Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules ☆

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ABSTRACT

Chitosan (CS), a cationic polysaccharide, is widely regarded as a safe and efficient intestinal absorption enhancer of therapeutic macromolecules, owing to its inherent mucoadhesive feature and ability to modulate the integrity of epithelial tight junctions reversibly. By using CS-based nanoparticles, many studies have attempted to protect the loaded macromolecules against acidic denaturation and enzymatic degradation, prolong their intestinal residence time, and increase their absorption by the intestinal epithelium. Derivatives of CS such as quaternized CS, thiolated CS and carboxylated CS have also been examined to further enhance its effectiveness in oral absorption of macromolecular drugs. This review article describes the synthesis of these CS derivatives and their characteristics, as well as their potential transport mechanisms of macromolecular therapeutics across the intestinal biological membrane. Recent advances in using CS and its derivatives as carriers for oral delivery of hydrophilic macromolecules and their effects on drug transport are also reviewed.

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Contents

1. Introduction

Recent advances in biotechnology and genetic engineering have made the mass production of therapeutic macromolecules, such as proteins, nucleic acids and polysaccharides, feasible [1]. As is generally assumed, the oral route is the most convenient and comfortable means of administration of these hydrophilic macromolecules for patients. However, the oral bioavailability of these therapeutic agents is severely limited by their low stability in the gastrointestinal (GI) tract and their poor permeability across intestinal biological membranes [2–5]. Hence, vehicles that can protect the loaded macromolecules from destruction in the GI tract and increase their intestinal absorption capacity are highly desired.

As is widely assumed, chitosan (CS)-based nanoparticles (NPs) are a promising vehicle for oral delivery of therapeutic macromolecules. CS is a nontoxic and biocompatible polysaccharide derived from the shells of crustaceans and insects [6–8], with its safety demonstrated in both animal models and humans [9–12]. Available in an oral pill form, CS can reduce dietary fat and cholesterol absorption [9,10,12]. In addition to protecting the loaded therapeutics against acidic denaturation and enzymatic degradation, CS-based NPs exhibit a mucoadhesive feature, capable of prolonging their residence time in the small intestine [13,14]. Moreover, CS can mediate the opening of tight junctions (TJs) between epithelial cells reversibly, thus facilitating the paracellular transport of hydrophilic macromolecules [15–17]. Therefore, many studies have focused on using CS-based NPs to improve the oral bioavailability of macromolecular agents [18–21].

Recent studies have demonstrated that the mucoadhesive feature of CS and its ability to mediate the opening of TJs depend strongly on its protonation degree [22–25]. As the pKa value of the amine groups on CS is approximately 6.5, CS is protonated and thus soluble at acidic pH, yet aggregates at neutral pH [26,27]. This finding suggests that CS can be effective as a mucoadhesive agent and an absorption enhancer only in a limited area of the intestinal lumen where the pH values are below or close to its pKa (e.g., in the duodenum) [28].

Many studies modified the chemical properties of CS to improve its solubility and increase its absorption enhancement capability in an environment where the pH value is neutral [28–31]. For instance, quaternized CS has been synthesized to improve the solubility of CS and its effectiveness as an absorption enhancer in the intestinal pH environments [28]. Additionally, according to previous studies, immobilization of the thiol groups on CS can significantly enhance its mucoadhesive capability [29–31]. This review article discusses the synthesis of various CS derivatives and their inherent properties. The potential transport mechanisms of therapeutic macromolecules orally delivered by CS-based NPs across the intestinal epithelium are also described. Finally, recent advances in using CS and its derivatives as carriers for oral delivery of active macromolecules are described as well.

2. Transport mechanisms of macromolecules across intestinal epithelium

Intestinal epithelium is the major barrier for the absorption of macromolecules from the intestinal lumen to the systemic circulation [32]. This epithelial cell layer consists of enterocytes, goblet cells and M cells (Fig. 1A) [33,34]. As the most abundant epithelial cells in the intestine, enterocytes are specialized cells responsible for transporting nutrients by active transport or passive diffusion [35]. The second most abundant cells in the intestinal epithelium are goblet cells, which can secrete mucus, a physical barrier against pathogens [36,37]. Characterized by their flattened apical surfaces and lack of mucus layer, M cells are specialized epithelial cells that reside predominantly in Peyer's patches in the ileum, the distal portion of the small intestine. In contrast to enterocytes, M cells can take up antigens and microorganisms from the

intestinal lumen and then deliver them to the underlying immune system of mucosa [38].

Intestinal absorption of macromolecules occurs either through the transcellular or paracellular route (Fig. 1B) [39]. As an efficient absorption enhancer and drug carrier [40], CS increases both transcellular and paracellular transports of macromolecules across the intestinal epithelium [41].

2.1. Transcellular route

Transcellular uptake of CS-based NPs occurs by transcytosis, a process by which carriers are taken up by enterocytes or M cells, mimicking the entry of pathogens (Fig. 1B) [42]. By using two in vitro cellular models to partially reproduce the characteristics of intestinal enterocytes and M cells, Kadiyala et al. demonstrated that NP transport through the M-cell co-culture model is 5 folds higher than that of the intestinal Caco-2 cell monolayers [43].

Transcellular transport can be enhanced by adapting the physicochemical properties of NPs, such as their particle size and mucoadhesivity [44]. NPs under 100 nm are likely to be absorbed by the enterocytes, while particles larger than 500 nm are likely to be taken up by the M cells of the Peyer's patches [45–47].

Improving the mucoadhesive ability of NPs is also an effective means of increasing their uptake by epithelial cells [3,42,48–50]. CS is a well-known mucoadhesive polymer, whose mucoadhesive property is due to an electrostatic interaction between the positively charged CS and the negatively charged sialic-acid residues on the mucosal surface [22]. This interaction can provide a prolonged contact between the CS-based NPs and the absorptive surface, thereby promoting their oral absorption capacity. CS mucoadhesion is also supported by the observation that CS increases significantly the half time of its clearance from the GI tract [51].

Several studies have established the effectiveness of the mucoadhesive CS-based NPs in increasing their transcellular permeability in the intestinal mucosa [42,52–54]. Woitiski et al. found that multilayered NPs containing CS and alginate facilitate the insulin permeation across rat intestinal mucosa by internalizing insulin-loaded NPs into epithelial cells [54].

2.2. Paracellular route

Absorption via the paracellular route (i.e., uptake through the interstitial space between epithelial cells) is normally restricted by the relatively narrow width of the paracellular channels and the presence of TJs. The TJs form a barrier that allows the absorption from the lumen of needed water and electrolytes, but prevents the passage of inflammatory and infectious agents into the systemic circulation [55]. They are composed of a complex combination of transmembrane integral proteins, including claudins (CLDNs), occludin and junctional adhesion molecules along with several intracellular plaque proteins and several regulatory proteins, which anchor the transmembrane proteins to the actin cytoskeleton [56,57]. Transmembrane proteins, especially CLDNs, play a major role in forming the seal between adjacent cells. Plaque proteins are necessary to form a structural support for TJs, and regulatory proteins regulate signal transductions involving TJ permeability and cell differentiation [57–59].

