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Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo

Yan Pan [a], Ying-jian Li [a], Hui-ying Zhao [a], Jun-min Zheng [a,*], Hui Xu [a], Gang Wei [a], Jin-song Hao [b], Fu-de Cui [a]

[a] *School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, PR China*
[b] *Department of Pharmacy, National University of Singapore, Singapore 119260, Singapore*

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Abstract

There are many ongoing investigations to improve the oral bioavailability of peptide and protein formulations. Bioadhesive polysaccharide chitosan nanoparticles (CS-NPs) would seem to further enhance intestinal absorption of them. In this study, Insulin-loaded CS-NPs were prepared by ionotropic gelation of CS with tripolyphosphate anions. Its particle size distribution and zeta potential were determined by photon correction spectroscopy and laser Dopper anemometry. The ability of CS-NPs to enhance intestinal absorption of insulin and increase the relative pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats after oral administration of various doses of insulin-loaded CS-NPs. CS-NPs had a particle size in the range of 250–400 nm and its polydispersity index was smaller than 0.1, positively charged, stable. Insulin association was found up to 80% and its in vitro release showed a great initial burst with a pH-sensitivity property. CS-NPs enhanced the intestinal absorption of insulin to a greater extent than the aqueous solution of CS in vivo. Above all, after administration of 21 I.U./kg insulin in the CS-NPs, the hypoglycemia was prolonged over 15 h and the average pharmacological bioavailability relative to SC injection of insulin solution was up to 14.9%.

Keywords: Insulin; Chitosan-nanoparticles; Oral administration; Relative pharmacological bioavailability

1. Introduction

Mucosal delivery of insulin is one of the most intensively studied subjects, among which, achieving oral delivery of insulin has been an elusive goal for many investigators. The use of colloidal carriers made of hydrophilic polysaccharides, e.g. chitosan, has arisen as a promising alternative for improving the transport of macromolecules such as peptides, proteins, oligonucleotides and plasmids across biological surfaces (Lueben et al., 1996; Calvo et al., 1997a,b, 1998; Rog et al.,

* Corresponding author. Tel.: +86-24-238437113661; fax: +86-24-23891576
E-mail address: chryms2002@yahoo.com.cn (J.-m. Zheng).

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1999). Many different strategies have been applied to develop a bioactive oral insulin formulation. A successful formulation would have to bypass two main barriers against the oral delivery of proteins: the enzymatic barrier of the intestinal tract and the physical barrier made of the intestinal epithelium (Lee, 1988). Surfactants, permeation enhancers, protease inhibitors, enteric coatings and bioadhesive microparticles or nanoparticles have all been used in an attempt towards developing oral insulin formulation (Morishita et al., 1993; Yamamoto, 1994; Takeuchi et al., 1996; Carino et al., 2000).

Chitosan nanoparticles (CS-NPs) can be obtained by a very mild ionotropic gelation procedure, and have been reported with an excellent capacity for the association of proteins. From a biopharmaceutical point of view, CS has the special feature of adhering to the mucosal surface and transiently opening the tight junction between epithelial cells. It has proved that CS could enhance insulin absorption across human intestinal epithelial (Caco-2) cells without injure to them (Artursson et al., 1994; Schipper et al., 1996, 1997; Thanou et al., 2001).

Therefore, we chose chitosan as polymer vehicle and associated insulin to CS-NPs efficiently. Insulin-loaded CS-NPs were prepared and characterized for their physicochemical properties and in vitro release behavior. The effect of CS-NPs to enhance the intestinal absorption of insulin was studied by measuring the decrease of the plasma glucose levels following oral administration and the relative pharmacological availability was calculated.

2. Materials and methods

2.1. Materials

The hydrophilic chitosan (viscosity 45 mPa s, degree of deacetylation 88.9%, made by Physical Chemical Laboratory of Shenyang Pharmaceutical University, Shenyang, PR China) was generously supplied by Dr Zhao R.L. working in Guangzhou. Insulin (27.6 I.U./mg) was purchased from Xuzhou biochemical plant (China). Tianjing Tianhe Reagents Company (China) produced polyanion tripolyphosphate sodium (TPP). Poloxamer188 was purchased from BASF Corporation (USA), All the other reagents were of chemical grade.

2.2. Animals

Wistar male rats weighing 220–280 g, 12–13 weeks old, were provided by pharmacological laboratory of our school. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Shenyang Pharmaceutical University, PR China.

