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Bioadhesion and oral absorption of enoxaparin nanocomplexes

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ABSTRACT

Polyelectrolyte complexes (PEC) formed between chitosan derivatives and enoxaparin were prepared by a self-assembly process and were characterized in terms of particle size and surface charge. The morphology was observed by atomic force microscopy (AFM). The colloidal stability and bioadhesion of the PEC were characterized by dynamic light scattering (DLS). The absorption of enoxaparin in rats was evaluated by activated partial thromboplastin time (APTT) assay. It was shown that the prepared PEC had a spherical shape with positive charge and a mean diameter in the range of 200–600 nm. An increase in temperature led to a decrease in particle size (ca. 10%) with an increased kcps value (ca. 10–20%) for the PEC studied, depending on the polymer structure. Thiolation and methylation of chitosan could significantly improve the corresponding PEC's bioadhesion and hence the oral absorption of enoxaparin. A good relationship between bioadhesion and *in vivo* absorption was established. However, PEC of PEGylated chitosan did not display a significantly enhanced permeation of enoxaparin compared with unmodified chitosan. In conclusion, the oral bioavailability of enoxaparin can be enhanced by improving the bioadhesive properties of PEC via the chemical modification of chitosan employed.

1. Introduction

Over the years, polymeric nanoparticle delivery systems have drawn significant attention as carriers for hydrophilic macromolecular drugs (Jung et al., 2000). The main advantages of nanosize as an oral medication are that (1) nanoparticles as carriers of drugs can offer protection against *in vivo* acid and enzymatic degradation; (2) increase the membrane permeability; (3) increase the contact and absorption area (Jung et al., 2000). Particle size has been recognized as a crucial parameter for bioadhesion to and adsorption to the gastrointestinal tissue. The paracellular and endocytotic pathway for absorption of nanoparticles are both size-dependent. Numerous authors have demonstrated that the uptake of particles of nanosize in the intestine was significantly increased compared to larger particles of 1–10 μm (Desai et al., 1996). Particularly, polyelectrolyte complexes (PEC) formed by self-assembly through electrostatic interactions have recently emerged as an efficient class for the delivery of various charged bioactive macromolecules, such as polysaccharides, nucleic acids and proteins (Sun et al., 2008; Germershaus et al., 2008; Mao et al., 2006). The PEC formation leads to particles with dimensions on a colloidal level, generating optically homogeneous and stable nano-dispersions. In addition, such method has the advantage of not necessitating sonication and organic solvents during preparation, therefore minimizing possible damage to drug candidates.

A critical parameter influencing properties of the polymeric nanoparticle delivery system is the characteristics of the polymer employed (Krauland and Alonso, 2007). Chitosan (poly [β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a natural cationic polysaccharide derived by partial deacetylation of chitin isolated from crustacean shells. As a biocompatible, biodegradable and low toxic biopolymer, its inherent mucoadhesive nature and the capacity to open the tight junctions in the mucosa have been wildly investigated (Gaserod et al., 1998; Illum et al., 2001). Due to its specific properties, chitosan has been extensively exploited as a promising absorption enhancer of hydrophilic macromolecular drugs and a non-viral gene delivery vehicle (Hejazi and Amiji, 2003). However, the application of chitosan in the biomedical field is limited by its poor solubility in physiological media. To further increase the solubility of chitosan and improve the mucoadhesiveness, various chitosan derivatives were developed, such as trimethyl chitosan (TMC), PEGylated TMC copolymers and thiolated chitosans, which have shown promising characteristics in the development of polymeric nanoparticle delivery system (Sandri et al., 2005; Mao et al., 2005a,b; Bravo-Osuna et al., 2006).

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Enoxaparin, a low molecular weight heparin (LMWH), is a highly sulfated and acidic glycosaminoglycan composed of chains of alternating residues of D-glucosamine and uronic acid. It is used mainly for the prevention and treatment of venous thrombo-embolism (Hirsh et al., 1998). Enoxaparin is almost ineffective by oral administration presumably due to its high negative charge density and large molecular size, consequently has to be administered via the parenteral route. Numerous efforts to develop an oral enoxaparin formulation have been reported, including lipidization of the drug or coadministration with penetration enhancers (Ross and Toth, 2005; Schmitz et al., 2005; Kim et al., 2006), but have met with limited success.

In this study, we presented the development of enoxaparin/chitosan derivatives PEC, which were expected to combine the distinctive advantages of nanoparticle delivery system and chitosan derivatives employed, thus enhancing the transport of enoxaparin across the intestinal epithelium. Previously, we have evaluated the feasibility of PEC formation between enoxaparin and chitosan derivatives by self-assembly and investigated various factors influencing the PEC formation process in detail (Sun et al., 2008). Thus, the aim of the present study was to focus on the evaluation of the bioadhesion and oral absorption of enoxaparin PEC based on various chitosan derivatives (chitosan–cysteine conjugates, TMC and PEGylated TMC copolymer). The colloidal stability and bioadhesion of the PEC were characterized by dynamic light scattering (DLS). The absorption of enoxaparin in rats was evaluated by activated partial thromboplastin time (APTT) assay.