Our recent study elucidated the mechanism and consequences of CS-mediated reversible TJ opening in Caco-2 cell monolayers [60]. According to those results, following CS treatment, redistribution of CLDN4 from the cell membrane to the cytosol is related to its degradation in lysosomes, subsequently decreasing TJ strength and ultimately increasing paracellular permeability [60]. Importantly, TJ disruption likely occurs through multiple mechanisms. This study further demonstrates that reconstruction of TJs during recovery depends on CLDN4 synthesis (Fig. 2) [60]. Moreover, elucidating the mechanism of CS-induced TJ opening in intestinal cells significantly contributes to
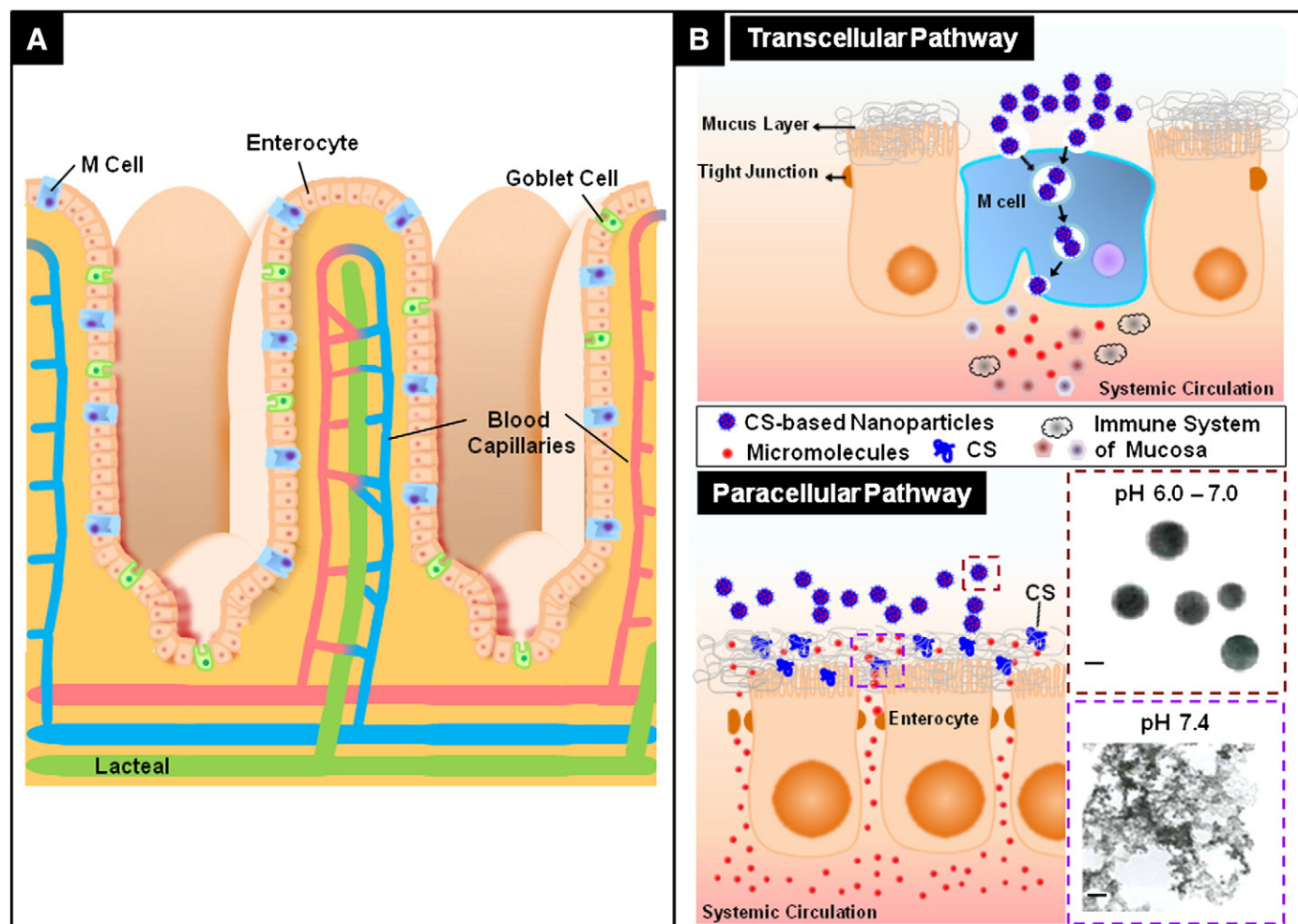
Fig. 1. Schematic illustrations of (A) the structure of the intestinal epithelium comprising enterocytes, goblet cells and M cells in Peyer's patches; (B) the presumed mechanisms of the transcellular and paracellular transport of macromolecules.

efforts to use CS and its derivatives as paracellular permeation enhancers.

Our recent investigation examined the mucoadhesion of CS and its subsequent effects on the opening of epithelial TJs and their paracellular permeability in rats by using microscopic and ultra-structural approaches [61]. In this study, the CS labeled by quantum dots (CS-QD) is administered to ICR mice that had been fasted overnight. Light microscopic images of the intestinal segments revealed that the CS-QD complex could adhere to and infiltrate the intestinal mucosa (Fig. 3A, blue arrows) [61].

Transmission-electron microscopic examination revealed that several electron-dense CS aggregates (blue arrows) dotted the brush border and were retained in the microvilli scaffold of the intestinal villi (Fig. 3B) [61]. Despite the occasional observation of a few electron-dense particulates over the TJs (Fig. 3C) [61], the particulates were seldom found in the paracellular spaces. Moreover, the particulates were absent in the basal region of the intestinal villi and in the lacteal lumen. Since the pKa value of CS is around 6.5 [62], CS molecules can aggregate when brought to the physiological pH of approximately 7.4 (as in the mucus layer), possibly explaining the poor permeation of CS molecules in the paracellular spaces.

This study investigated the lanthanum (an electron-dense tracer) staining of the exposed intestinal segments to visualize the TJ opening activity of CS. In the control groups, without the opening of TJs (white arrow), lanthanum was observed mainly on the microvilli surface (Fig. 3D) [61]. However, after the oral administration of CS, paracellular

surfaces were stained (red arrows), revealing the opening of TJs following CS treatment (Fig. 3E) [61]. Additionally, a lateral cell surface was occasionally found to exhibit TJ opening with segmental lanthanum-staining (red arrows, Fig. 3F) [61]; meanwhile, the other paracellular cell surface remained unstained (white arrows). Above data verify the mucoadhesive characteristics of CS and provide definitive evidence of its TJ-opening activity.

Theoretically, paracellular transport of NPs is infeasible because the interstitial space between epithelial cells in natural state ranges from 0.3 to 1 nm, which is too narrow for most NPs to permeate [48,63]. Even in a fully-opened state, the width of TJs is less than 20 nm [64], which remains extremely limited for the transport of intact CS NPs across the paracellular space. Therefore, the paracellular permeability of macromolecules must be more likely attributed to the anticipated release of drugs from the disintegrated CS NPs [16,65]. The presumed mechanism is that CS NPs can adhere to and infiltrate the mucus; the infiltrated NPs become unstable and disintegrate near the epithelial cell surface due to their pH sensitivity and subsequent release of the loaded drugs. The released drug can then enter the systemic circulation due to the CS-mediated TJ opening (Fig. 1B) [65].

Whether CS can also enhance the absorption capacity of endotoxins in the small intestine when TJs are opened remains a contentious issue. We have investigated how CS NPs affect the absorption of lipopolysaccharide (LPS), the most commonly found toxin in the GI tract [16]. Experimental results indicate that the insulin loaded in
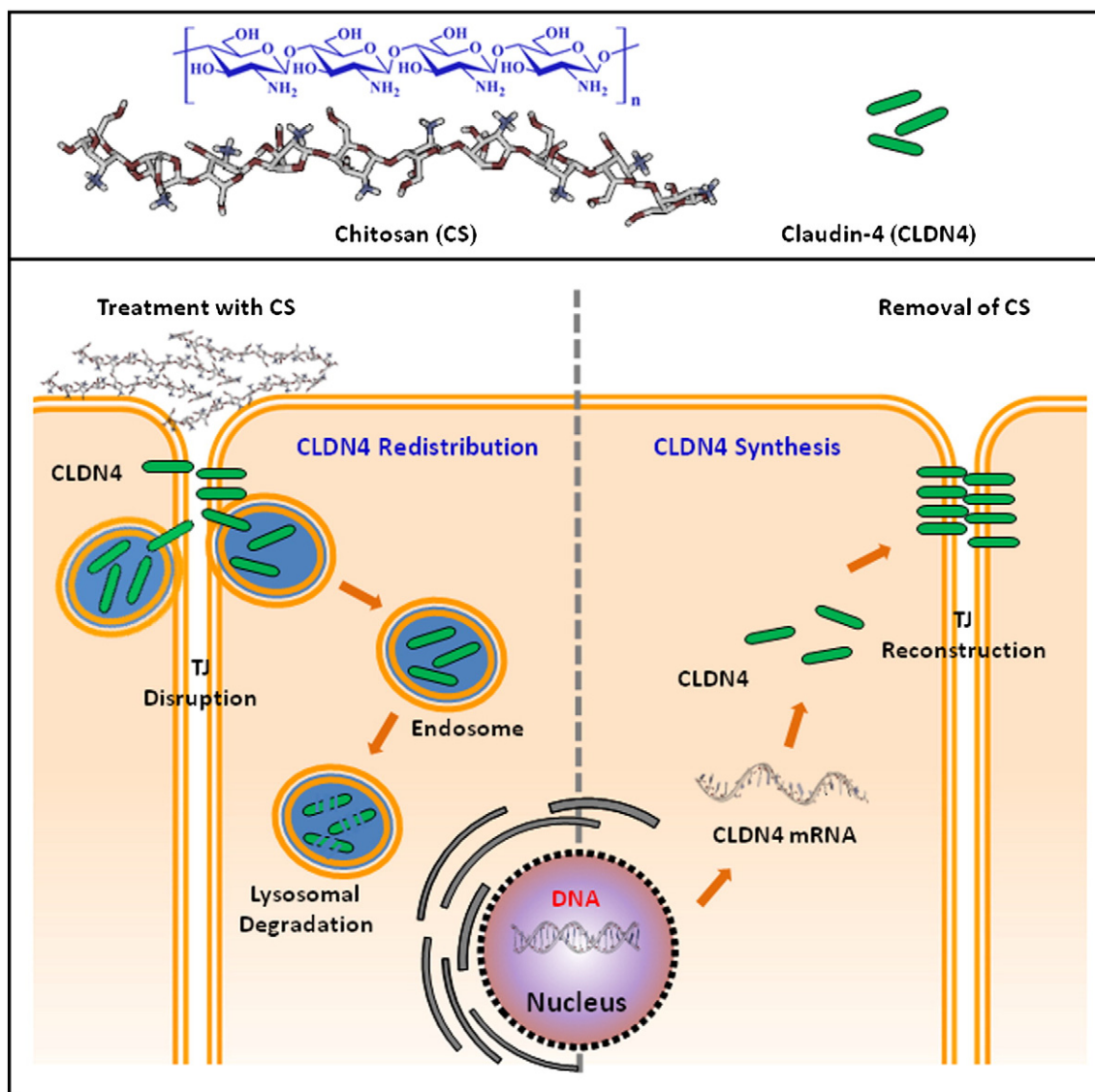
Fig. 2. Schematic illustrations showing the mechanism and consequence of chitosan (CS)-mediated reversible tight junction (TJ) opening. Reproduced with permission from Ref. [60], Copyright 2011 Elsevier.

