Disulfide cross-linked biodegradable polyelectrolyte nanoparticles for the oral delivery of protein drugs

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Polyelectrolyte nanoparticles were prepared by mixing chitosan (CS) and polyaspartic acid functionalized with cysteamine (PASP–CA) under mild conditions. The nanoparticles (NPs) included thiol moieties that were cross-linked to render the NPs stable at physiological pH. It was demonstrated that reacting the thiol moieties to form disulfide bonds led to improved stability. NPs without disulfide bonds were readily deconstructed at physiological pH. The disulfide bond cross-linked NPs can remain more stable in physiological pH solution and decrease the loss of protein drugs caused by simulating the gastric pH environment, and can release the drugs in the simulated pH environment of the intestine. This approach has potential for the *in vivo* application of NPs for the oral delivery of protein drugs.

Introduction

Macromolecule drugs such as peptides and proteins are normally restricted to intravenous administration because they are easily hydrolyzed in the acidic conditions of the stomach, degraded by proteolytic enzymes in the gastrointestinal (GI) tract and metabolized *via* enterohepatic circulation. Although significant advances in the areas of biotechnology have offered the possibility of working with large quantities of biological agents, among them only a few therapeutic peptide agents are currently marketed and in clinical use. Therefore, the development of an oral administration system of macromolecules is a challenging opportunity for researchers.[1–3]

The successful oral administration of protein drugs requires that the drugs be protected in the harsh environment of the stomach, to then be slowly released at the uptake site in the intestine. One possibility to overcome this problem is to encapsulate them in colloidal NPs that can protect the peptide from being degraded in the GI tract and facilitate their transportation into systemic circulation.[4–5]

Chitosan (CS), a weak cationic polysaccharide, produced by deacetylation of the natural polymer chitin, has many useful biological properties, such as biocompatibility, biodegradability, and bioactivity. Because of the existence of amine groups, CS is a polycation and is able to form intermolecular complexes with a wide variety of polyanions including hyaluronic acid,[6] poly(galacturonic acid),[7] alginate,[8] gelatin,[9] dextran sulfate,[10] and poly(acrylic acid).[11] But these NPs were readily deconstructed at physiological pH and led to burst release.

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PASP is a poly(α-amino acid), which can be classified as a weak synthetic polyelectrolyte. Poly(amino acids) would seem to have considerable advantages over other polymers owing to their protein-like structure.[12] PASP is easy to modify and is of potential interest for use as a biodegradable water soluble poly(carboxylic acid).

In the present study, to improve the oral delivery of protein drugs, investigations were made toward oral drug delivery systems using insulin as a model drug. The polyaspartic acid functionalized with cysteamine (PASP–CA)–chitosan (CS) nanocomplexes were prepared under quite mild conditions and stabilized *via* the formation of inter- and intra-chain disulfide bonds within these particles. The disulfide bond cross-linked NPs can remain stable and decrease the loss of protein drugs in a low pH environment, and can release the drugs in high pH environments. This is very significant to the oral delivery of not only protein drugs like insulin, but also other drugs such as aspirin.

Experimental

Materials

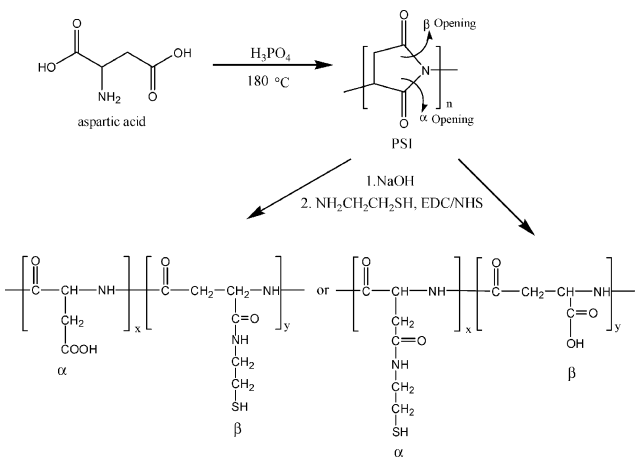
Chitosan with an M_w = 50 kDa was purchased from Yuhuan Ocean Biochemical Co., Ltd. (Zhejiang, China) and the degree of deacetylation was 0.93. L-Aspartic acid was purchased from Beijing Xing Jing Ke Biotechnology Co., Ltd. Insulin, with an M_w of 5.7 kDa, was purchased from Beijing Jun Yao WeiYe Biotechnology Co., Ltd. Coomassie brilliant blue G-250 was obtained from Fluka (USA). Cysteamine (CA) was purchased from Bai Ling Wei (Beijing, China). 1-Ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd. and used as received. All other reagents were of analytical grade and were not purified before use.

