been obtained by grafting a polymerizable group onto chitosan, leading to the formation of polymer chains. Then, polymerization 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 polymeriz-

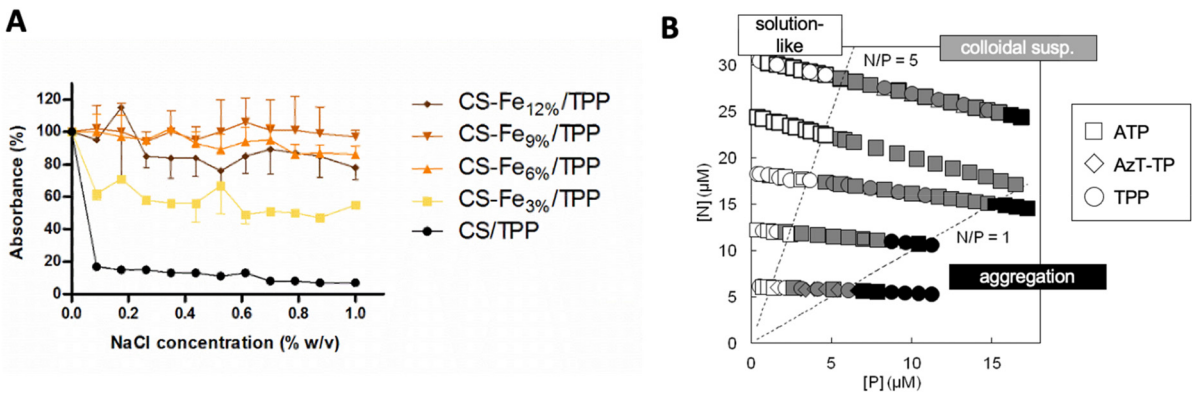

Fig. 3 Illustration of drug delivery challenges and opportunities associated with the ionic gelation of chitosan. (A) Dilution of CS/TPP nanogels in electrolytes may cause their rapid disassembly, as shown by turbidity measurements following nanogel dilution in a 0–150 mM (~0–0.9% w/v) NaCl medium. This effect can be reversed by the introduction of Fe^{3+} coordination complexes within the nanogels (reproduced from ref. 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 formulations, as shown in the case of the nucleotide adenosine triphosphate (ATP) and the nucleotide analogue azidothymidine triphosphate (AzT-TP) (reproduced from ref. 298 with permission from the American Chemical Society, copyright 2013).

ation 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 its numerous possible chemical modifications, is important for 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 to date.

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 the nano-formulation types based on them. The understanding of their *in vivo* fate after administration is not only 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 their biodistribution in H-22 tumor-bearing mice (Fig. 4). To do this, two types of chitosan conjugated with rhodamine, carboxymethyl-chitosan (RhB-CMC) and chitosan hydrochloride (RhB-CH), were associated with 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] Overall, this study demonstrates that the surface charge influences the biodistribution of chitosan-based nanoparticles, with negatively charged nanoparticles