CS NPs can traverse across the intestinal epithelium and enter the systemic circulation, whereas LPS fails to do so, probably, owing to the charge repulsion between the anionic LPS in the form of micelles and the negatively charged mucus layer [16]. The in vivo toxicity study further confirms that the improvement of paracellular permeation by CS fails to increase the absorption of LPS [16]. Results of this study demonstrate that CS NPs are highly promising for use as a safe carrier for oral drug delivery.

## 3. Synthesis and characterization of CS derivatives

Chemical modification of CS is feasible because it has reactive amino and hydroxyl groups that can be readily modified with a diverse array of ligands, functional groups and moieties [14]. Various chemical modifications, including quaternization, thiolation, carboxylation, alkylation, acylation, PEGylation and graft copolymerization, have been performed to further improve the beneficial properties of CS such as its aqueous solubility, mucoadhesivity as well as enzymatic inhibitory and TJ opening abilities for oral drug delivery [7,8,15,44, 66–70].

3.1. Quaternized CS

Quaternization of CS can conserve its positive charge at a neutral pH value, thus increasing its aqueous solubility significantly [71]. Various quaternized CS derivatives, trimethyl CS (TMC), dimethylethyl CS (DMEC), diethylmethyl CS (DEMC) and triethyl CS (TEC), have been synthesized [7,8,15,69,70]. TMC is a partially quaternized derivative of CS prepared by the reductive methylation of CS, in which methyl iodide and sodium iodide are inserted in an alkaline solution of *N*-methyl pyrrolidinone (NMP) [72]. TMC-based NPs have been extensively studied for its potential to increase the absorption for oral delivery of macromolecules, owing to the effectiveness of TMC in mucoadhesion as well as in mediating a TJ opening at distinct intestinal pH environments [71–76].

DMEC, DEMC and TEC with different substituted *N*-alkyl groups can be made based on TMC synthesis with some modifications (Fig. 4) [77–81]. The TJ opening ability of quaternized CS derivatives depends on their degree of quaternization [28]; a higher degree of quaternization (or higher charge density) implies a higher transport capability [82]. Length of the substituted *N*-alkyl groups on quaternized CS also plays an important role in profoundly impacting
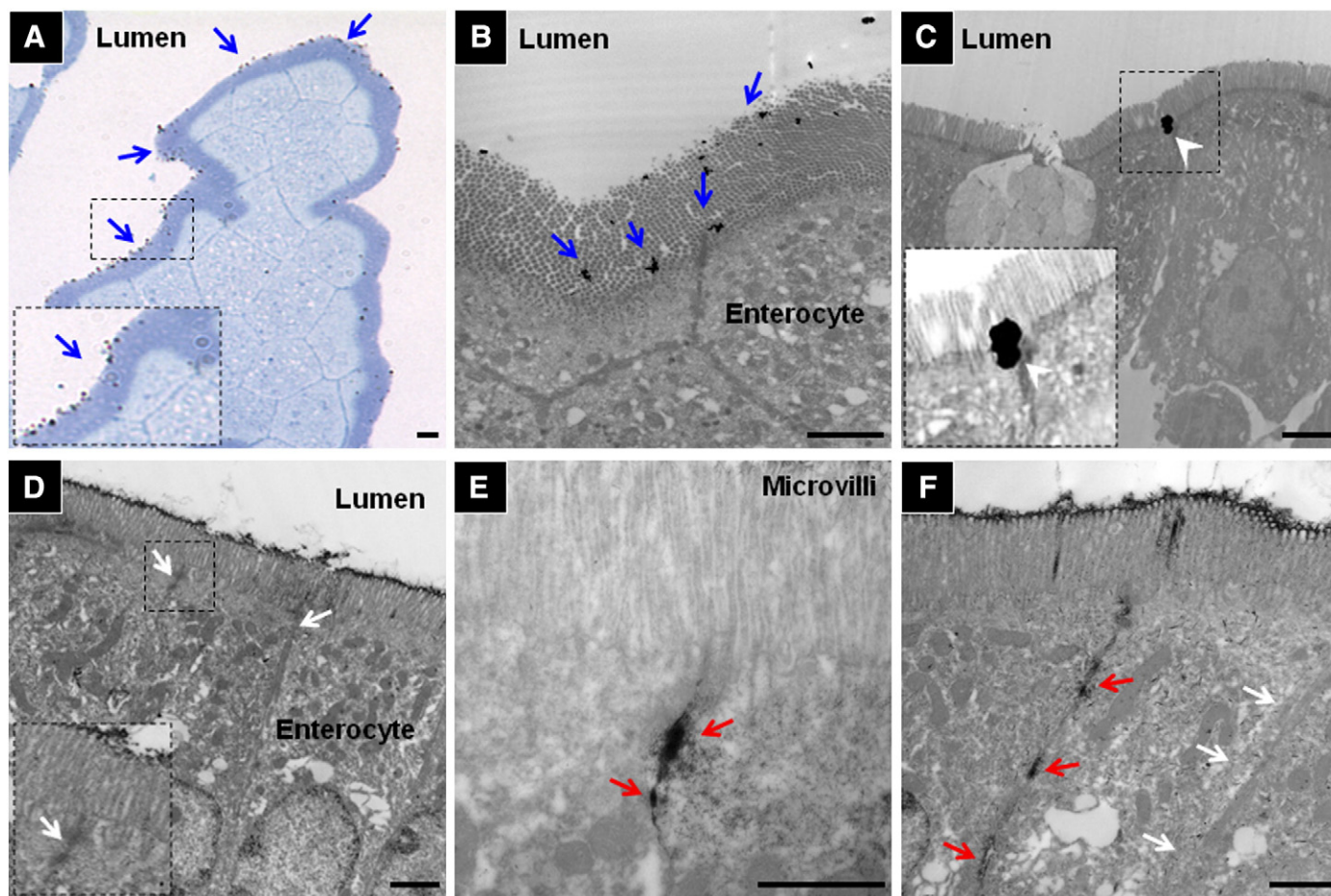
Fig. 3. (A) Light microscopic image and (B) TEM micrograph showing the mucoadhesion and infiltration of silver-enhanced QD-CS (blue arrows) in the intestinal villi 3 h after oral administration to mice; (C) an evidence of the inability of QD-CS aggregates to pass through the apical opening of the intercellular space (white arrowhead). TEM micrographs showing the lanthanum-stained intestinal segments (D) before and (E, F) after chitosan treatment in mice. The lanthanum staining of intercellular surfaces (red arrows) indicated the opening of tight junctions. Scale bars: 1 μm.
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their paracellular permeability. An ethyl group, which is longer than a methyl group, substituted on the nitrogen atoms of CS may shield the positive charge of the quaternary amines due to the steric hindrance, subsequently lowering the efficiency of quaternized CS in their transport enhancement [78]. The effectiveness of NPs prepared by quaternized CS derivatives on the opening of TJs and transport of insulin across Caco-2 cell monolayers was found to be in the following order: $TMC > DMEC > DEMC > TEC > CS$ [78].

Another quaternized CS [quaternary ammonium palmitoyl glycol CS (GCPQ), an amphiphilic polymer] was synthesized to facilitate the dissolution of hydrophobic drugs by forming polymeric micelles [83,84]. A previous study demonstrated that GCPQ micelles can
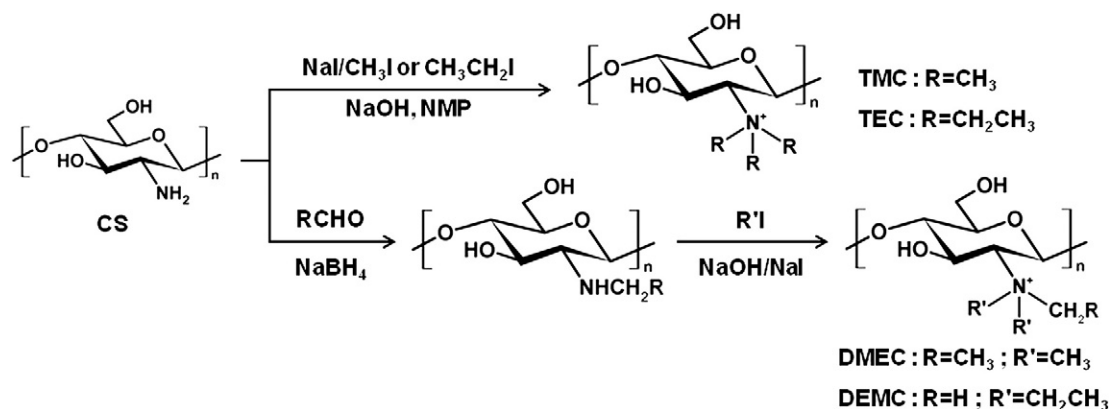
Fig. 4. Schematic illustrations of the synthesis of quaternized chitosan (CS) derivatives. Trimethyl CS (TMC), dimethylethyl CS (DMEC), diethylmethyl CS (DEMC) and triethyl CS (TEC).

improve the ability of encapsulated hydrophobic drugs to adhere to and penetrate the mucus layer, subsequently increasing their permeability across the intestinal epithelium [84].