2.3. Methods

2.3.1. Investigation of the conditions for the formulation of CS-NPs

CS-NPs were prepared according to the procedure first reported by Calvo et al. (1997b) with little modification based on the ionotropic gelation of chitosan with TPP anions. Preliminary experiments were done to determine the formation zone of the nanoparticles. For this purpose, CS was dissolved in pH 4 acetic acid aqueous solution at various concentrations: 1, 2, 3, 4 and 5 mg/ml, TPP was dissolved in purified water at concentrations of 0.5, 1, 1.5, 3 and 5 mg/ml. Finally, a various volume of TPP solution was added to 4 ml of the CS solution through a syringe needle under magnetic stirring at room temperature. Then samples were visually analyzed and three different systems were identified: solution, suspension and aggregates. As illustrated in Fig. 1, the zone of the suspension should be a suspension of nanoparticles we hope to acquire, It was found that when the concentrations of CS and TPP were in the range of 0.9–3.0 mg/ml, 0.3–0.8 mg/ml, respectively, different sizes of nanoparticles could be obtained. For the numbers of experiments we have done were limited, the area between two dotted lines and a solid line was not enough to define, it may be a transition area including solution, nanoparticles suspension and aggregates state.

Nevertheless, when the concentrations of chitosan used in our experiments and TPP were in the ranges mentioned above, nanoparticles could be firmly acquired.

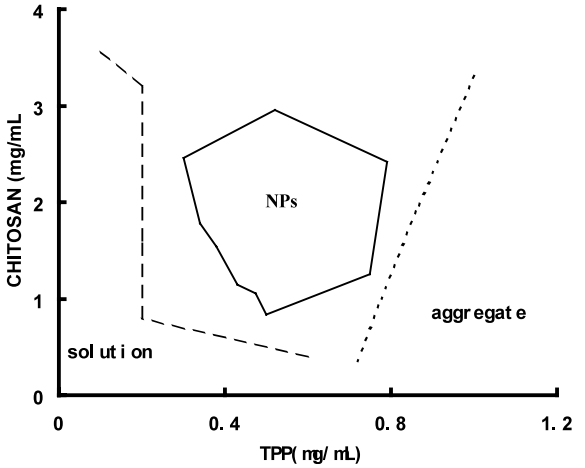

Fig. 1. Investigation on the conditions for the formation of chitosan nanoparticles (CS-NPs).

2.3.2. Optimum formulation of insulin-loaded CS-NPs by orthogonal design

Insulin-loaded CS-NPs was formed spontaneously upon incorporation of TPP aqueous solution containing insulin to pH 4 CS acetic acid solution according to the methods above. Finally, poloxamer188 was dissolved in the nanoparticles suspension so as to make the final concentration of poloxamer188 1.0% (w/v).

As has proved that the weight ratio of CS to insulin would affect the association of insulin (Fernanedz-Urrusuno et al., 1999), in order to associate insulin to CS-NPs efficiently, we have done the following experiments to determine the optimal formulation. Tablet 1 was the result of orthogonal design (L_9 (3^4)). When the final concentrations of TPP and CS were 2.6 and 0.45 mg/ml, insulin could be loaded efficiently while with relatively lower loading capacity.

2.3.3. Characterization of CS-NPs

The size and zeta potential of the nanoparticles were analyzed by photon correction spectroscopy and laser Dopper anemometry, respectively using a Zetasizer® III (Malvern Instruments, UK). For determining the electrophoretic mobility, samples were diluted with KCl 0.1 mol/l and placed in the electrophoretic cell where a potential of $\pm$150 mv was established. Each batch was analyzed in triplicate.

2.3.4. Insulin loading capacity of CS-NPs

The association efficiency of the process was determined upon separation of nanoparticles from the aqueous medium containing non-associated insulin by ultracentrifugation at 40 000 rpm, 10 °C for 30 min. The amount of free insulin in the supernatant was measured using HPLC. Twenty microliters was injected into a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Skimadzu SPD-10A) and reversed phase column (Kromasil C-18, 5μ, 4.6 × 200 mm^2, Zirchrom, TIANHE-Chromatography Industry, China). The mobile phase was a mixture of acetonitrile: 0.1 M NaH_2PO_4: 0.05 M Na_2SO_4 = 30:35:35 (adjusted to pH 2.5 by adding phosphate acid). The flow rate was 1.0 ml/min; the wavelength was set at 214 nm and the column operated at 35 °C. Insulin association efficiency and loading capacity of the nanoparticles were calculated according to the equation established by Fernanedz-Urrusuno et al. (1999).