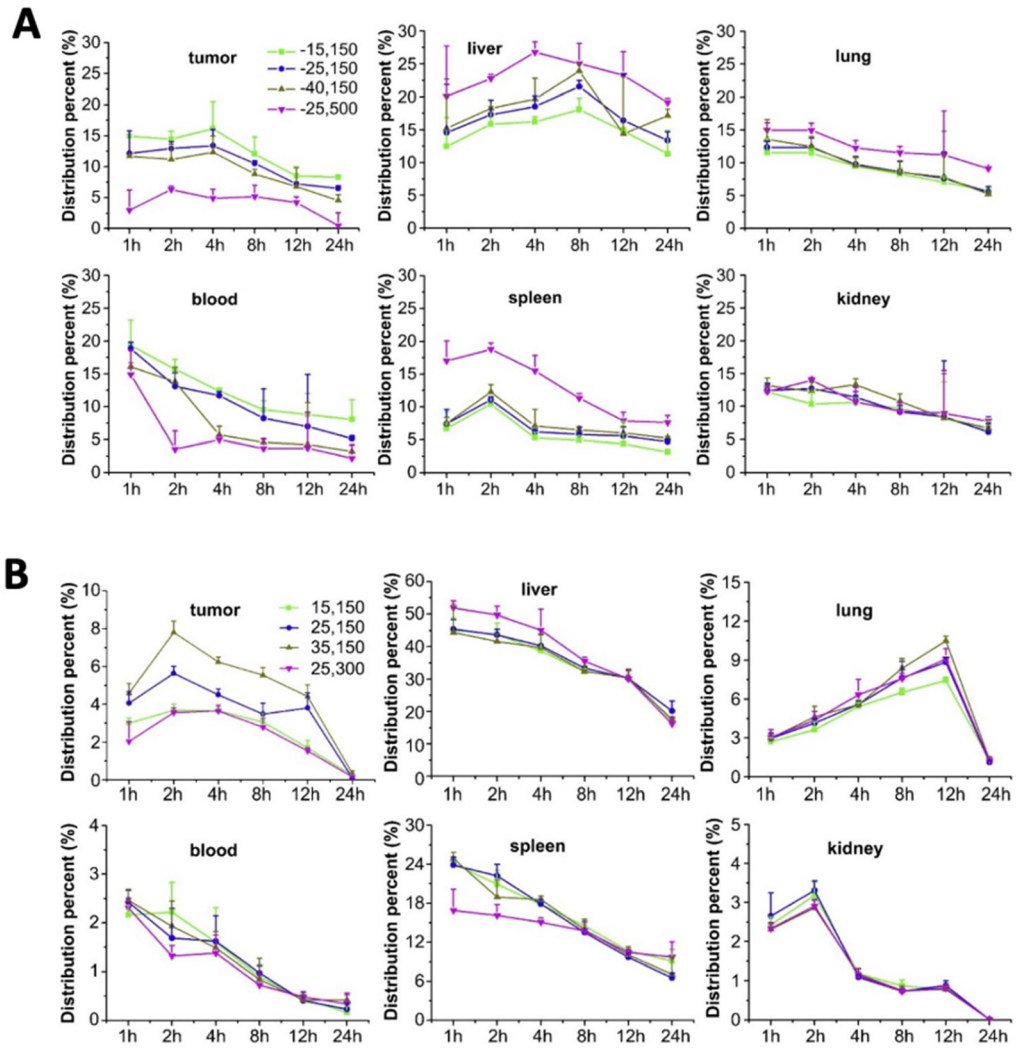

Fig. 4 Biodistribution of chitosan-based nanoparticles displaying various size and surface charge (legend: zeta potential [mV], size [nm]) obtained from (A) carboxymethyl-chitosan and (B) chitosan hydrochloride, following intravenous administration to H-22 tumor-bearing mice (reproduced from ref. 353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

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, where 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 their increased circulation time in the bloodstream. Higher surface charges and nanoparticle size caused a decrease in the circulation time in the blood and a higher accumulation in the liver and spleen. The renal elimination of nanoparticles was not found to be influenced by their 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 their load, probably resulting from the formation of aggregates in the pulmonary capillaries.

The biodistribution of chitosan glycol-based nanoparticles with different anti-cancer agents (cisplatin,[44] doxorubicin,[48] and docetaxel[46]) has also been described in the literature. Again, the 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 the liver, lung, kidneys, spleen, and heart, and gradually decreasing over time. All types of nanoparticles showed accumulation at the tumor level depending 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 with 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 remained unchanged. The nanoparticles accumulated preferentially at the level of the tumor, and few have been found in the organs (kidney, spleen, liver, and lungs). The amounts determined in the blood and the tumor were higher after IV administration, where 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 that 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 slow absorption from the injection site and better accumulation in the lymph nodes than a solution of the 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 the accumulation of nanoparticles in the tumor due to their 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, and molar mass) remains to be fully understood.

5 Biodegradation

Once in the form of a polymer in solution, chitosan is degraded by hydrolysis of 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 the correlation between the *in vitro* and *ex vivo* results has also been demonstrated.[357,358]

From study to study, the 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, the chemical modification of chitosan can limit the accessibility of its amino groups for the 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 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 their 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 on 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 its β(1-4)-glycosidic bonds. McConnell *et al.* evaluated this with different chitosan films prepared in the presence or absence of different concentrations of chemical or ionic crosslinking agent, glutaraldehyde, or TPP, respectively. The non-crosslinked films were degraded by pancreatic and colonic enzymes from human colonic bacteria and porcine pancreatic enzymes in less than 4 h, while the films crosslinked with glutaraldehyde resisted any type of degradation. In contrast, the films crosslinked with only TPP resisted degradation by pancreatic enzymes. This difference is linked to the nature of the crosslinking, where glutaraldehyde forms covalent bonds, while TPP forms ionic and more 'flexible' bonds between chitosan chains.[365]

Therefore, the rate of degradation of chitosan depends on the molar mass, the DA, and chemical modifications. Thus far, the *in vivo* degradation pathways of chitosan and its derivatives have not been 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 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, its modulation on its 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} (ref. 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 their cytotoxicity depending on their concentration and incubation time.[266,367]

The impact of molar mass and degree of substitution (deacetylation, trimethylation, and 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 its toxicity may increase 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 identical DTMs, 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}) 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}) (IC_{50} = 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 the higher degree of PEGylation, *i.e.*, 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 its cytotoxicity.[266]