3.2. Thiolated CS

Thiolated CSs are synthesized by covalently coupling with sulfhydryl bearing agents such as cysteine, thioglycolic acid and glutathione onto the backbone of CS; in addition, they can be prepared through either a ring opening reaction of 2-iminothiolane or a direct imidoester reaction of isopropyl-S-acetylthioacetimidate [85,86]. These reactions lead to the formation of different thiolated CS derivatives, including CS-thioglycolic acid (CS-TGA) [87], CS-cysteine (CS-Cys) [88], CS-glutathione [89], CS-4-thio butyl-amidine (CS-TBA) [22,90] and CS-thioethylamidine (CS-TEA) (Fig. 5A) [91].

In contrast to the unmodified CS, the mucoadhesive strength of thiolated CS derivatives to the intestinal mucosa increases significantly, making them especially attractive for oral delivery of macromolecules [50,92–98]. This feature may be attributed to the formation of disulfide bonds between their thiol groups and the cysteine-rich subdomains of glycoproteins in the mucus layer, which is stronger than the ionic interaction between cationic CS and anionic mucosal substances [88,99]. According to a previous study, the extent to which the mucoadhesive strength of thiolated CS is increased is correlated with its degree of thiolation [30].

The thiolated TMC, TMC-cysteine (TMC-Cys), is synthesized via amide bond formation between the residual primary amino groups on TMC and the activated esters of carboxyl groups on cysteine (Fig. 5B) [50,100]. The TMC-Cys derivative was synthesized to combine the mucoadhesion- and permeation-enhancing effects of TMC and thiolated polymers related to different mechanisms for oral absorption [50]. Mucoadhesion and permeation enhancing effects of TMC-Cys NPs were significantly higher than those of TMC NPs [50]. Since particle translocation necessitated a preliminary phase of attachment of the particles to the mucosal surface [89], the more significantly enhanced permeation level by TMC-Cys NPs than that of TMC NPs could be ascribed to the ameliorated contact with epithelial cells favored by concentrating the particles in the mucus layer through disulfide bonding.

3.3. Carboxylated CS

CS can be chemically modified by negatively charged carboxyl groups to increase its water solubility. Carboxymethyl CS derivatives can be prepared by introducing the $-CH_2COOH$ groups onto 6-O and 2-N atoms on CS [101,102]. Another carboxylated CS derivative, N-succinyl CS, can be obtained by introducing succinyl groups into the glucosamine units of CS via ring-opening reactions with succinic anhydride (Fig. 6) [101]. Owing to their ability to contain both cationic ($-NH_3^+$) and anionic ($-COO^-$) groups, carboxymethyl and succinyl CS derivatives can be regarded as polyampholytes [101]. A previous study demonstrated that carboxylated CS derivatives decrease the transepithelial electrical resistance (TEER) and increase the paracellular permeability of heparin in epithelial cell monolayers, which is an indication of permeation enhancers [6].

Carboxylated CS can be grafted with poly(methyl methacrylate) by using ammonium persulfate as a radical initiator to prepare carboxylated CS grafted NPs (CCGN), which have pH-sensitive properties [103,104]. In simulated gastric fluid, the release of insulin from CCGN was relatively slow, while its release in the simulated intestinal fluid at pH 7.4 was instant [104]. A minimal drug release in the gastric fluid is desired for oral delivery of macromolecules to avoid their gastric degradation.

3.4. Amphiphilic CS

Amphiphilic CS derivatives can be prepared by grafting hydrophobic compounds such as aliphatic acids (C6–C16) via N-acylation [105,106]
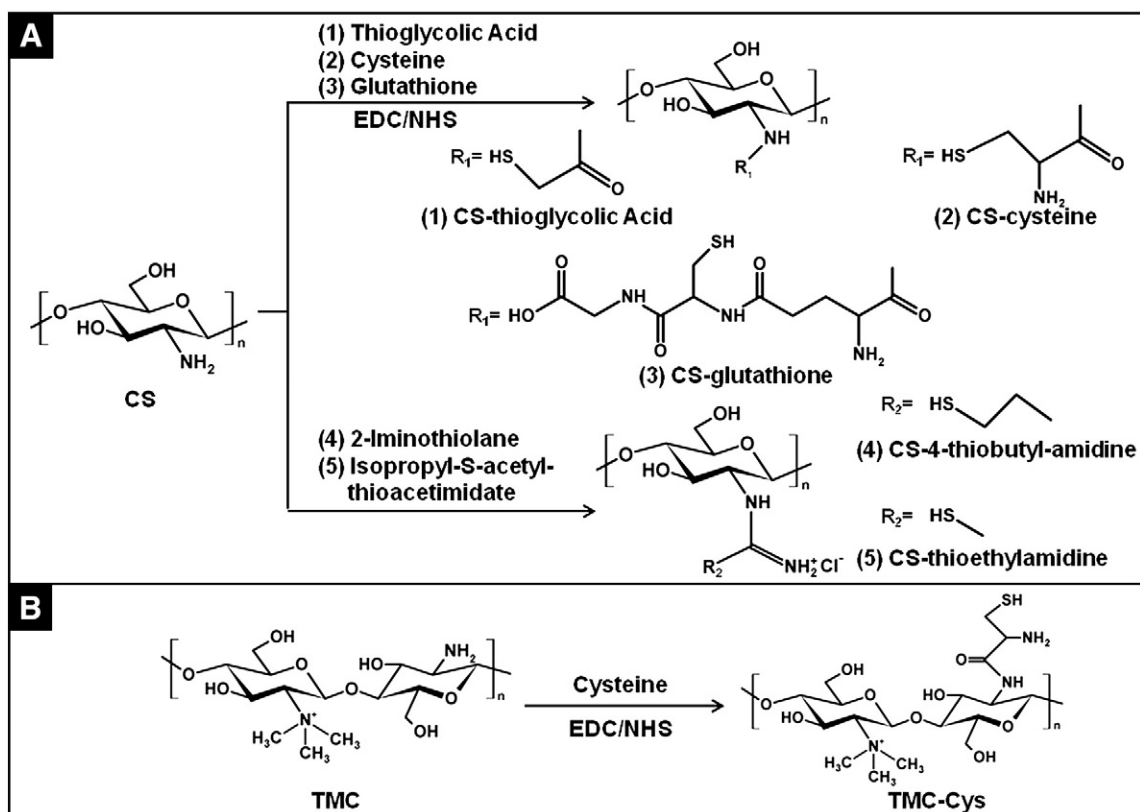

Fig. 5. Schematic illustrations of the synthesis of thiolated CS derivatives (A) through covalently coupling methods: CS-thioglycolic acid (CS-TGA), CS-cysteine (CS-Cys), CS-4-thio butyl-amidine (CS-TBA), and CS-thioethylamidine (CS-TEA); (B) the synthesis of TMC-cysteine (TMC-Cys).
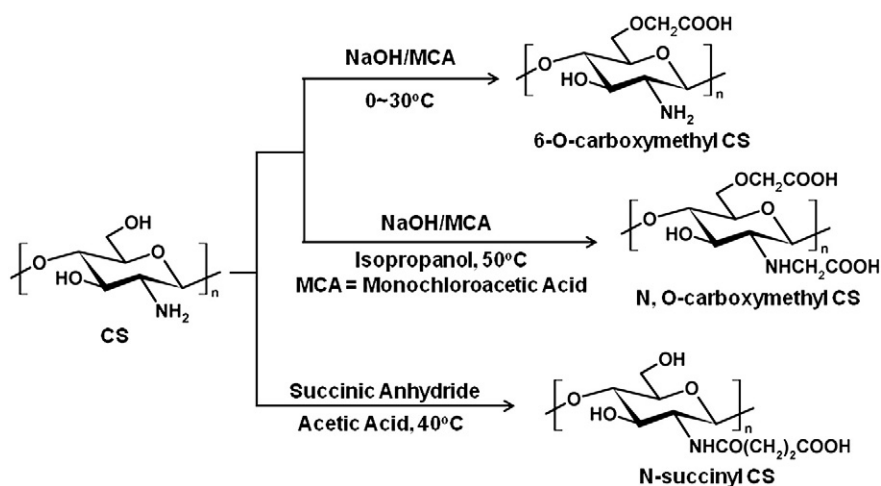
Fig. 6. Schematic illustrations of the synthesis of carboxymethyl CS and succinyl CS derivatives.

or bile acids/fatty acids through amidating reaction on CS [107–109]. In an aqueous environment, polymeric amphiphiles can self-assemble into NPs due to the interaction between their hydrophobic and hydrophilic segments [110]. As is well known, two residues are present in the mucin for mucoadhesive interaction: the charged acidic groups on sialic acid and sulfonated residues and the hydrophobic methyl groups on fucose residues [111]. Therefore, carriers containing hydrophobic segments may have a better affinity to the mucin [112,113].

A previous study prepared an amphiphilic CS derivative, lauryl succinyl CS (LSC), by chemical modification of CS with the hydrophobic lauryl and hydrophilic succinyl moieties (Fig. 7) for oral insulin delivery [112]. The hydrophobic lauryl group on LSC can assist its mucoadhesivity, while its carboxyl group of succinyl moiety can chelate calcium ions, subsequently inhibiting the proteolytic enzymes and disrupting the structure of TJs [112].