Methods

Synthesis of polyaspartic acid–cysteamine. Poly(succinimide) (PSI) was synthesized by the acid catalyzed thermal polycondensation of L-aspartic acid as previously reported.[13] Upon hydrolysis of PSI in aqueous NaOH, poly(L-aspartic acid) was obtained. To introduce thiol functionality, PASP was conjugated with cysteamine in the presence of 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC)–*N*-hydroxysuccinimide (NHS).[14] Briefly, to an aqueous (50 mL) solution of PASP (1.0 g), were added EDC, NHS and CA under an atmosphere of N_2. After stirring for 7 h at 277 K, the reaction solution was placed into a dialysis bag for 6 days at 277 K and then freeze-dried for 24 h. This PASP consists of L-aspartic acid repeating units in the α- and β-form (Scheme 1). Weight average molecular weights of PASP were measured to be 22.9 kDa by gel permeation chromatography (Waters 510, USA), M_w = 22.9 kDa, PDI = 1.402.

Preparation of the disulfide bond cross-linked PASP–CA–CS NPs. PASP–CA–CS NPs were prepared by mixing negatively charged PASP–CA and positively charged CS with a dropping method.[15] To explain the procedure briefly: 100 mg chitosan was dissolved in 100 mL of acetic acid (1 mg mL^{-1}, w/v) under magnetic stirring at room temperature and the solution was stirred for 12 h, and filtered through filter paper for use. 100 mg PASP–CA was dissolved in 100 mL of deionized water, stirred for 12 h, and filtered through filter paper for use. Afterwards, the CS solution was dropped into PASP–CA solution in a different weight ratio under magnetic stirring. The pH of the solution for the preparation of NPs was 4.0. The cross-linked NPs were prepared by the formation of intra-chain disulfide bonds with the addition of chloramine-T. These NPs were isolated by ultracentrifugation at 38 000 rpm for 30 min at 277 K.

Preparation of insulin loaded PASP–CA–CS NPs. The drug-loaded NPs were prepared by dropping a mixture of CS and insulin into a PASP–CA solution. PASP–CA was dissolved in 4 mL deionized water at a specified concentration (0.1, 0.5, 1.0, 1.5 and 2.0 mg mL^{-1}). Then this solution was dropped into a solution of 4 mL of a specified concentration (0.1, 0.5, 1.0, 1.5 and 2.0 mg mL^{-1}) of CS solution and 2 mL deionized water, while sonicating with an ultrasonic sonicator at 160 W. Then the insulin loaded cross-linked NPs were prepared *via* the addition of chloramine-T.



Scheme 1 Synthesis of PASP–CA.

Characterization of nanospheres and nanoparticles. Particle size was measured by photon correlation spectroscopy (PCS) at 298 K with a detection angle of 90° and zeta potential was measured by ZetaPlus measurement (Brookhaven, America).

The morphologies and approximate sizes of nanospheres and the composite nanospheres were studied with a TEM (Tecnai G2 T20ST).