Table 3 Cytotoxicity of chitosan and its derivatives measured by the MTT test

Chitosan (DD, M_w)	IC_{50} (cells, incubation time)	Ref.
Trimethylated chitosan, PEGylated	L929 cells, 24 h	266
84.7% DD, 40% DTM		
400 kg mol^{-1} PEG 5000 g mol^{-1}		
0% DP	0.015 mg mL^{-1}	
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, 2 h	367
87% DD, 20–460 kg mol^{-1}		
Aspartate	0.67–0.72 mg mL^{-1}	
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, 72 h	368
78% DD, <50 kg mol^{-1}	2.50 mg mL^{-1}	
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, 72 h	368
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}	
Chitosan glycol		
100% DD, 152 kg mol^{-1}	2.47 ± 0.15 mg mL^{-1}	
Trimethylated chitosan	MCF-7 cells, 24 h	366
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}	
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^{-1}	

Adapted from ref. 356. 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 charge, such as PEGylation, may reduce the toxicity of chitosan. The 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 (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, and composition) based on chitosan on the cytotoxicity, knowing that these nano-formulations have already demonstrated a significant role in 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 a decrease in the polymer DD from 88% to 61%.[369]

Qi *et al.* 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 an IC_{50} of 15 and 6 μg mL^{-1}, respectively. The surface charge of the nanoparticles increased the electrostatic interactions with the 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 cytotoxicity towards hepatic cells[370] (Fig. 5D and 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 with hyaluronic acid (HA), and nanoparticles of 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 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, which is 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 including L929 cells[266] (Fig. 5B and 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 their surface charge density resulting from amine groups, the size of the nanoparticles and the three-dimensional arrangement of the polymer. Depending on the conformation and the cat-

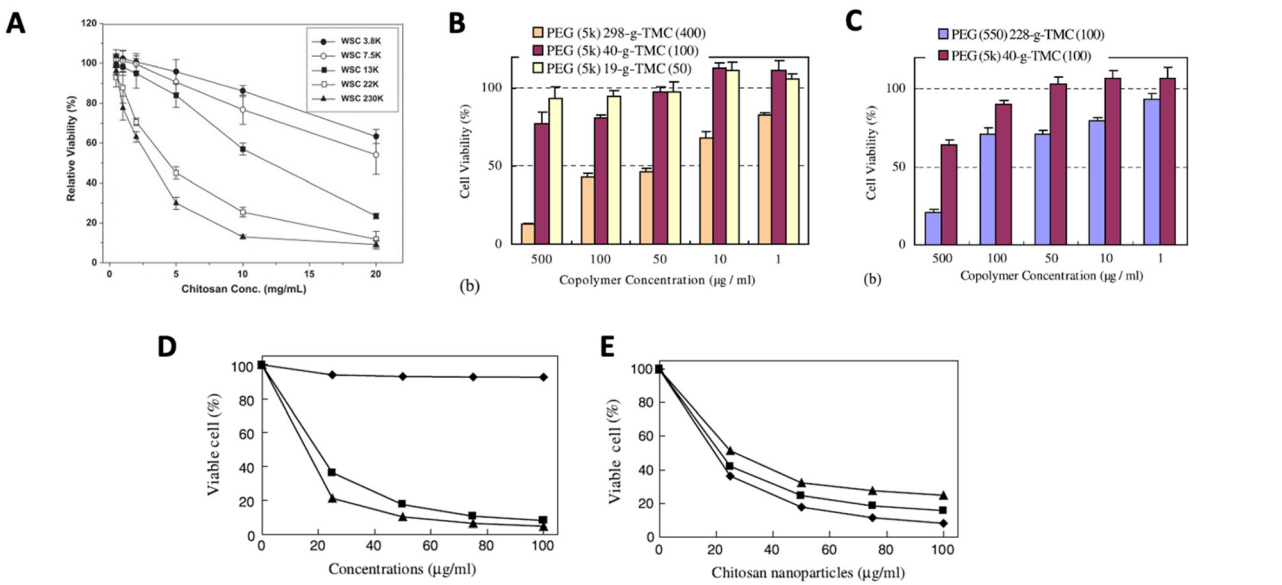

Fig. 5 Impact of chitosan chemistry and supramolecular organization on *in vitro* toxicity. (A) Cytotoxicity of soluble chitosan of various M_w: ● 3.8 kg mol^{-1}; ○ 7.5 kg mol^{-1}; ■ 13 kg mol^{-1}; □ 22 kg mol^{-1} and ▲ 230 kg mol^{-1} (reproduced from ref. 373 with permission from Elsevier, copyright 2005). (B and C) Cytotoxicity of trimethyl chitosan-poly(ethylene glycol) (TMC-PEG) conjugates displaying various M_w of the TMC and PEG segments (reproduced from ref. 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 ref. 370 with permission from Elsevier, 2005).

ionic charge of the nanoparticles, a decrease or 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.