2. Materials and methods

2.1. Materials

Chitosan (400 kDa) was purchased from Weifang Kehai Chitin Co., Ltd. (China) with a degree of deacetylation of 86.5%. Enoxaparin (mean MW 4500 Da) was kindly provided as a gift by Dongying Tiandong Biochemical Co., Ltd. (China). L-Cysteine was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N*-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). Activated partial thromboplastin time (APTT) assay kits were purchased from Shanghai Sunbio Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade.

2.2. Chitosan derivatives preparation and characterization

Chitosans of different molecular weights were prepared by depolymerization with sodium nitrite without changes in the degree of deacetylation as described previously (Mao et al., 2004). The products obtained were named as CS 400, CS 100, CS 50 and CS 10 depending on their measured molecular weight. *N*-Trimethyl chitosan (TMC) with quaternization degree of 40% were prepared by reductive methylation of the parent chitosan with CH_3I in the presence of NaOH using the procedure described previously (Sieval et al., 1998). Grafting of TMC with poly (ethylene glycol) 5 kDa was achieved to yield copolymer $PEG(5)_{19}$-g-TMC(50) as described elsewhere (Mao et al., 2005a). The following nomenclature was adopted for the copolymer: $PEG(X)_n$-g-TMC(50), where X denotes the molecular weigh of PEG in kDa and the subscript n represents the average number of PEG chains per TMC macromolecule of 50 kDa. Thiolated chitosans were synthesized according to a method reported previously with little modification (Bernkop-Schnürch et al., 2004). Briefly, cysteine was covalently attached to chitosan via the formation of an amide bond mediated by carbodiimide and *N*-hydroxysuccinimide to obtain chitosan–cysteine conjugates. Iodine titration was used to determine the thiol group content.

2.3. Preparation of CS/enoxaparin nanocomplexes

CS/enoxaparin PEC were prepared from positively charged chitosan and negatively charged enoxaparin by self-assembly. Briefly, 1 mg/ml chitosan solution was prepared by dissolving chitosan powder in 0.25% acetic acid solution and filtered through a Millipore Millex 0.45 μm filter. Then an equal volume of enoxaparin solution (0.5 mg/ml) was added into the chitosan solution under magnetic stirring. The system pH was further adjusted to 6.5 and the solution was incubated for further 0.5 h at room temperature. Freshly prepared solutions were used in each experiment.

2.4. Characterization of CS/enoxaparin nanocomplexes

The mean particle size (z-ave) and the polydispersity index (PI) of the PEC were determined by photon correlation spectroscopy (PCS) with a Malvern Zetasizer 4 (Malvern Instruments, UK) at 25 °C with a scattering angle of 90°. The zeta potential measurements were carried out using the Malvern Zetasizer 4 by electrophoretic laser doppler anemometry at 25 °C.

To determine enoxaparin encapsulation efficiency, the PECs were centrifuged at 20,000 rpm for 1 h at room temperature. The quantity of enoxaparin in the supernatant was determined with Azure II colorimetric method (Jiao et al., 2002). The drug encapsulation efficiency was expressed as the percentage of enoxaparin entrapped with respect to the initial value.

A PicoPlus AFM (Agilent Technologies, USA) was employed to characterize the morphology of PEC. The samples were diluted with ultra pure water and 10 μl of the diluted sample was applied to a freshly cleaved mica surface and allowed to adhere to the surface for a few minutes. Then the supernatant was removed and the samples were allowed to air-dry (ca. 10 min). Commercially available silicon tips attached to I-type silicon cantilevers with a length of 230 μm, a resonance frequency of about 140 kHz and a scan frequency of 0.8–1.1 Hz were used. All measurements were performed in tapping mode in order to avoid damage of the sample surface.

2.5. Colloidal stability of PEC

2.5.1. Temperature stability studies

A number of different PEC were incubated at 25, 37, and 50 °C for 6 h, and the integrity of the PEC was subsequently characterized at predetermined time intervals (0, 15, 60, 145, and 360 min) using a Malvern Zetasizer 4 (Malvern Instruments, UK) by dynamic light scattering.

2.5.2. Influence of the system pH

In order to investigate the stability of PEC in GI fluid, the PEC formed with various chitosan derivatives were diluted with pH 1.2 simulated gastric fluid (SGF) or pH 6.8 simulated intestinal fluid (SIF) in the volume ratio of 1:5 and incubated at room temperature for 6 h. The integrity of the PEC at predetermined time intervals (0, 15, 60, 145, and 360 min) was monitored by dynamic light scattering.

2.6. Bioadhesion ex vivo assay

Ex vivo bioadhesion studies on rat intestinal mucosa were carried out with a Malvern Zetasizer 4 (Malvern Instruments, UK) by dynamic light scattering, kcps (kilo count per second), which is the intensity of the light scattering signal measured in count/s, was investigated during the measurements and has been demonstrated to be a measure of particle concentration in a sample (Mao et al.,

Table 1
Properties and characteristics of the polymers and the PEC.