Determination of insulin loading capacity and encapsulation efficiency of the NPs. Bound and unbound insulin were separated by ultracentrifugation of the nanosuspension at 38 000 rpm at 277 K for 30 min (Optima LE-80k Ultracentrifuge, Beckman). The amount of free insulin in the clear supernatant was measured by a Bradford protein assay using a UV spectrometer at 595 nm (Shimadzu UV 2550, Japan).[16] The insulin encapsulation efficiency (EE) and loading capacity (LC) of the NPs were calculated using the following equations:

$$\mathrm{EE}\% = \frac{\text{total insulin} - \text{free insulin}}{\text{total insulin}} \times 100\%$$

$$\mathrm{LC}\% = \frac{\text{total insulin} - \text{free insulin}}{\text{nanoparticles weight}} \times 100\%$$

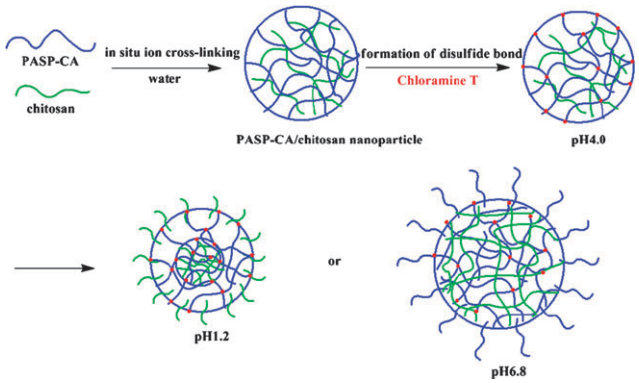
All measurements were performed in triplicate and averaged.

***In vitro* release studies.** Typically, insulin-loaded NPs (20 mg) were placed in 1 mL of pH 1.2 hydrochloric acid for 120 min with horizontal shaking at 310 K. Then, the NPs were isolated by ultracentrifugation and put in 1 mL of pH 6.8 PBS solution with horizontal shaking at 310 K. At appropriate time-points, samples were centrifuged and aliquots of 200 μL were withdrawn and replenished by fresh buffer solution. The amount of free insulin was determined by Bradford assay.[16] A calibration curve was made using non-loaded NPs in order to correct for the intrinsic absorption of the polymer.

Circular dichroism (CD) measurements. Circular dichroism (CD) spectroscopy (Jasco 715 spectropolarimeter) was used to measure the conformational change of the released insulin with respect to the native insulin. A solution of the native insulin or the released insulin was diluted to 0.1 mg mL^{-1} and scanned over the wavelength range 200–260 nm, using a 1 mm quartz cylindrical cell.[17]

Results and discussion

In the study, a novel nanoparticle system composed of PASP–CA and CS was prepared using a mild ionic-gelation method under magnetic stirring at room temperature, then making thiol cross-links to prepare disulfide. The disulfide bond cross-linked NPs are intended to remain stable in physiological pH and decrease the loss of protein drugs in a pH environment of 1.2, and release the drugs in a pH environment of 6.8 (Scheme 2).



Scheme 2 Schematic representation of the pH-responsive characteristics of PASP–CA–CS NPs in different pH environments.

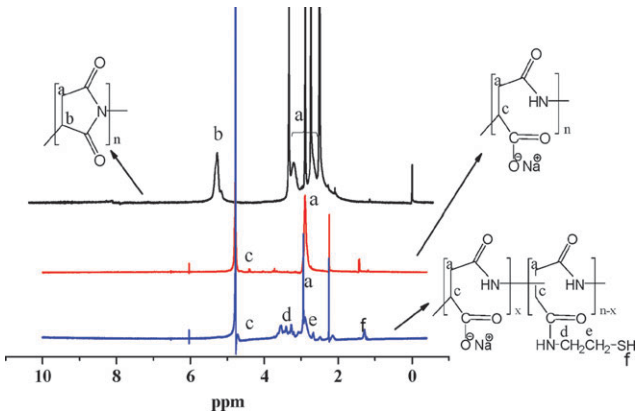


Fig. 1 ^{1}H NMR spectra (400 MHz) (top) PSI in DMSO-d6; (middle) PASP in D_2O; (bottom) PASP–CA in D_2O.