3.5. CS derivatives bearing chelating agents

Although CS exhibits mucoadhesive and TJ opening capabilities, its efficacy as an absorption enhancer for macromolecule delivery is limited, owing to its lack of enzyme inhibitory ability [113]. Intestinal metallo-peptidases can be divided into luminally secreted enzymes and brush-border-membrane bound enzymes [114]. Chelating agents, including nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA), can inhibit these peptidases owing to their deprivation of essential divalent cations (e.g., Ca^{2+} and Zn^{2+}) out of the enzyme structures [115]. Additionally, according to a previous study, chelating agents disrupt adherens junctions by chelating the extracellular Ca^{2+} [116]. The assembly of TJs between epithelial cells requires the previous formation of adherens junctions; thus, disruption of the adherens junctions modulates TJ formation and their paracellular permeability [117]. CS can be endowed with the metallo-peptidase inhibiting ability and improvement of paracellular permeability through the conjugation of one of these chelating agents (Fig. 8) [118–120].

3.6. PEGylated CS

As a highly hydrophilic, flexible polymer, poly(ethylene glycol) (PEG) has an inherently long circulating property, making it an attractive polymer for modifying the surfaces of drug carriers to increase their hydrophilicity and circulating half-life [121]. PEGylated CS (CS-PEG) can be synthesized by using PEG-succinimidyl succinate or activated esters of PEG carboxylic acids capable of reacting with the primary amine groups on CS to form stable amides (Fig. 9) [122–125]. Nanocapsules coated by CS-PEG improve their stability in simulated GI fluids and reduce their cytotoxicity [126–128].

4. Efficacy of CS-based NP systems for oral delivery of macromolecules

4.1. Protein delivery

Therapeutic proteins such as insulin, calcitonin and cyclosporine A have become the drugs of choice for treating distinct diseases owing to their high selectivity and ability to provide effective and potent physiological actions [129]. CS-based NPs have been extensively studied for their use in orally delivering these protein drugs, demonstrating their potential to improve oral bioavailability. This section reviews recent advances in using CS-based NPs for oral protein delivery and their efficacy in drug transport (Table 1).

4.1.1. Insulin and its analogs

Exogenous insulin is essential in treating diabetes mellitus. Normally transported to the liver via the portal circulation, endogenously secreted insulin suppresses hepatic glucose production and causes cells in the liver, muscle and fat tissue to take up glucose from the blood, resulting in a hypoglycemic effect [130]. Oral delivery of exogenous insulin is a preferred route owing to its ability to reproduce the physiological profile of insulin undergoing the first hepatic bypass [131,132]. This route can improve glucose homeostasis and also avoid hyperinsulinemia induced
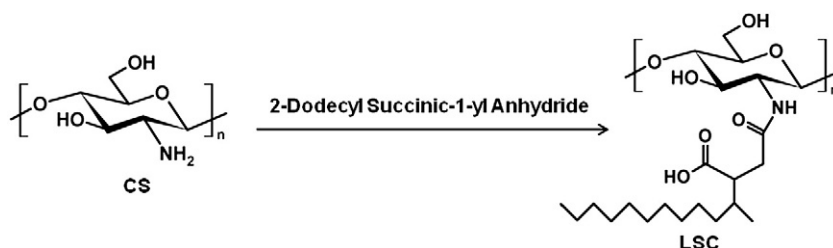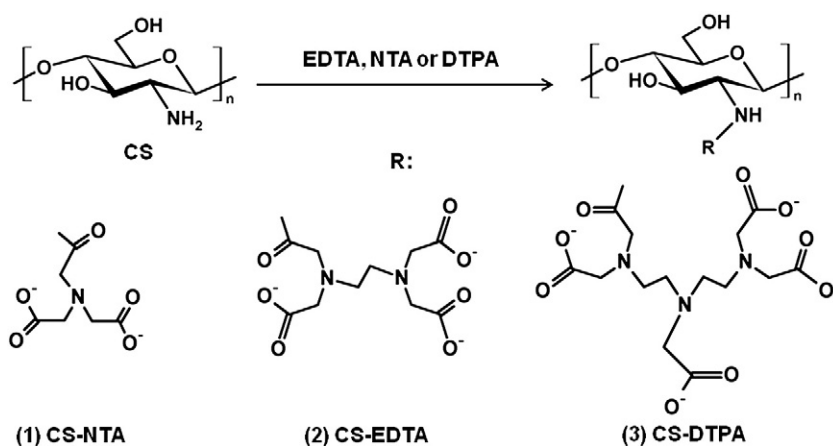

Fig. 7. Schematic illustration of the synthesis of lauryl succinyl CS (LSC).

Fig. 8. Schematic illustrations of the synthesis of CS derivatives bearing chelating agents nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTPA).

by subcutaneous injection (SC) [133]. Nevertheless, delivering insulin orally often leads to a low bioavailability, owing to its presystemic degradation and inadequate permeation through the intestinal epithelium [134,135].

Recent studies described a pH-responsive NP system shelled with CS, capable of increasing the oral absorption of insulin and producing a hypoglycemic effect in diabetic rats [65,66,136,137]. Insulin-loaded NPs were obtained by adding an aqueous poly(γ-glutamic acid) (γ-PGA, negatively charged) blended with insulin into an aqueous CS (positively charged) solution under magnetic stirring. This ionotropic gelation method is promising, since CS/γ-PGA NPs can be prepared under aqueous-based conditions at room temperature, thus preventing the denaturation of protein drugs [19,138,139].

According to the in vivo toxicity study, CS/γ-PGA NPs were well tolerated, even at a dose 18 times higher than that used in the pharmacodynamic (PD) and pharmacokinetic (PK) studies [65]. The in vivo fluorescence-microscopic results demonstrated that insulin could be absorbed into the systemic circulation, while most CS was retained in the microvilli scaffolds (Fig. 10A) [61]. These observations were verified in a biodistribution study following the oral administration of isotope-labeled nanoparticles by single-photon emission computed tomography (Fig. 10B) [61]. Oral administration of the insulin-loaded CS/γ-PGA NPs showed a significant hypoglycemic effect for at least 10 h in diabetic rats; in addition, the relative bioavailability of insulin compared to SC was 15% [65].

A follow-up biodistribution study showed that some of the orally administered CS/γ-PGA NPs were retained in the stomach for a long duration, which might lead to the loss of insulin from test NPs [136]. An attempt was made to further increase the bioavailability of insulin by

freeze-drying and filling CS/γ-PGA NPs in an enteric-coated capsule [136]. The in vivo dissolution study revealed that the enteric-coated capsule could prevent the loaded NPs from contacting the highly acidic gastric media, but liberated them rapidly in the proximal segment of the small intestine. Consequently, all test NPs loaded in the capsule were brought into the small intestine, subsequently increasing the absorption capacity of insulin into the systemic circulation; in addition, the relative bioavailability of insulin was approximately 20%.

As is well known, complexing agents such as DTPA inhibit intestinal proteases and interfere with epithelial TJs by chelating divalent metal ions [115–117]. A recent study attempted to maintain the complexing agent concentrated on the intestinal mucosal surface, where enzyme inhibition and paracellular permeation enhancement are required, by covalently conjugating DTPA on γ-PGA and forming functional NPs with CS for oral insulin delivery (Fig. 11) [134]. The γ-PGA-DTPA conjugate inhibited the intestinal proteases substantially, as well as produced a transient and reversible enhancement of paracellular permeability. An earlier study found that intestinal absorption by CS improved mainly in the duodenum [65]. Results of this study demonstrate that CS/γ-PGA-DTPA NPs can increase the absorption of insulin throughout the entire small intestine. The improved absorption in the jejunum and ileum may be attributed to the mediation of TJ opening by γ-PGA-DTPA. Additionally, oral intake of the insulin-loaded CS/γ-PGA-DTPA NPs by diabetic rats produced a prolonged reduction in blood glucose levels revealing a maximum insulin concentration 4 h after treatment. The relative oral bioavailability of insulin was approximately 20%.

A previous study also demonstrated that CS/γ-PGA NPs are a promising vehicle for oral delivery of aspart-insulin, i.e. a rapid-acting insulin analog [137]. Results of a biodistribution study indicated that
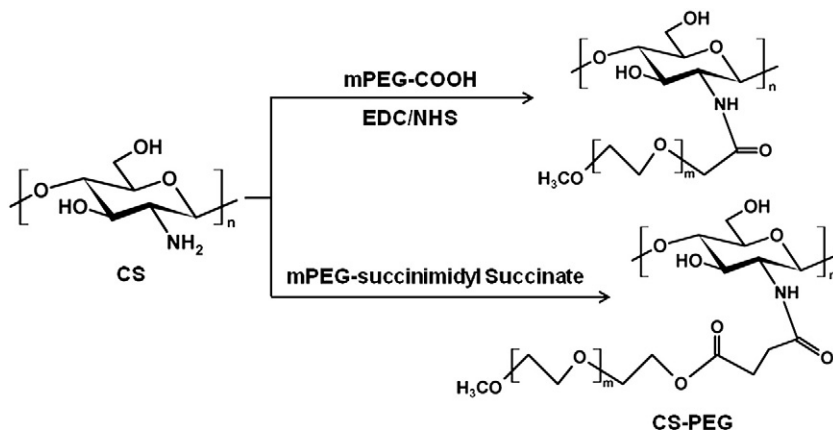
Fig. 9. Schematic illustrations of the synthesis of PEGylated CS derivatives.