Polymer (kDa)	Substitution	TMC content [%, (w/w)]	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Drug loading (%)
CS 400			681.2 ± 15.1	0.392 ± 0.078	8.1 ± 1.2	91.2 ± 1.7
CS 100			425.3 ± 11.8	0.353 ± 0.061	7.2 ± 1.1	90.5 ± 1.0
CS 50			276.1 ± 12.4	0.259 ± 0.051	5.6 ± 0.7	90.9 ± 0.7
CS 10			235.6 ± 10.1	0.233 ± 0.047	4.5 ± 0.6	89.2 ± 1.2
CS(50)-Cys-L	49.5[a]		319.3 ± 12.7	0.356 ± 0.069	4.0 ± 0.3	87.9 ± 1.2
CS(50)-Cys-H	151.2[a]		332.5 ± 15.9	0.362 ± 0.070	3.1 ± 0.4	86.6 ± 1.9
TMC 50	39.0[b]	100	220.8 ± 8.7	0.221 ± 0.041	15.5 ± 0.7	93.5 ± 1.4
$PEG(5)_{19}$-g-TMC(50)	6.1[c]	34.2 ± 0.9	207.1 ± 7.6	0.209 ± 0.034	14.1 ± 1.1	92.5 ± 1.1

[a] Thiol group content (μmol/g), measured by iodine titration.
[b] Degree of quaternization (%), calculated by ^{1}H NMR analysis.
[c] Based on the primary amino group content in chitosan (%), calculated by ^{1}H NMR analysis.

2006). Fresh small intestine (jejunum) of sacrificed male Wistar rats was excised, rinsed with physiological saline and cut into segments of 4 cm length. Each segment was opened lengthwise along the mesentery and spread into 0.5 ml of freshly prepared solutions of enoxaparin PEC based on various chitosan derivatives, and then incubated for 2 h at 37 °C. The bioadhesiveness of different PEC was characterized by the corresponding reduced kcps percentage with respect to the initial value, which was attributed to the particle immobility at the intestinal surface by adhesion.

2.7. In vitro release studies

Enoxaparin release studies were performed by incubating 5 ml enoxaparin PEC in 30 ml of pH 1.2 SGF (in the first 2 h) or pH 6.8 SIF (in the followed 4 h) at 37 °C. At various time intervals, 2 ml samples were withdrawn and centrifuged at 20,000 rpm for 1 h. The supernatant was removed and assayed for enoxaparin according to the Azure II colorimetric method.

2.8. Oral absorption studies in rats

All animal studies were approved by the University Ethics Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male Wistar rats (weighting 250 ± 20 g) were administered at a single oral dose (1070 IU/kg) of enoxaparin PEC based on various chitosan derivatives (n = 5). Blood samples (about 300 μl) were withdrawn from the tip of the tail at 0, 2, 4, 5, 6, 8, 10 and 12 h in citrated microcentrifuge tubes and subsequently centrifuged at 1600 × g for 10 min to obtain plasma samples for further analysis. The extent of absorption was monitored by measuring the activated partial thromboplastin time value of the plasma sample with a standard kit (Lee et al., 2001). Main pharmacokinetic parameters were calculated from the APTT–time profiles of various enoxaparin PEC with the linear trapezoidal method. The absolute bioavailability (F) was calculated by comparing the AUC of oral administration with that of intravenous injection corrected by the administered dose.

2.9. Statistical analysis

Results are depicted as the mean value ± standard deviation (S.D.) from at least three measurements. Significance of difference was evaluated using one-way ANOVA at the probability level of 0.05.

3. Results and discussion

3.1. Chitosan modification and characterization

The MW of chitosan has a major influence on its biological and physicochemical properties, and it is considered to influence the biomolecule/chitosan complexes' surface characteristics, bioadhesive ability and the efficiency of cell uptake (Bravo-Osuna et al., 2007a). Therefore, a series of chitosans with different molecular weights were prepared using the oxidative degradation method without modifying the degree of deacetylation.

The mucoadhesive and permeation enhancing properties of chitosan could be significantly improved by the immobilization of thiol groups, due to the formation of disulfide bonds with mucus glycoproteins and the regulation effect of tight junctions, respectively (Bernkop-Schnürch et al., 2004). Previously, we demonstrated that the mucoadhesion of chitosan increased significantly with the increase of thiolation degree (Mei et al., 2008). Therefore, chitosan–cysteine conjugates with two degrees of thiolation (low 49.5 μmol/g and high 151.2 μmol/g) were prepared for the following studies, taking the influence of thiol group content into consideration.