2.3.5. In vitro release studies

Insulin release from CS-NPs was determined by incubating the nanoparticles in 5 ml of pH 7.4, pH 5.8 phosphate buffer solution (PBS) or pH 4.0 acetate buffer solution, respectively, at 37 °C. The amount of nanoparticles in the release media was adjusted in order to assess sink conditions for insulin. An exception was made to guarantee the sink condition in the pH 5.8 PBS (such as add some excipients to increase the solubility of insulin in pH 5.8 PBS). At appropriate time intervals, individual samples were centrifugated and the amount of insulin released in the supernatant was evaluated by HPLC.

2.3.6. In vivo studies

Wistar male rats were made diabetic prior to the study by intravenous injection of 40 mg/kg alloxan in isotonic saline solution. They were considered to be diabetic when the base line glucose levels were in the range of 180–250 mg/dl. The diabetic rats were fasted for 12 h before experiments. Formulations following were administered to them orally: (1) control insulin solution; (2) insulin–chitosan solution (insulin dose 14 I.U./kg); (3) insulin-loaded CS-NPs suspension containing 1.0% polox-

amer188 (insulin dose 7, 14, 21 I.U./kg, number mean diameter 289.3 nm, insulin loading (9.5 ± 4.0)%); (4) SC injection of insulin solution (dose 1 I.U./kg) as well as a hungry group allowed drinking during the experiment. All above formulations were in the medium of physiological saline before oral administration. The oral administration volume of the suspension and solution was in the range of 1–2 ml/100 g.

Blood samples were collected from the retro-orbital plexus of the rats prior to oral administration to establish baseline glucose levels, and at different times after dosing blood samples were collected in the same way. Glycemia was determined in plasma sample by glucose-oxidase method (Glucose GOD-PAD kit, Beijing Ruikang Biochemical Reagent Industry, Beijing, China). Results were shown as the mean values of plasma glucose levels (±standard deviations) of animals of each group.

2.3.7. Statistical analysis of in vivo data

The mean plasma glucose levels determined in samples collected before oral administration were taken as the baseline levels. The percentage of glucose reduction at each time after dosing was calculated and plotted against time. Data from different experimental groups were compared with the corresponding control groups by the t-test with significant level of $^{*}P < 0.01$.

Relative pharmacological bioavailability was calculated by utilizing Eq. (1):

$$f = \frac{\mathrm{AAC}_{\mathrm{oral}} \times (\mathrm{dose})_{\mathrm{SC}}}{\mathrm{AAC}_{\mathrm{SC}} \times (\mathrm{dose})_{\mathrm{oral}}} \quad (1)$$

f was the pharmacological efficacy of the dose vs. SC, $\mathrm{AAC}_{\mathrm{oral}}^{0-38\ \mathrm{h}}$ and $\mathrm{AAC}_{\mathrm{SC}}^{0-38\ \mathrm{h}}$ were the area above the curve of administration various doses of insulin-loaded CS-NPs and SC injection of insulin solution. The administered doses were determined experimentally. The average standard deviations of plasma glucose levels measured in the 8 experimental rats were graphed vs. time and the trapezoid rule was used to calculate the AAC.

3. Results and discussion

The main goal of this work was to investigate the potential of CS-NPs for oral delivery of insulin. Therefore, we expected to have information not only about the potential of CS-NPs for increasing insulin intestinal absorption, but also about the suitability of the NPs formulation process for preserving the insulin activity and the hypoglycemic effect as well as the relationship between doses of insulin and pharmacological availability.

3.1. Association of insulin to CS-NPs

Results of preliminary investigations on the experimental conditions for the formation of CS-NPs showed that nanoparticles could be obtained by varying the concentrations of TPP and chitosan. As concluded in Table 1, association efficiency and loading capacity of the nanoparticles were affected by the insulin concentration in the TPP solution and the amount of insulin incorporated, with increasing amount ratio of insulin to chitosan leading to a slight decrease of association efficiency and an enhancement of loading capacity. These results were coincident with the conclusion first drawn by Calvo et al. (1997b). The mechanism of proteins association to CS-NPs was mediated by an ionic interaction between both macromolecules. The electrostatic interactions between the acidic insulin groups and the amino groups of CS played a role in association of insulin to the CS-NPs. The same conclusion was also drawn by other authors (Calvo et al., 1998).