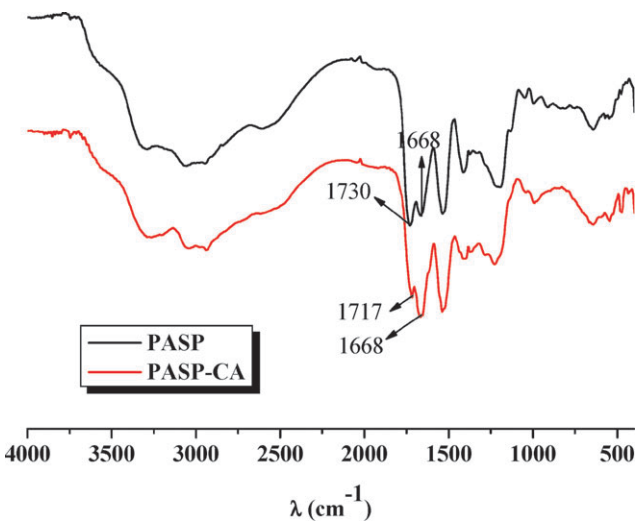


Fig. 2 FT-IR spectra of PASP and PASP–CA.

Characterization of PASP–CA

Fig. 1 shows the ^{1}H NMR and Fig. 2 shows the FT-IR spectra of PASP and synthesized PASP–CA, respectively. As shown in Fig. 1, PASP–CA showed three new signals at 1.60, 2.68 and 3.41, which represent the SH, CH_2 and CH_2 of cysteamine, respectively. In the FT-IR spectra, the peak at 1730 cm^{-1} of C═O moved to 1717 cm^{-1}; it was confirmed that a carboxyl group was converted to an amide group. The result indicated that PASP–CA was successfully synthesized. Degrees of modification of the synthesized PASP–SH were estimated using ^{1}H NMR spectra. The degree of functionalization of PASP–SH was approximated at 20%. The degree of functionalization was estimated from elemental analysis of the polymer, which corresponds to 24.5% modification. The two methods had similar results.

As shown in Table 1, the mean size and encapsulation efficiency both increased as the weight ratio of PASP–CA/CS increased. As the weight ratio increased, the concentration of thiol moieties increased. More thiol moieties reacted with each other to form disulfide bonds, so many insulin molecules were encapsulated in the NPs. The mean size slow growth was due to more disulfide bonds making the NPs stack tightly.[15] The weight ratio of 3/1 was chosen as the research sample.

From Fig. 3, as the salt concentration increased, the size of the NPs increased. When the salt concentration increased to 0.020 mol L^{-1}, the size of the NPs kept the same along with the addition of salt. The addition of salt interferes with the electrostatic attraction between the polymer chains with opposite charges, which reduces the interaction. Therefore at low salt concentrations, the effect on the formation and growth of nanoparticles may exceed the screening of the interaction, which leads to the enhancement of the final interaction. At higher salt concentration, the dominant screening of the interaction leads to the total reduction of the interaction. However, due to the cross-linked disulfide bonds, the NPs could not break apart and were more stable.

In the study, the NPs were prepared in deionized water (pH 4.0). At pH 4.0, CS and PASP–CA were ionized. The ionized CS and PASP–CA could form polyelectrolyte complexes, which resulted in a matrix structure with a spherical shape. When the thiol moieties reacted with each other, the disulfide cross-linked NPs had small diameters compared with those of the non-cross-linked NPs (Fig. 4a and 4b). At pH 1.2 (simulating the pH in the stomach), if there was no disulfide in the NPs, they would break apart, but the cross-linked NPs have a core–shell structure (Fig. 4c). From this picture, the successful fixed structure could be confirmed. At pH 6.8 (simulating the pH in the intestine), the electrostatic interaction between PASP–CA and CS was therefore relatively weaker as compared to that at pH 4.0, thus leading to a relatively larger size of the NPs (Table 2). At pH 1.2 and 6.8, the NPs displayed different morphology. Both cross-linked and non-cross-linked NPs were stable when exposed to pH 4 buffer solution. The NPs swelling and the diameter was obviously increased. From Table 2, we also can find the same result. The covalently cross-linked NPs were more firm as they remained stable when incubated in a hydrochloride solution. As shown by TEM, the covalently cross-linked NPs showed a compact core–shell separation structure at pH 1.2 (Fig. 4c) and a loop conformation leading to an increase in particle size at pH 6.8 (Fig. 4d). The swelling degree of NPs was higher in a solution with pH 6.8 than in that of pH 1.2.[18,19]