Since chitosan is only soluble in acid environment, an attempt was made to make chitosan soluble over a wide pH range by synthesizing the partially quaternized derivative *N*-trimethyl chitosan chloride (TMC), which is soluble irrespective of pH and has proved to be a potent penetration enhancer of hydrophilic macromolecules across the intestinal epithelium (van der Merwe et al., 2004). Since the absorption enhancing effect of TMC is quaternization degree dependent (Hamman et al., 2003), TMC with optimum quaternization degree of 40% was employed in the present work. Moreover, to further improve the solubility of chitosan at physiological pH and the biocompatibility of TMC, polyethylene glycol (PEG) grafted trimethyl chitosan copolymers were synthesized in our laboratory. Our previous study showed that PEGylated TMC could significantly enhance the uptake of insulin in Caco-2 cells by adsorptive endocytosis (Mao et al., 2005b). Properties of chitosan derivatives employed in the present study are listed in Table 1.

3.2. Preparation and characterization of CS/enoxaparin PEC

In the present study, self-assembled enoxaparin PEC were prepared by electrostatic interaction between the positively charged chitosan derivatives and the negatively charged enoxaparin. The particle size, zeta potential and drug loading of various enoxaparin PEC are presented in Table 1. Results are in agreement with our previous study.

The particle size of PEC was chitosan MW-dependent and decreased significantly from 600 nm to 200 nm when CS MW decreased from 400 kDa to 10 kDa (p < 0.05). Moreover, compared with unmodified chitosan 50 kDa, the particle size of PEC prepared with TMC and PEGylated TMC copolymer of comparable molecular weight was much smaller, which can be explained by the stronger ionic interaction between the polymer and enoxaparin due to the high positive charge of the polymers. In contrast, a remarkable particle size increase was observed with the PEC formed with the

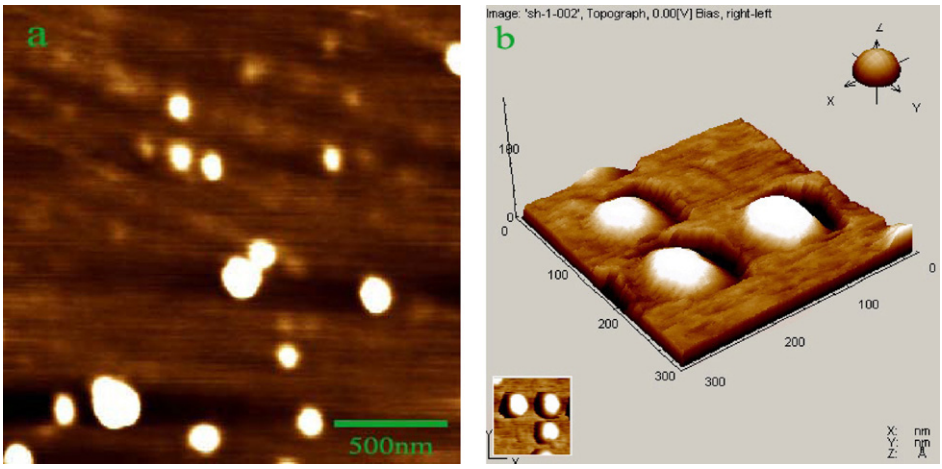

Fig. 1. Atomic force microscopy images of polymer/enoxaparin PEC. (a) Chitosan 50 kDa, (b) three-dimensional image of chitosan(50k)–cysteine conjugates.

thiolated chitosan, which can be attributed to the decreased charge density of the polymer due to amino group substitution.

The free amino groups of chitosan were responsible for the measured positive zeta potential of all the PEC, which might ensure the electrostatic interaction with the anionic groups of the mucus and facilitate enoxaparin uptake. For the CS 50 derivatives investigated in this paper, the zeta potentials were in the order of TMC 50 > PEG(5)$_{19}$-g-TMC(50) > CS 50 > CS(50)-Cys-L > CS(50)-Cys-H, which could be explained by the different charge density of the polymers due to the structure modification as described above.

Since enoxaparin is a highly negatively charged molecule, it is expected to be able to interact with chitosan derivatives very efficiently. As anticipated, the association efficiencies of enoxaparin were considerably high, at approximately 90%, regardless of polymer structure. As shown in AFM images (Fig. 1), the nanosized PEC were spherical and well separated from each other, suggesting that the PEC were stabilized against agglomeration in aqueous solution. Kinetically, the process of agglomeration depends on the activation energy. Both electrostatic and steric stabilization are possible mechanisms for providing a barrier to agglomeration of nanoparticles. In this paper, the steric effect of chitosan chains at the surface of the complex can significantly improve the stability and prevent aggregation of the complex even when the zeta potential is less than 10 mV as indicated in Table 1. Similar phenomenon was also observed by other researchers (Bae et al., 2009). The particle size values observed in the microphotographs were in the same range as measured by PCS technique.