Table 1
Selected examples of CS-based NP systems for oral delivery of macromolecules.

Formulation	Drug	Observation	Ref.
CS/γ-PGA NPs	Insulin	Hypoglycemic effect lasted for at least 10 h; BA[a] was 15%	[65]
CS/γ-PGA NPs in an enteric-coated capsule	Insulin	BA[a] was 20%	[136]
CS/γ-PGA-DTPA NPs	Insulin	BA[a] was 20%	[134]
CS/dextran sulfate NPs	Insulin	Hypoglycemia effects prolonged over 24 h; PA was 5.6%	[19]
CS/alginate NPs	Insulin	Hypoglycemia effects prolonged over 18 h; PA was 6.8%	[138]
CCGN	Insulin	PA was 9.7%	[104]
LSC NPs	Insulin	Hypoglycemia effects lasted for 6 h	[112]
CS/γ-PGA NPs	aspart-insulin	Biodistribution study indicated that the aspart-insulin was absorbed into the systemic circulation, while the CS carrier was mainly restricted in the gastrointestinal tract; BA[a] was 15%	[137]
CS/γ-PGA NPs in an enteric-coated capsule	Exendin-4	BA[a] was 15%	[20]
CS-PEG coated nanocapsules	sCT	Reduction in the serum calcium levels for at least 24 h	[127]
CS NPs	CsA	BA was increased to about 73% when compared with the reference Neoral® microemulsion orally	[149]
GCPQ NPs	CsA	Maximum plasma concentration of CsA was increased 2-fold when compared with the commercial Neoral® formulation orally	[84]
CS/heparin complexes	Heparin	0.2 − 0.3 IU/mL of Anti-Factor Xa activity in plasma was maintained for up to 12 h; BA[b] was 20.5%	[18]

Abbreviations: CS, chitosan; γ-PGA, poly(γ-glutamic acid); NPs, nanoparticles; DTPA, diethylenetriaminepentaacetic acid; CCGN, carboxylated CS grafted NPs; LSC, lauryl succinyl CS; PEG, poly(ethylene glycol); GCPQ, quaternary ammonium palmitoyl glycol CS; sCT, salmon calcitonin; CsA, Cyclosporin A; BA, bioavailability; PA, relative pharmacological bioavailability. BA was determined based on the serum drug level relative to that achieved via the subcutaneous[a] or intravenous[b] drug injection. PA was determined based on the extent of the hypoglycemic response relative to that achieved via the subcutaneous insulin injection.

the orally administered aspart-insulin was absorbed into the systemic circulation, while the drug carrier (CS) was retained mainly in the gastrointestinal tract. Orally administering test NPs exhibited a

slower but more prolonged absorption of aspart-insulin than that of SC injection, implying that it is likely to reduce the risk of hyperinsulinemia commonly observed via SC injection [137]. Additionally, the relative
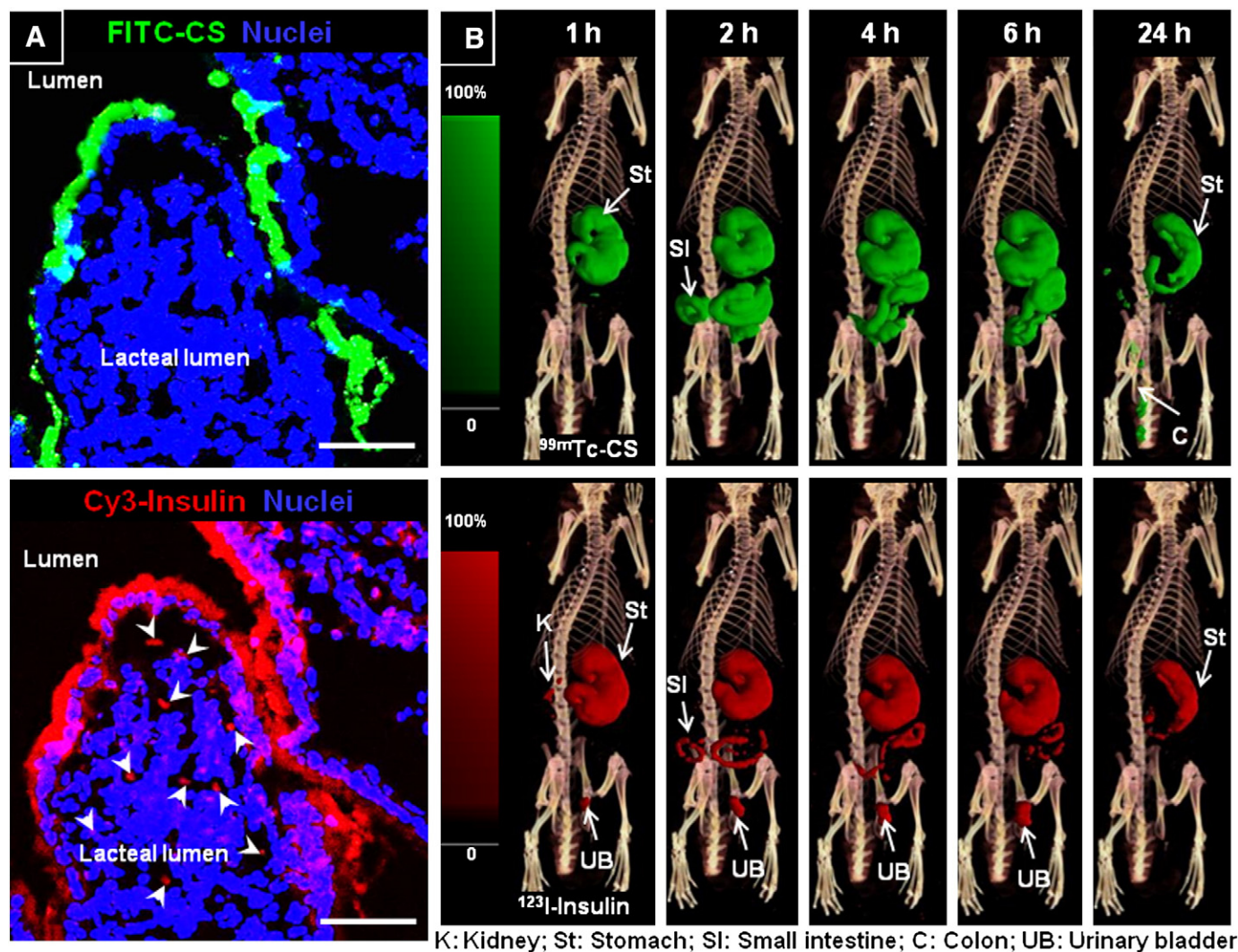
Fig. 10. (A) Fluorescence images showing the intestinal villi retrieved from a rat model 3 h after being orally administered with fluorescent nanoparticles; (B) reconstructed 3D single-photon emission computed tomography images showing the biodistribution of ^{99m}Tc-CS and ^{123}I-insulin in rats orally treated with the isotope-labeled nanoparticles. Reproduced with permission from Ref. [61], Copyright 2012 American Chemical Society.
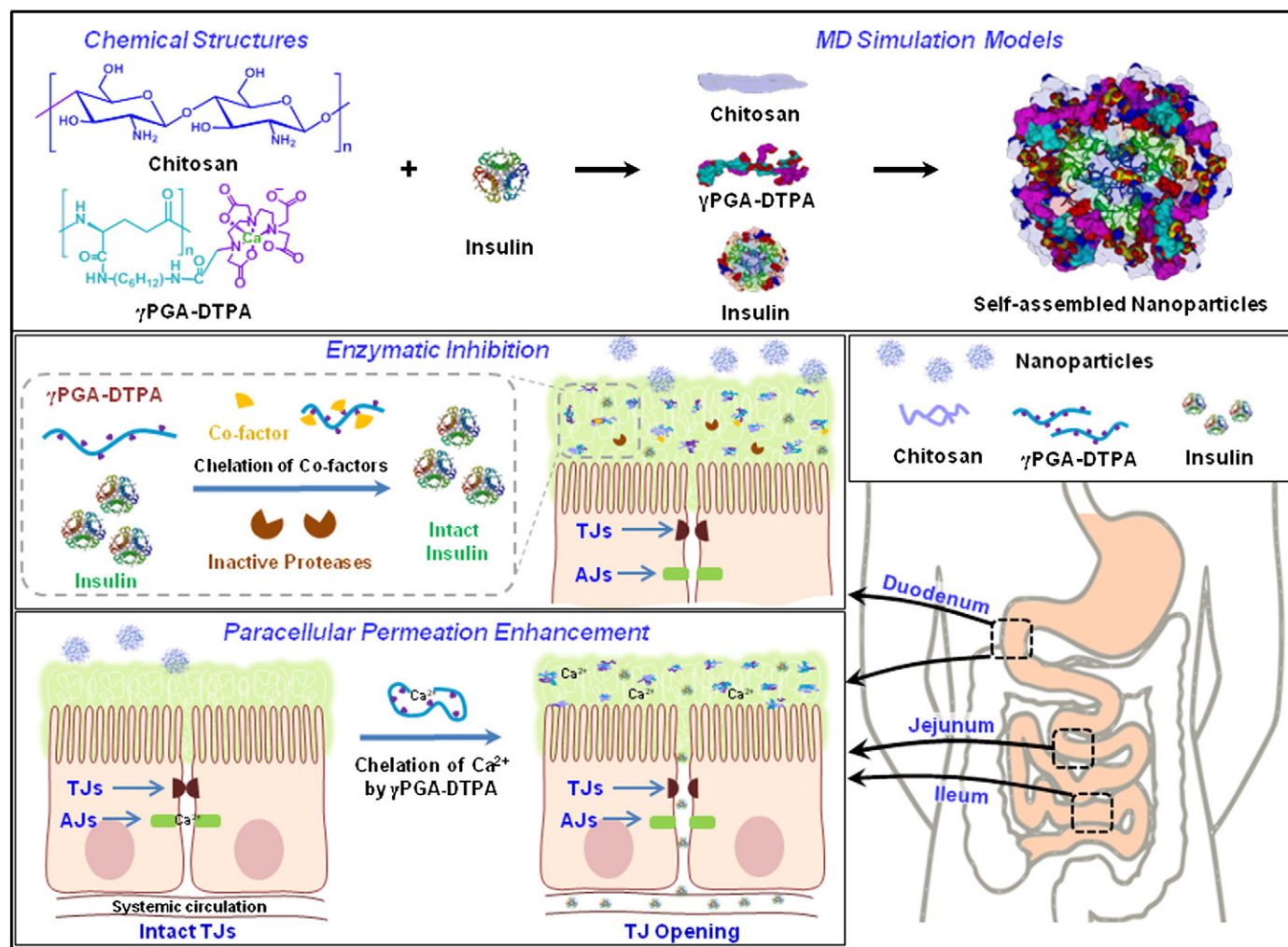
Fig. 11. Schematic diagrams showing the compositions of CS/γPGA-DTPA nanoparticles (NPs) and their mechanisms for inhibition of intestinal proteases and enhancement of paracellular permeation. TJs: tight junctions; AJs: adherens junction.
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bioavailability of aspart-insulin orally delivered by test NPs was approximately 15%. Comparing the PD/PK profiles of the orally administered aspart-insulin with those of the SC injection of NPH-insulin, an intermediate-acting insulin, implies the feasibility of the CS/γ-PGA NP system for use as a noninvasive alternative for basal insulin therapy.