As shown in Fig. 5a, the non-cross-linked NPs released insulin so fast that it leads to burst release. While the cross-linked NPs have a slow rate of insulin release.

Table 1 Characterization of PASP–CA–CS NPs and encapsulation efficiency of NPs[a]

PASP–CA/CS sample	Mean size/nm	PI	Encapsulation efficiency (%)
1/3	142.3 ± 3.3	0.14	62.6 ± 2.2
1/2	145.2 ± 2.7	0.17	72.9 ± 4.5
1/1	145.5 ± 3.2	0.31	88.1 ± 6.2
2/1	147.7 ± 1.7	0.11	90.2 ± 6.9
3/1	155.8 ± 2.4	0.08	95.7 ± 6.2

[a] The concentrations of PASP–CA and CS are both 1 mg mL^{-1}. Insulin concentration is 1 mg mL^{-1}.

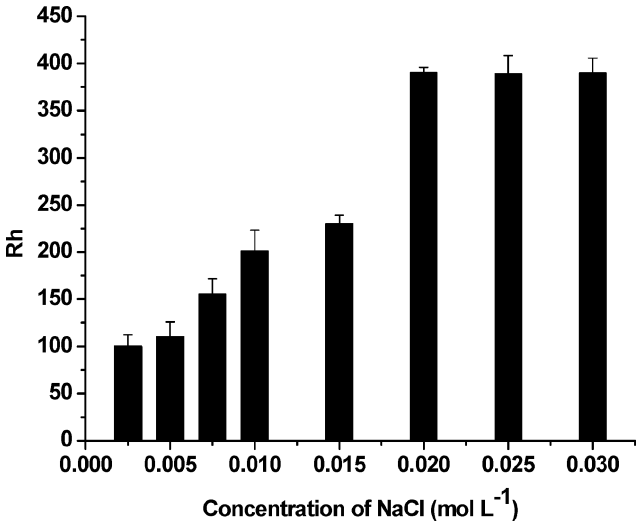


Fig. 3 Effect of salt concentrations on NPs.

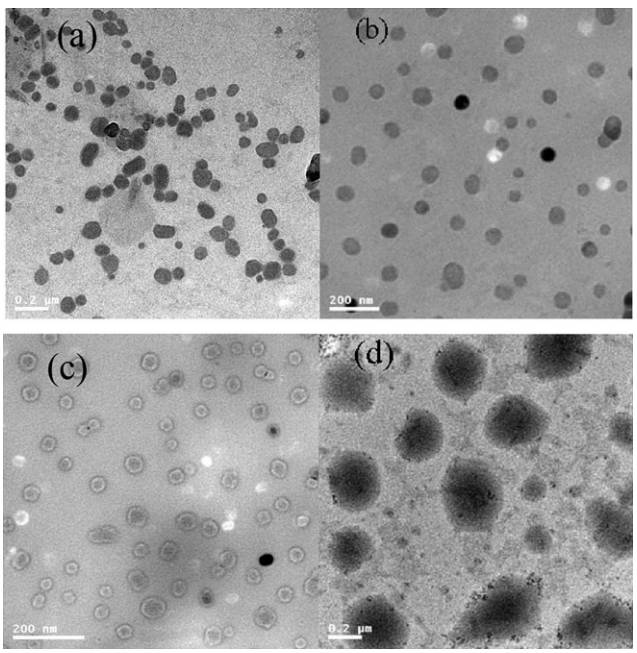


Fig. 4 TEM micrographs of PASP–CA–CS NPs in different environments. (a) PASP–CA–CS NPs at pH 4.0 (b) PASP–CA–CS (CL) NPs at pH 4.0 (c) PASP–CA–CS (CL) NPs at pH 1.2 (d) PASP–CA–CS (CL) NPs at pH 6.8 (CL: cross-linked).