3.3. Colloidal stability of PEC

3.3.1. Effect of temperature

Different PEC based on various chitosan derivatives were incubated at 25, 37, and 50 °C for 6 h, and subsequently characterized at predetermined time intervals at room temperature. All PEC were quite stable over 6 h at 25 °C without any change in particle size or kcps value (data not shown). Also, the PEC based on TMC 50 kDa and PEG(5)$_{19}$-g-TMC(50) showed no significant change in the particle properties at 37 °C for at least 6 h. In contrast, a decrease in particle size (ca. 10%) with an increased kcps value (ca. 10–20%) was observed for the PEC of CS 50 and CS(50)–cysteine conjugates at 6 h at 37 °C. And this tendency was more significant at 50 °C as shown in Fig. 2. For instance, the particle size of PEC prepared with unmodified chitosan 50 decreased from 285.4 ± 6.9 nm to 254.3 ± 8.6 nm with an increased kcps value of 15.2% over 6 h. This suggests that elevated temperature may have a profound influence on the kinetics of complex formation, thus facilitating nanocomplexes formation with a low aggregation tendency. Additionally, a rise in temperature increases the entropy of the system which is associated with the release of small counterions initially bond to the polymers, resulting in compaction of the particles.

Similar changes were found for the PEC formed with thiolated chitosans. In addition to the explanations stated above, it should also be noted that higher temperature might enhance the oxidation behavior of free thiol groups in the polymer, which may promote the formation of inter-chain disulfide bonds and therefore facilitate the formation of compact particle structure with smaller size. Compared with CS 50 and CS (50)–cysteine conjugates, the PEC formed with TMC 50 and PEGylated TMC copolymer seemed to be less sensitive to thermal change. This is probably due to the relatively compact nanocomplexes structure caused by the stronger ionic interaction among the polymer chains.

3.3.2. Effect of system pH

It was found that all PEC were stable in SGF and SIF with no significant dissociation over 6 h (data not shown). As reported previously, the PEC prepared with chitosan and insulin were not stable in acidic environment and the dissociation appeared immediately after adding SGF, which may be a main obstacle for the consideration of peroral administration (Jintapattanakit et al., 2007). However, unlike insulin (with apparent isoelectronic point of 6.4), enoxaparin employed in the present study is known as a highly

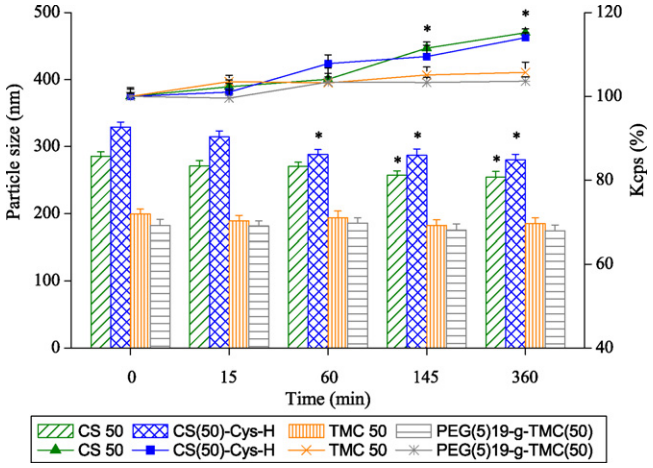

Fig. 2. Mean particle size (bars) and kcps value (lines) obtained from PEC based on CS 50, CS(50)-Cys-H, TMC 50 and PEG(5)$_{19}$-g-TMC(50) at 50 °C for 360 min. *Statistically significant differences with the values at 0 min ($p < 0.05$).

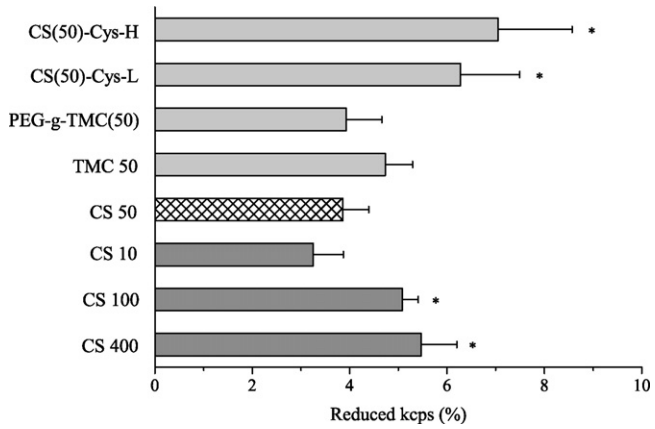

Fig. 3. Bioadhesion (reduced kcps percentage) of PEC based on various MW chitosans and chitosan derivatives, including chitosan(50k)–cysteine conjugates (CS-Cys-H 151.2 μmol/g, CS-Cys-L 49.5 μmol/g), TMC 50 kDa, PEG(5)$_{19}$-g-TMC(50), measured by dynamic light scattering. *Statistically significant differences with CS 50 ($p < 0.05$).