3.2. Effect of poloxamer188 on the characterizations of CS-NPs

It has been proved that the size and zeta potential of CS-NPs could be conveniently modulated by varying the ratio of CS to poloxamer188 (from 200 nm up to 1 μm and from +20 up to + 60 mv, respectively). According to this, we made a further investigation on the incorporation of poloxamer188 into the nanoparticles suspension. As shown in Table 2, higher concentration of poloxamer188 would lead to a slightly enlarge-

Table 1
Investigation on the optimal conditions for the formulation of CS-NPs by orthogonal design (L_9 (3^4))

No.	C_{cs} (mg/ml)[a]	C_{TPP} (mg/ml)[a]	m_{ins}/m_{cs} (w/w)[b]	Association efficiency (%)	Loading capacity (%)
1	1.40	0.30	0.1	66.8±2.1	7.0±3.5
2	1.40	0.45	0.2	64.5±1.8	13.4±4.0
3	1.40	0.60	0.3	59.6±2.0	18.9±2.7
4	2.00	0.30	0.2	78.3±2.3	24.5±2.9
5	2.00	0.45	0.3	70.6±3.1	23.7±3.7
6	2.00	0.60	0.1	81.3±2.5	9.7±4.0
7	2.60	0.30	0.3	80.3±1.7	26.3±4.6
8	2.60	0.45	0.1	88.6±2.4	9.7±4.0
9	2.60	0.60	0.2	71.3±1.9	15.3±4.1

[a] Chitosan and TPP final concentration in the nanoparticles suspension.
[b] m_{ins}/m_{cs} is the final ratio of amount in the nanoparticles suspension.

ment of the CS-NPs and a decrease on their surface charge. This made us to accept the fact that poloxamer188 was incorporated into the structure of the CS-NPs, which had been analyzed by Calvo et al. who attempted to evaluate their surface chemical groups by X-ray photoelectron spectroscopy (1997a).

On the other hand, it could be seen that the entrapment of insulin was not limited greatly by the presence of poloxamer188 incorporated after the formation of insulin-loaded CS-NPs. This fact also agreed with the discovery made by Calvo et al. (1997b). Insulin and poloxamer188 may compete in deed in their interaction with CS; however, insulin loading was not modified if it was incorporated subsequently to the entrapment of insulin in the CS-NPs suspension. For these reasons, in our research we dissolved poloxamer188 to the nanoparticles suspension eventually so as to guarantee the loading capacity as high as possible.

3.3. Release of Insulin from CS-NPs

Fig. 2 displayed the release profiles of insulin from CS-NPs at different pH values. It was apparent that insulin release in vitro showed a very rapid initial burst. It could be seen that insulin release from NPs occurred very rapidly at

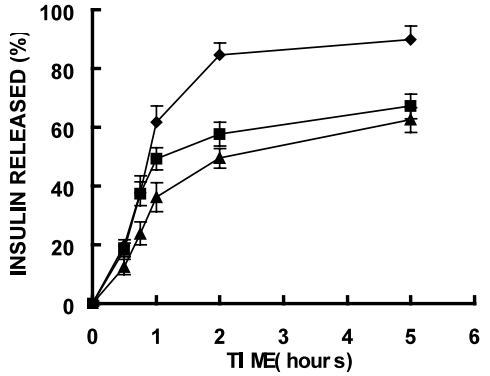

Fig. 2. Insulin release profiles from CS-NPs containing 9.63% insulin at pH 7.4 (◆—◆), pH 5.8 (▲–▲) and pH 4.0 (■–■) (mean±SD, $n=4$).

Table 2
Influence of poloxamer188 on the characteristics of insulin-loaded CS-NPs subsequently incorporated after the formation of NPs

$C_{poloxamer188}$ (w/v)	Mean particle size (nm)[a]	Zeta potential (mV)[a]	Association efficiency (%)[a]
0	265.3±34.1	+40.71±0.69	88.6±2.4
0.5%	273.1±21.7	+38.42±1.21	86.9±3.1
1.0%	289.3±31.2	+32.56±2.10	87.3±2.0
10.0%	387.4±35.6	+27.31±0.98	85.4±1.3

[a] Data shown are the mean±standard deviation, ($n=3$).