Sarmento et al. developed CS/dextran sulfate NPs [19] and CS/alginate NPs [138] for oral insulin delivery. Confocal microscopic examination of the FITC-labeled insulin orally delivered by the developed NPs to diabetic rats clearly showed its adhesion within the intestinal mucosa and absorption into the epithelium. The hypoglycemia effects were prolonged over 24 h and 18 h; in addition, the pharmacological availabilities were 5.6% and 6.8% for the groups treated with CS/dextran sulfate NPs and CS/alginate NPs, respectively.

Quaternized CS derivatives, such as TMC, with an improved aqueous solubility over that of native CS are considered potential intestinal absorption enhancers to increase the paracellular transport capability [118]. TMC can open the TJs of intestinal epithelium at physiological pH values, where CS is insoluble and therefore ineffective [28]. A recent study demonstrated that self-assembled TMC/γ-PGA NPs opened the TJs of Caco-2 cells to facilitate the transport of insulin along the paracellular pathway at all test intestinal pH environments [74]. This finding suggests that TMC/γ-PGA NPs may be a promising carrier for transmucosal delivery of insulin within the entire intestinal lumen.

Effectiveness of thiolated CS as an oral delivery vehicle can be attributed to its pronounced mucoadhesiveness [140,141]. Thiolated CS,

however, is insoluble at a neutral pH value, thus limiting its efficiency in mucoadhesion [99]. To address this concern, Yin et al. synthesized TMC-Cys to increase the aqueous solubility of thiolated CS while maintaining its mucoadhesive feature for oral insulin delivery [50]. In contrast to their TMC counterparts, TMC-Cys NPs promoted Caco-2 cell permeability by $1.7 - 3.0$ folds, subsequently increasing insulin transport through the rat intestine by $1.7 - 2.6$ folds and increasing the uptake in the Peyer's patches by $1.7 - 5.0$ folds, respectively.

Cui et al. prepared pH-sensitive carboxylated-CS grafted poly(methyl methacrylate) NPs (CCGN) as an insulin carrier [104]. The release of insulin from CCGN strongly depended on the protonation degree of their carboxyl groups. Moreover, the pH-sensitivity of CCGN resulted in a slow release of insulin at acidic pH (pH 2.0) and a fast release at neutral pH (pH 6.8–7.4). This observation implies the protection of the loaded insulin against an acid environment in the stomach. Oral administration of insulin-loaded CCGN led to a significant hypoglycemic effect in rats; in addition, the pharmacological bioavailability was 9.7%.

Another study developed a NP system composed of a CS derivative LSC and sodium tripolyphosphate (TPP) for oral delivery of insulin [112]. The NPs prepared by this CS derivative with both the hydrophobic (lauryl) and hydrophilic (succinyl) moieties more significantly improved both the mucoadhesivity of CS and the characteristics of insulin release as well as its paracellular permeability than those of the native CS particles. The increased mucoadhesive capacity of LSC NPs can

be attributed to the lauryl groups, which could develop a hydrophobic interaction with the protein domains in mucins. Their results further demonstrated that LSC NPs can reduce the blood glucose levels in diabetic rats for about 6 h.

4.1.2. Exendin-4

Exendin-4, a 39-amino-acid peptide found in lizard saliva, is a potent insulinotropic agent in diabetic patients [142]. However, its therapeutic utility is limited due to the frequent injections required [143]. This peptide shares several glucoregulatory activities with the mammalian incretin hormone glucagon-like peptide-1 (GLP-1), such as glucose-dependent enhancement of insulin secretion, suppression of glucagon secretion, as well as reduction of gastric mobility and food intake [144]. The Food and Drug Administration (FDA) has approved exenatide, a synthetic version of exendin-4, as adjunctive therapy for type 2 diabetic patients failing to achieve glycemic control with oral antidiabetic agents [145]. Gedulin et al. elucidated the biological activity of exenatide delivered via distinct non-injection routes. According to their results, the bioavailability of exenatide, relative to the SC administration, was minimal: enteral (intraduodenal) route of ~0%, sublingual route of 0.6%, intranasal route of 2.7%, and pulmonary aerosol route of 0.2% [143].

Nguyen et al. developed an orally available exendin-4 formulation by using an enteric-coated capsule containing CS/γ-PGA NPs [20]. Their dissolution study on rats found that the enteric-coated capsule remained intact while in the stomach; whereas it was dissolved promptly in the proximal segment of the intestine and then released its loaded content (Fig. 12). Oral administration of the capsule containing exendin-4-loaded NPs showed a maximum plasma concentration 5 h after treatment; the bioavailability, relative to its SC counterpart, was approximately 15%. The absorbed exendin-4 could then stimulate the insulin secretion and provide a prolonged glucose-lowering effect. Above results suggest that this orally available exendin-4 formulation warrants further exploration as a potential therapy for diabetic patients.

4.1.3. Salmon calcitonin (sCT)

Calcitonin, a peptide hormone of 32 amino acids, has been administered for many years to lower blood calcium and treat bone diseases, including osteoporosis and Paget's disease [146]. Notably, sCT is the most widely used preparation in clinical practice due to its 40−50 times higher intrinsic potency than that of human calcitonin, as well as its improved analgesic properties [147]. According to a previous study, CS nanocapsules prepared by a solvent displacement method are an effective means of enhancing the oral absorption of sCT and reducing calcemia levels in rats [21]. These positive results were attributed mainly to the unique role of mucoadhesive CS in improving the interaction of the nanocapsules with the absorptive epithelium.

A related study highlighted the crucial role of CS-PEG coating in stabilizing nanocapsules and its ability to maintain the oral absorption of sCT [127]. In vivo studies indicated a significant reduction in the serum calcium levels for at least 24 h, as well as the ability of CS-PEG coated nanocapsules to increase and prolong the intestinal absorption of sCT, depending on the degree of PEGylation on CS.