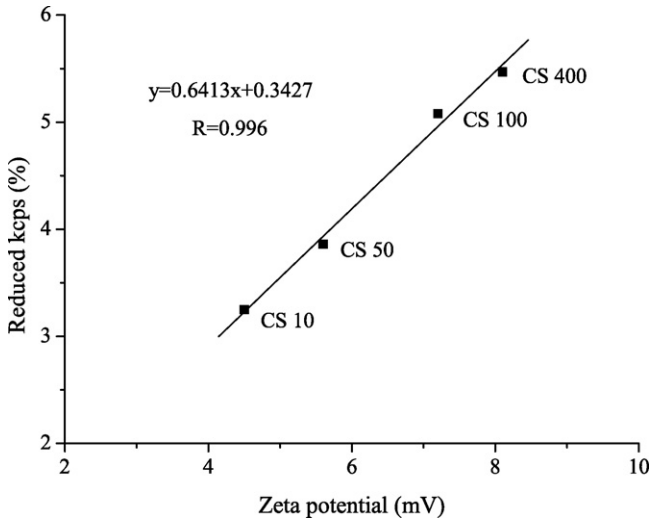

Fig. 4. The relationship between the measured zeta potential and reduced kcps percentage of different MW chitosan based PEC.

acidic mucopolysaccharide, which can keep the electrostatic attraction with oppositely charged chitosan derivatives in a relatively wider pH range, and consequently enable the PEC to keep stable even in SGF, which will facilitate the oral administration. It was noticed that the particle size of the PEC incubated in SGF was significantly larger than that in SIF (359.6 ± 15.2 nm versus 281.1 ± 9.2 nm for CS 50 after 6 h incubation), which can probably be attributed to the fact that in acidic medium, chitosan is highly positively charged and the relatively strong mutual electrostatic repulsion of free ammonium groups on the polymer chains caused a highly stretched structure of the PEC.

3.4. Bioadhesion ex vivo assay

Here the bioadhesion of the PEC was investigated by the decrease of kcps upon incubation of the PEC with the small intestine of rats. As shown in Fig. 3, significantly reduced kcps percentage (3.25–7.05%) with respect to the initial value was observed for all the PEC investigated. This could be explained by the strong mucoadhesive interactions between positively charged chitosan derivatives and the negatively charged mucin gycoproteins, due to the formation of ionic and hydrogen bonds.

A significant increase in the bioadhesion of the PEC was observed when CS MW increased from 10 kDa to 400 kDa ($p < 0.05$), probably due to the increase of the positive charge density with molecular weight as presented in Table 1. Interestingly, a linear relationship between the measured zeta potential (x) and reduced kcps percentage (y) of chitosan based PEC was established and was shown in Fig. 4. In addition, the higher tendency of longer chains chitosan to interpenetrate and entangle with the mucus protein chains may also contribute to the bioadhesion (Bravo-Osuna et al., 2007b).

In addition to molecular weight and charge density, polymer structure could also strongly influence the bioadhesion of the corresponding PEC. Compared to unmodified chitosan and other derivatives, thiolated chitosans showed significantly higher bioadhesion despite of the relatively low surface charge. Previously we also measured the bioadhesion of thiolated chitosans using rotating cylinder method and got the same conclusion (Mei et al., 2008). This could be explained by the formation of covalent disulphide bonds between thiol groups of the polymer and cysteine rich subdomains of glycoproteins in the mucus (Bernkop-Schnürch, 2005). Additionally, bioadhesion of the PEC was influenced by the thiolation degree. When the thiol group content increased from 49.5 to 151.2 μmol/g, there was a slight increase in bioadhesion. Moreover, TMC and PEGylated TMC copolymer also exhibited relatively higher adhesion tendency compared to unmodified chitosan 50 kDa, which could be explained by the increased number of positive charges on the polymer chains caused by chemical modification, forming more electronic non-covalent bonds within the polymer–mucus interface. Compared with TMC, the PEGylated copolymer showed a reduced bioadhesion, which could be attributed to the steric effect of PEG chains, weakening the interaction with mucin.

It is noticed that the bioadhesion of PEC with small intestine of rats was less than 10% in this study. This is because we just took 4 cm length segment of intestine for the study. In the *in vivo* condition, the intestine is much longer with larger surface area and then the intestine targeted function can be more effective. In order to test this point, we also investigated the PEC adhesion on the parts of stomach and colon using the same experimental method. For all the complexes investigated, the reduced kcps percentage was less than 1.5%, indicating that the bioadhesion in stomach and colon was 4–5 times weaker than that in the intestine, emphasizing the intestine targeting effect of the PEC. This result can be explained by the fact that the mucoadhesive capacity of chitosan polyelectrolyte complexes depends on the pH of GIT, which can influence the ionization degree of chitosan (p*Ka* 6.5) and sialic acid residues on mucin (p*Ka* 2.6) (Peppas and Sahlin, 1996). Therefore, at pH lower than 2.6 (stomach, pH 1.2–2.1) or higher than 6.5 (colon pH 7–8), the ionization degree of either sialic acid residues or chitosan would be reduced, causing the decreased electrostatic interaction of the polyelectrolyte complexes with the mucin.