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 complement activation.[374,375]

Regarding the toxicity of chitosan in the form of nano-objects, Hu *et al.* studied it with chitosan nanoparticles cross-linked with TPP in a zebrafish embryo model, which is 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, malformations were only observed in the embryos treated with the 200 nm nanoparticles, showing an impact on the size of the nanoparticles. At the cellular level, the nanoparticles caused physiological stress. These toxic effects were observed only for high concentrations of nanoparticles. The 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 after 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, where the median

Table 4 *In vivo* toxicity of chitosan and its derivatives after parenteral and oral administration

Chitosan (DD, M_w)	Route	Study	Observations	Ref.
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^{-1} for 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^{-1} Suc-CS/MMC: LD_{50} = 25 mg kg^{-1} No clinical sign	345
N-Octyl-nanoparticles Chitosan *O*-sulfate Paclitaxel	Parenteral route	Mouse, IV injection Rats, IV injection Rabbit, IV injection 6 mg per kg per day for 3 days Rabbit, IV injection 2 mg kg^{-1}	92% DD, 65 kg mol^{-1} IV: LD_{50} = 72.16 mg kg^{-1} PI: LD_{50} = 81.28 mg kg^{-1} 97% DD, 65 kg mol^{-1} IV: LD_{50} = 102.59 mg kg^{-1} PI: LD_{50} = 130.53 mg kg^{-1} No pathological changes observed No hypersensitivity	42 and 380
Chitosan 3 kg mol^{-1}	Oral route	Rabbits, hens 700–800 mg kg^{-1} for 239 days	No pathological changes	378
Chitosan 80% DD, 30–40 kg mol^{-1}	Oral route	Mouse Food containing between 0.5–5% chitosan for 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 1000–10 000 mg kg^{-1} Rats 750–3000 mg kg^{-1} for 30 days	LD_{50} > 10 000 mg kg^{-1} No mutagenicity No clinical or pathological signs LD_{50} > 3000 mg kg^{-1} No clinical, hematological or pathological signs	381
Chitosan n/a	Oral route	Rats 0–2545 mg kg^{-1} for 364 days 0–2545 mg kg^{-1} for 728 days	LD_{50} > 2323–2545 mg kg^{-1} 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^{-1} for 14 days	No clinical signs (diarrhea, fever, weight loss, *etc.*), pathological and inflammatory Non-significant hematological and biochemical abnormalities	383

Adapted from ref. 376. DD: degree of deacetylation; M_w: molar mass; LMW: low molecular weight IV: intravenous; IP: intraperitoneal; LD_{50}: median lethal dose; DS: degree of *N*-succinylation.

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 route, for which a lack of sufficient hemocompatibility and the formation of deadly emboli, despite the safe administration in several animal models, have been raised as concerns.[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 ability to modulate the 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, and chickens) (Table 4). Absorption through the intestinal barrier is dependent on the molar mass of chitosan, which increases as its molar mass decreases and its water solubility increases.[391] Therefore, the oral route is not the 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 its route of administration, which encourages its use as a biomaterial for the administration of active drug substances.

Many examples can be found in relation to the permeability enhancement of drugs. Yin *et al.* developed micelles of *N*-octyl-*O*,*N*-carboxymethyl chitosan to investigate their role in enhancing the 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 a difference in the 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 min, while the encapsulated insulin showed a prolonged reduction in blood glucose. The presence of insulin in the plasma was also modified by the nanoparticles. After 1 h injection of free insulin, the maximum was reached, and in the case of the insulin-loaded nanoparticles, this maximum was observed at 2–3 h after administration.[394]

However, each *in vivo* toxicity study uses its own experimental conditions; thus, the 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 itself. Chitosan forms nano-objects in the presence of other components such as a chemical and ionic crosslinking agents or active substance, which may have their own toxicity.

7 Conclusions and future perspectives

Nanoparticles composed of chitosan have emerged as an excellent option to compose biodegradable, biocompatible and versatile drug delivery systems with overall low toxicity. Restrictions in chitosan solubility draws attention and chitosan derivatives were developed to become excellent alternatives to overcome this issue. The modification of chitosan may lead to more suitable and less toxic delivery systems, improving the properties of chitosan. 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 can be a promising perspective in the use of polymers in different fields, such as food, agriculture, and especially health care.

Several challenges remain to be addressed for the 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, including the scaling-up and economic feasibility of chemical modifications and processes of nano-formulations under GMP conditions,[29] in addition to the cost of the chitosan raw material depending on its source,[395] and 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 by several European initiatives.[397]

Despite these hurdles, it should be noted that several commercial products have received approval for use as wound dressings, hemostatic sealants, and even as nerve conduits.[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 an adjuvant in vaccines, but should be taken into account for other drug delivery purposes.

Conflicts of interest

There are no conflicts to declare.

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