At pH 1.2, there were little electrostatic interactions between non-cross-linked PASP–CA and CS, and the NPs became unstable and subsequently broke apart. After the cross-linking, disulfide bond inter-nanoparticles exist, so they become stable and release insulin slowly. Apart from the pH, the release of the insulin could be modulated by varying the cross-linking degree and composition of the NPs. As shown in Fig. 5b, when the cross-linking degree decreases, the chain of NPs does not stack tightly, so the NPs release insulin a little faster than those of more cross-linked degree. The disulfide bond cross-linked NPs remain more stable in physiological pH solution and decrease the loss of protein drugs caused when simulating the gastric pH environment, and can release the drugs in the simulated pH environment of the intestine.

Circular dichroism spectroscopy was used to examine the conformation and self-association of insulin. There are three common secondary structures in insulin, namely α-helices, β-sheets, and turns. Native insulin has two extreme valleys at 208 and 222 nm.[20] Since α-helices are one of the elements of secondary structure, the quantitative analysis of the structural change of insulin could be evaluated by the content of the preserved α-helices. The α-helical content of protein is estimated according to the following equation:[17]

$$\%\ \alpha\text{-helical content} = \frac{\theta_{\mathrm{mrd}} - 4000}{33\,000 - 4000}$$

Where, θ_{mrd} is the mean molar ellipticity per residue at 208 nm (deg cm^2 $dmol^{-1}$). Usually the raw data from the experiment are expressed in terms of θ_{d} (the ellipticity in units of mdeg). However, it can be converted to mean molar ellipticity per residue, using the following equation:[21]

$$\theta_{\mathrm{mrd}} = \frac{\theta_{\mathrm{d}} M}{10CLN}$$

Where, M is the insulin molecular weight (Da), C is the insulin concentration (mg mL^{-1}), L is the sample cell path length (cm), and N is the number of amino residues. As indicated by the circular dichroism spectra (Fig. 6), the calculated percentage of α-helix in the native insulin and the released insulin are 34% and 33%, respectively. In other words, no significant conformation change was noted for the released insulin by using chloramine-T as compared with the native insulin. The presence of chloramine-T did not disrupt the normal pattern of insulin assembly and lead to conformational changes. The insulin released from cross-linked NPs also has no significant conformation. The disulfide bond cross-linked NPs retained the structure and biological activity of encapsulated insulin. Mentioned above all, the results suggest the disulfide bond cross-linked NPs are promising carriers for oral delivery of protein drugs. Studies on the properties of the

Table 2 Mean particle size and zeta potential values of NPs in distinct pH environments (n = 3)

Sample	pH of nano-suspension	Particle size/nm	Zeta potential/mV
PASP–CA–CS NPs	4.0	145.7 ± 2.1	−17.48 ± 0.66
PASP–CA–CS (CL) NPs	4.0	134.4 ± 3.2	−22.54 ± 0.82
PASP–CA–CS (CL) NPs	1.2	127.2 ± 1.9	15.92 ± 0.67
PASP–CA–CS (CL) NPs	6.8	172.8 ± 2.4	−24.57 ± 0.36
PASP–CA–CS/insulin NPs	4.0	179.5 ± 3.2	−20.69 ± 0.92
PASP–CA–CS (CL)/insulin NPs	4.0	158.4 ± 4.2	−17.59 ± 1.24