pH 7.4 and 4.0 while release was slightly slowed down at pH 5.8. Since the fact that the solubility of insulin at pH 5.8 (pI 5.3) is lower than the others, this fact made us to accept the discovery that in vitro release behavior of insulin from CS-NPs was accorded with a dissociation mechanism. This was also putforward by other authors (Tokumitsu et al., 1999). In our experiment, we also found that nanoparticles with sizes smaller than 120 nm in diameter dissolved in pH 1.2 HCl in several min, however, it became more and more difficult to dissolve for the nanoparticles with the increase of the size. The explanation for this phenomenon was not clear and the detailed release study is still in research until now. From these properties of the NPs, we may believe that CS-NPs is pH-sensitive delivery systems, a quality that renders them new prospects in the field of gene therapy (Erbacher et al., 1998). Since poloxamer188 was contained in the CS-NPs, the effect of it on the release behavior in vitro could not be neglected. These results have not been shown in this paper.

3.4. In vivo studies

Figs. 3 and 4 exhibited the behavior of different formulations administered orally to diabetic rats. The efficacy of the formulations was assessed by

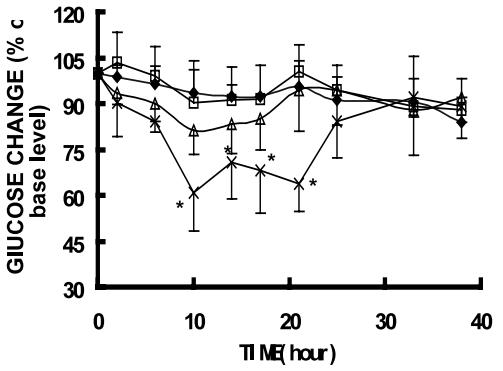

Fig. 3. Hypoglycemic effect of oral administration of: control (drinking saline without oral administrating anything, ◆—◆); insulin–chitosan solution (△—△); insulin solution (□—□); 14 I.U./kg insulin-loaded CS-NPs suspended in pH 5.5 saline (×—×). Data represents the mean ± S.D., $n = 8$ per group. Statistically significant difference from control (*$P < 0.01$).

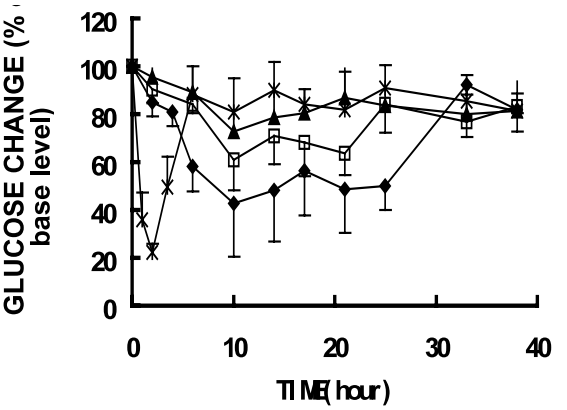

Fig. 4. Hypoglycemic effect of administration different doses of insulin-loaded CS-NPs to diabetic rats orally: 7 I.U./kg (▲—▲); 14 I.U./kg (□—□); 21 I.U./kg (◆—◆); subcutaneous injection of insulin solution in physiological saline (×—×). Data represents the mean ± S.D., $n = 8$ per group.

measuring the plasma glucose concentration and the relative pharmacological availability of various doses of insulin-loaded CS-NPs was calculated. As shown in Figs. 3 and 4, three dosages of insulin-loaded CS-NPs were found orally effective. It indicated that insulin was released from NPs in its active form. When insulin-loaded CS-NPs were orally administered to the diabetic rats at a dosage of 14 I.U./kg, no significant decrease of the plasma glucose was found during the first 6 h; 10 h later, the decrease in plasma glucose levels was significantly different from that induced by the insulin control solution and this hypoglycemic effect lasted at least 10 h (*$P < 0.01$). To our cheer, we found that most of the diabetic rats fed 21 I.U./kg of insulin in the CS-NPs had reduced glucose to normal range (80–120 mg/dl) for more than 8 h. These results clearly evidenced the ability of CS-NPs to enhance the intestinal absorption of insulin. This positive behavior of CS-NPs could also demonstrate the facts that the interaction of CS with the cell membrane resulted in a structural reorganisation of tight junction-associated insulin which was followed by enhanced transport through the paracellular pathway (Borchard et al., 1996).