4.1.4. Cyclosporin A (CsA)

As a hydrophobic peptide commonly used as an immunosuppressive drug, CsA prevents allograft rejection in organ transplantation.
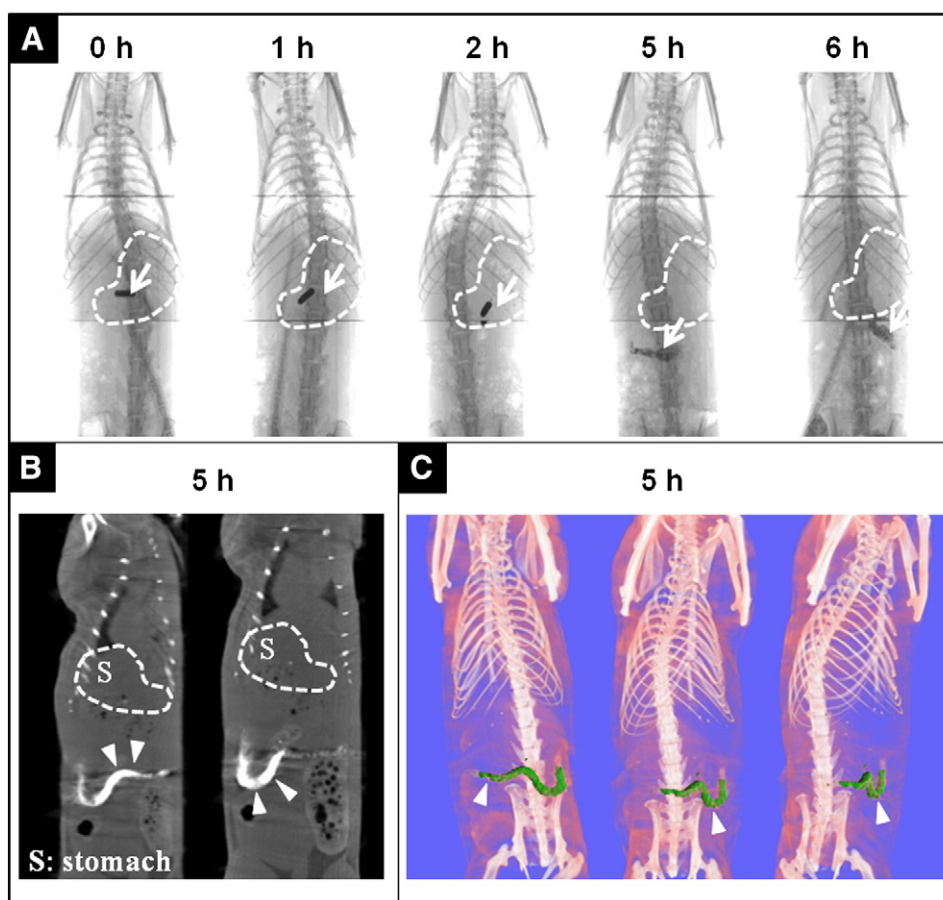
Fig. 12. (A) Sequential plain X-ray images; (B) CT-scan images; and (C) 3D-volume-rendering images of a rat orally treated with the barium sulfate-filled capsule (indicated by arrows). The released barium sulfate was located in the intestine (indicated by arrow heads).
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However, the bioavailability of CsA after oral dosage is low, owing to its relatively high molecular weight and lipophilicity and low intestinal permeability [148]. To address this concern, the poorly absorbable CsA was encapsulated within the positively charged NPs by an emulsification solvent diffusion method using emulsifiers and CS hydrochloride salts as charge inducing agents [149]. Following oral administration of CsA-loaded CS NPs to beagle dogs, the relative bioavailability was increased to about 73%, when compared with the reference Neoral® microemulsion. That study also demonstrated that the nature of cationic CS and its mucoadhesion effect on the permeability of GI mucosa significantly affects its ability to increase the absorption rate and the bioavailability of CsA.

Siew et al. recently designed a triple-action nanomedicine using a CS amphiphile, (GCPQ), which significantly enhanced the oral absorption of hydrophobic CsA [84]. Amphiphilic GCPQ bearing pendant alkyl and acyl chains formed self-assembled NPs in aqueous media with a critical micellar concentration in the µM range. The CsA-loaded GCPQ NPs were extremely stable, with drug remaining entrapped within the NPs for up to 6 months when the product was freeze-dried and stored as a liquid formulation. After the GCPQ formulations were administered to rats, the maximum plasma concentration of CsA increased 5-fold and 2-fold when compared to the drug alone in water and the commercial Neoral® formulation, respectively. Based on their results, that study postulated that the mechanism of absorption enhancement by GCPQ NPs involves increasing dissolution of the drug using NPs, mucoadhesion of NPs to the mucus layer, and transcellular transport of drug molecules across the GI epithelium.

4.2. Nucleic acid delivery

Gene therapy is highly promising for clinical applications involving the insertion of a functioning nucleic acid into cells to correct a cellular dysfunction or providing a new cellular function [150–153]. Nevertheless, nucleic acids fail to be effectively transported across the intestinal epithelium due to their characteristics of large molecular size, hydrophilicity and negative charge. Moreover, nucleic acids are extremely labile in the biological environment and can be readily degraded by nucleases, thus having a low transfection efficiency [154–156]. Therefore, the development of an efficient carrier that can orally deliver and protect nucleic acids to the target cells, as well as allow cellular internalization, endosomal escape and nucleus uptake, is a prerequisite to realize its therapeutic function [157,158].

The M cells of the Peyer's patches can transport a variety of materials, including NPs [43,159] and microparticles [43,160–162], making them attractive portals for oral delivery of therapeutics and for mucosal vaccination [43]. M cells, which cover the gut-associated lymphoid tissue, play a prominent role in initiating the cascade of mucosal immunity. These specialized antigen-sampling epithelial cells are characterized by a high endocytic rate and low degradation ability [159]. Particle uptake by M cells depends on various factors, such as particle size, surface charge, dosage, stability and ligand conjugation [163].

NPs prepared by CS or its derivatives have gained increasing attention as an oral delivery system for therapeutic genes and DNA vaccines. CS can condense and encapsulate plasmid DNA to form NP complexes, capable of adhering to the intestinal epithelium and being transported across the mucosal boundary by M cells and transfect epithelial and/or immune cells in the gut associated lymphoid tissue (Fig. 1A) [164–166]. According to recent studies, their transfection efficiency depends on a number of CS-based formulation parameters, including the molecular weight of CS, its deacetylation degree, complex formulation, and pH environment [158]. This section examines recent advances in oral nucleic acid delivery using CS-based NPs.

While describing oral gene therapy using CS/DNA NPs that carry the murine erythropoietin (Epo) gene, Chen et al. demonstrated their efficacy in delivering genes to intestinal epithelial cells, by an over 25% increase in hematocrit levels [165]. Epo is a glycoprotein,

which stimulates red blood cell production; it is used in patients with anemia associated with chronic renal failure, and in cancer patients for stimulation of erythropoiesis during autologous transfusion [165]. Bowman et al. demonstrated the feasibility of using CS NPs as a gene carrier for orally delivering Factor VIII DNA in a mouse model with hemophilia A [167]. According to their results, a high dose oral delivery of CS/DNA NPs yielded higher plasmid copy numbers in Peyer's patch tissue than naked DNA delivery did. Hemophilia A is a disorder of the blood coagulation cascade caused by defective Factor VIII, a protein that normally circulates in the plasma at 100–200 ng/mL [167]. The disease is a popular target for gene therapy due to its low threshold for therapeutic value and wide therapeutic window of Factor VIII [168].

Oral vaccination is a highly promising application of CS NPs. A previous study developed an immunoprophylactic strategy using oral allergen-gene immunization to modulate peanut antigen-induced murine anaphylactic responses [166,169]. Food allergy is a common and often fatal disease with no effective treatment. According to that study, orally administering test NPs prepared by complexing plasmid DNA with CS resulted in a transduced gene expression in the intestinal epithelium, indicating its prophylactic utility in treating peanut allergy [166]. Another study also indicated that orally administered CS/DNA complexes can stimulate an immune response to the principal peanut allergen Arah-2 in Swiss albino mice [169]. Using a similar approach, Chew et al. demonstrated that oral delivery of CS NPs containing DNA vaccine encoding house-dust-mite-allergen Derp 1 and 2 in mice could induce an immune response against dust allergy [170].

4.3. Polysaccharide delivery

As a highly sulfated glycosaminoglycan, heparin is a potent anticoagulant that binds to the enzyme inhibitor antithrombin, causing a conformational change that results in its activation through an increase in the flexibility of its reactive site loop [171]. The activated antithrombin then inactivates thrombin and other proteases involved in blood clotting, especially Factor Xa [171]. Clinically, heparin can prevent deep vein thrombosis and peripheral arterial embolism, as well as reduce the incidence of myocardial infarction and mortality in patients with unstable angina [172].

To facilitate oral delivery of heparin, a NP system shelled with CS was prepared via a simple ionic gelation method to form CS/heparin complexes [18]. The in vitro characterization study demonstrated that this CS NP system could open the TJs between Caco-2 cells and allow the transport of heparin by passive diffusion via the paracellular route. No significant anticoagulant activity was detected after oral administration of the free form heparin solution in rats, indicating the poor oral absorption of heparin without an appropriate delivery system. Alternatively, oral administration of the same dosage of CS/heparin NP complexes could maintain its anti-Factor Xa activity in plasma of $0.2 - 0.3$ IU/mL for up to 12 h. The minimum effective concentration to treat deep vein thrombosis and pulmonary embolism was 0.1–0.2 IU/mL [173]. Above results indicate that CS NPs significantly improved the intestinal absorption of heparin, thus markedly increasing its bioavailability to around 20.5%.

5. Conclusions

Although CS-based NPs are highly promising for use as carriers for oral delivery of therapeutic proteins, nucleic acids and polysaccharides, no clinical trials have been conducted. Additionally, toxicological issues of these newly emerging NP systems remain a major concern. Although CS alone is considered safe for oral administration, its properties may change completely after chemical modification. The toxicity of each derivative should be evaluated individually, both in the free form and NP form. Further pre-clinical studies are required to demonstrate the

acceptable efficacy and safety of CS-based NP systems. We believe that more pre-clinical studies in the future will expedite and initiate the need for clinical trials in humans, leading to the implementation of CS-based NPs for oral delivery of therapeutic macromolecules.

Acknowledgment

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