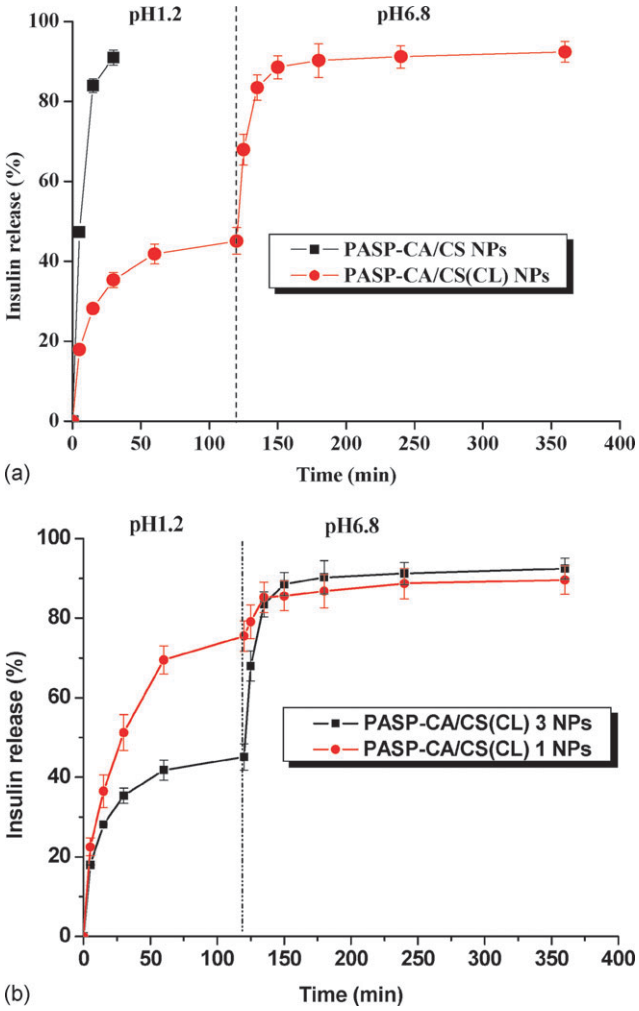


Fig. 5 (a) Release profiles of insulin from PASP–CA–CS and PASP–CA–CS (CL) NPs at pH 1.2 and 6.8; (b) release profiles of insulin from PASP–CA–CS (CL) NPs with different weight ratios at pH 1.2 and 6.8.

biodegradable NPs such as cell viability and the release kinetics are now in progress.

Conclusion

We have shown that cross-linking NPs using disulfide bonds provides a viable route to pH responsive NPs, which can release encapsulated materials. These NPs were more stable at physiological pH than non-cross-linked NPs. An *in vitro* release study showed that reacting the thiol moieties to form disulfide bonds did not damage the bioactivity of insulin. This offers new opportunities for applications in oral protein and peptide drug delivery.

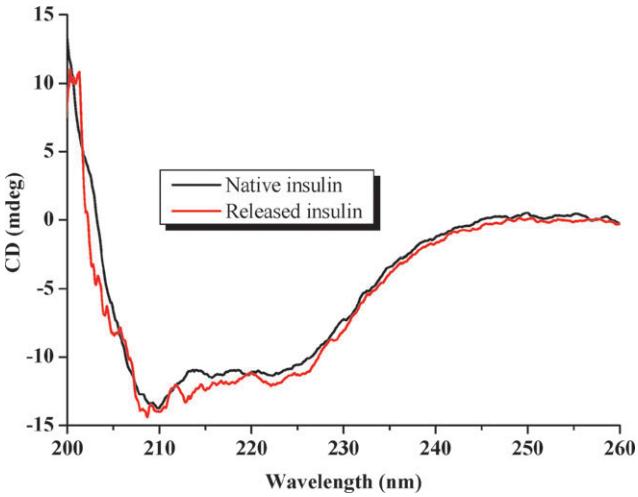


Fig. 6 Circular dichroism spectra of native insulin and insulin released from cross-linked NPs.

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