In order to investigate whether or not the nanoparticulate of CS played a role in improving intestinal absorption of insulin, the effect of CS-

NPs was compared to that of chitosan solution. Results depicted in Fig. 3 clearly showed that the decrease in plasma glucose levels induced by administration of insulin-loaded CS-NPs was much more ($^{*}P < 0.01$) than obtained following oral administration of insulin–chitosan solution. An explanation to this positive behavior of CS-NPs could be put forward on the demonstrated ability of NPs to make the entrapped protein more stable and protect it from degradation in harsh conditions of gastrointestinal tract (Vila et al., 2002). Theoretic evidence needs further research.

Since the penetration enhancing capacity of CS-NPs was demonstrated, it was important to determine if this capacity would be related to the amount of insulin administered. For this purpose in mind, we administered different doses of insulin-loaded into CS-NPs to diabetic rats (7, 14 and 21 I.U./kg). As shown in Fig. 4, the hypoglycemic effect appeared earlier with an increase doses of insulin-loaded CS-NPs as well as an elongation of hypoglycemic state. According to Eq. (1), the pharmacological availability of 7, 14 and 21 I.U./kg relative to SC injection were calculated to be 14.0, 15.6 and 15.3%, respectively and the mean availability was $(14.9 \pm 0.9)\%$.

Poloxamer188 contained in the CS-NPs was a surfactant; it may have an influence on the in vivo pharmacological action. It has been proved that poloxamer188 incorporated into the oral formulations could enhance the intestinal absorption by slowing the peristalsis of the intestinal and prolonging the resident time of the drugs in the intestinal tract (Ping and Sun, 2000). Furthermore, it has been reported that NPs smaller than 500 nm administered orally had greater capacity of being uptaken via peyer's patches in their original state (Jani et al., 1992). According to Muller and Wallis, 1993, the adsorption of hydrophilic compounds resulted from poloxamer188 probably on the surface of the NPs had the higher capacity of avoiding opsonization and subsequent reticuloendothelial system clearance. Wang and Zhang, 2001 had also proved that the surfactant coating of colloidal drug carriers decreased the uptake of cyclosporine A by mouse peritoneal macrophages in vitro. According to above, a conclusion could be made that the increased hydrophilicity and decreased zeta potential resulted from poloxamer188 contained in the CS-NPs rendered them protection from clearance and subsequently a much longer resident time in the circulation, which further led to a long-term hypoglycemic effect on diabetic rats once these NPs reached the circulation via peyer's patches.

From above, we had to accept the definite fact that CS-NPs could enhance the intestinal absorption of insulin. Different doses of insulin in the CS-NPs had different influences on the glucose level of the diabetic rats. Although the CS-NPs smaller than 120 nm appeared transparent several minutes later in pH 1.2 acidic medium, CS-NPs as large as 289.3 nm became difficult to dissolve in that medium, which rendered them a capability of greater hypoglycemic effect in vivo. Besides in our later experiments we found that particles of 345 nm in diameter resulted in a greater hypoglycemic effect than 123 nm nanoparticles in diabetic rats, this is coincident with the results reported by Tabata et al. (1996). From above, it could be concluded that NPs with different sizes had different capability of protecting insulin-loaded from destroying in the harsh gut condition and enhancing the absorption of it. Nevertheless, the mechanism of this protection was still unknown clearly until now. The reasons for these may be that the increased viscosity of the suspension containing CS-NPs with larger particles reduced the diffusion rates of molecules, or CS-NPs increased the stability of insulin in the presence of proteolytic enzymes in the gastrointestinal tract (Vila et al., 2002), or on the other hand these NPs made of mucoadhesive materials such as chitosan could adhere to the biomembrane tightly and transiently open the tight junctions between the epithelial cells so as to help the drug loaded transport through it quite easily. Kotze et al. (1998) have proved that chitosan at low pH showed a pronounced effect on the permeability of [^{14}C] mannitol, leading to 25-fold (glutamate salt) and 36-fold (hydrochloride salt) enhancement. These results may provide some evidence in support of the facts in our experiments. The adequate and complete explanation of CS-NPs enhancing the intestinal absorption of insulin needed more experiments to be verified.

4. Conclusion

CS-NPs depicted in this paper had shown an excellent capacity for the association of insulin. CS-NPs loading insulin showed a positive charge and rapid release kinetics in vitro. Furthermore, CS-NPs could release insulin in its active form in vivo and they were able to improve the intestinal absorption of insulin to a greater extent than insulin–chitosan solution. The diabetic glucose level could be reduced to normal level for a long time if modulating the doses of insulin-loaded CS-NPs was made.

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