Moreover, it should be noted that the surface area per unit length significantly varies within the gastrointestinal tract, and it is the highest in the jejunum and the ileum, accounting for more than 99% of the total. This can be explained by the highest density of villi and microvilli structures in the small intestine, and much lower density in other segments of the GIT (Avdeef, 2001).

3.5. In vitro release studies

The *in vitro* drug release of the PEC versus time was investigated according to the physiological pH change of the gastrointestinal tract, as shown in Fig. 5. At pH 1.2 (SGF) no drug release was observed. As mentioned in the part of 3.3.2, enoxaparin is known

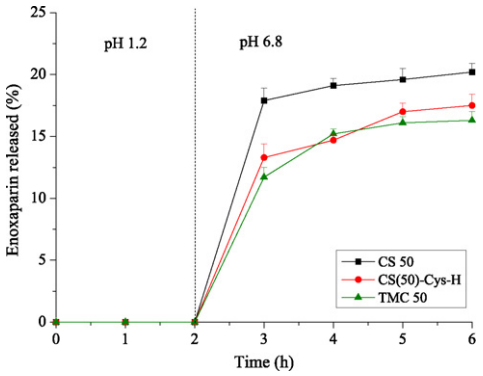

Fig. 5. Release profiles of enoxaparin PEC formed with chitosan and its derivatives at pH 1.2 and pH 6.8 ($n = 3$).

as a highly acidic mucopolysaccharide, which can keep the electrostatic attraction with oppositely charged chitosan derivatives in a relatively wider pH range, and consequently enable the PEC to keep stable even at pH 1.2 (SGF), which will facilitate the oral administration.

At pH 6.8 (SIF) the cumulative amount of drug release increased with time, but the drug release was very slow after 5 h. This may be related by the shortage of enzyme in the *in vitro* condition. A slow release behavior of enoxaparin from polyelectrolyte complexes at pH 6.8 (SIF) could be of interest for oral administration. In fact, it would be desirable that the release process starts only when the nanoparticles reach and interact with the absorbing intestinal epithelium. Furthermore, the followed *in vivo* studies in the part of 3.6 confirmed the usefulness of polyelectrolyte complexes for the oral delivery of enoxaparin. Similarly, previously reported heparin-loaded nanoparticles also displayed a slow *in vitro* release and led to very positive *in vivo* absorption results (Jiao et al., 2002).

3.6. In vivo oral absorption studies

After a single oral dose of enoxaparin PEC based on various polymer formulations, the absorption of the drug in rats was evaluated by APTT assay as shown in Fig. 6. Compared with enoxaparin formulated in saline, all CS/enoxaparin PEC induced a statistically significant increase in APTT value ($p < 0.05$). In terms of absolute bioavailability (Table 2), the PEC with chitosan MW 10 kDa, 50 kDa and 100 kDa, which exhibited varied bioadhesion in *ex vivo* assay, also showed different *in vivo* absorption, and a good correlation between bioadhesion and *in vivo* absorption could be established

Table 2
Main pharmacokinetic parameters of enoxaparin in fasted rats following a single oral administration of various enoxaparin-loaded PEC versus the enoxaparin solution administered intravenously.

Formulations	$APTT_{max}$ (s)	T_{max} (h)	$AUC_{0-12\,h}$ (s h)	F (%)
iv. solution	–	–	42.24	100
Oral solution	22.2	–	5.88	1.67
CS 10	23.7	6	20.68	5.87
CS 50	25.2	6	25.50	7.24
CS 100	25.4	6	29.42	8.36
CS 400	23.3	5	17.73	5.04
CS(50)-Cys-L	25.6	8	32.96	9.36
CS(50)-Cys-H	25.9	8	34.04	9.67
TMC 50	25.7	6	33.15	9.42
PEG-g-TMC	25.2	6	26.49	7.53

($r = 0.97$). However, $AUC_{0-12\,h}$ of the CS 400/enoxaparin PEC was much smaller than that of CS 100/enoxaparin PEC despite its high bioadhesion. This could be explained by the fact that *in vivo* absorption of PEC is not only related to the bioadhesion capacity but also the particle size. It should be noted that PEC formed with CS 400 had a relatively large particle size of 681.2 ± 15.1 nm. As demonstrated, particle size is an important parameter controlling the internalization of nanoparticle into epithelia of the GIT by endocytosis and as a rule of thumb, sizes smaller than 500 nm are required (Jung et al., 2000). Therefore, a fine balance must be achieved between bioadhesion (better with high MW) versus particle size of nanocomplexes (better with low MW) to obtain appreciable oral absorption of enoxaparin.

Moreover, the structure of chitosan derivatives used for enoxaparin PEC formation is also crucial for the *in vivo* absorption. As shown in Table 2, the enoxaparin PEC of thiolated chitosans exhibited comparatively high oral bioavailability. This can be explained by the presence of thiol groups in the polymer, which can lead to significantly improved mucoadhesion by the formation of covalent disulfide bonds with mucus, and the inhibition of protein tyrosine phosphatase resulting in opening of the tight junctions, thus further enhancing the permeation of enoxaparin (Bernkop-Schnürch et al., 2004). Increasing thiolation degree from 49.5 to 151.2 μmol/g caused only a slight increase in bioavailability from 9.36% to 9.67%. This is in agreement with the slight bioadhesion increase with the increase of thiolation degree, as described in part 3.4. For chitosan and thiolated chitosans of comparable molecular weight (50 kDa), a correlation was established between bioadhesion and absolute bioavailability, as shown in Fig. 7.

Compared with unmodified chitosan, a higher bioavailability was also obtained from PEC of TMC, and no remarkable difference

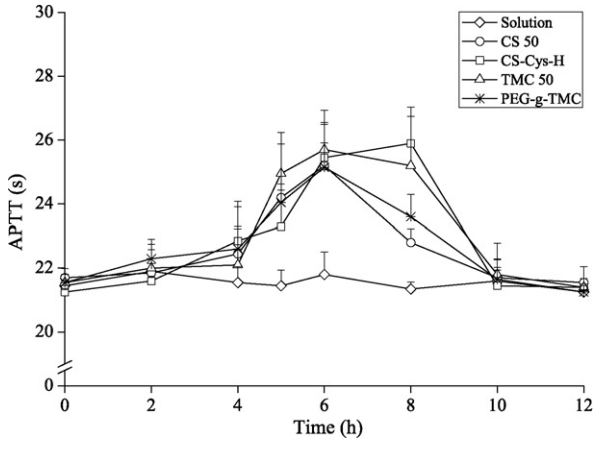

Fig. 6. Mean APTT over time after a single oral administration of enoxaparin-loaded PEC (1070 IU/kg) based on various chitosan derivatives in rats ($n = 5$).

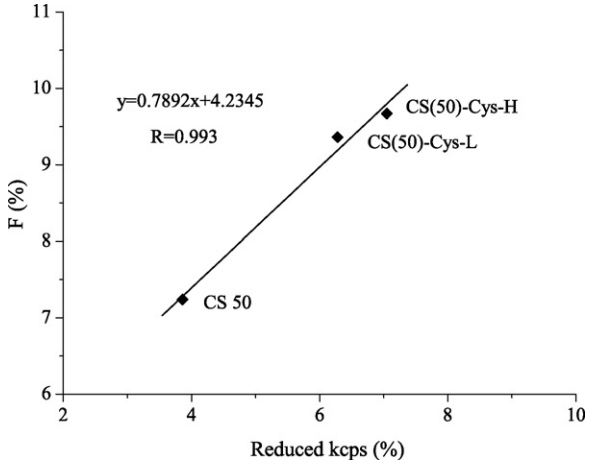

Fig. 7. The relationship between the bioadhesion and absolute bioavailability of PEC formed with chitosan and thiolated chitosans of 50 kDa.

in bioavailability between TMC based PEC and thiolated chitosan based PEC was found, although TMC had a weaker bioadhesion compared to thiolated chitosan (Fig. 3). This may be explained by the relatively high charge density on the TMC molecules in a neutral pH environment, which influences the interaction of the polymer with the negatively charged sites on the cell membranes and facilate the opening of tight junctions (Jonker et al., 2002). Similarly, the absorption enhancing effect of TMC was also reported by other groups (Thanou et al., 2001; Chen et al., 2008). However, compared with TMC, PEC of PEGylated TMC copolymer showed a decreased absorption of enoxaparin and the absolute bioavailability was only slightly higher than that of unmodified chitosan. This can probably be attributed to the shielding effect of the PEG chains on the polymer, which not only causes the decreased electrostatic interaction of the PEC with the mucin, but also prohibits the opening of the tight junctions by TMC due to the steric effect.

Based on these findings, it is reasonable to hypothesize that enoxaparin uptake was a consequence of a strong bioadhesion between the positively charged PEC and the negatively charged mucus, which subsequently facilitated the endocytosis. In addition, the interaction of chitosan derivatives with the cell membrane resulting in a structural reorganization of tight junction-associated proteins could also play an important role in drug absorption via paracellular route in the gastrointestinal tract.

4. Conclusions

In this work, self-assembly chitosan derivatives/enoxaparin polyelectrolyte complexes were developed to enhance the oral absorption of enoxaparin. The PEC had a spherical shape with positive charge and a mean diameter in the range of 200–600 nm. We demonstrated that chemical modification of chitosan could significantly influence the resultant PEC's bioadhesion and consequently the oral absorption of enoxaparin. The PEC formed with TMC and thiolated chitosan had significantly improved bioadhesion and bioavailability compared to unmodified chitosan. However, PEC of PEGylated chitosan did not show a significantly enhanced permeation of enoxaparin. The current strategy using polyelectrolyte complexes based on chitosan derivatives as drug carriers seems to have potential applications for the oral delivery of enoxaparin.

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