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Effective protection and controlled release of insulin by cationic β-cyclodextrin polymers from alginate/chitosan nanoparticles

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ABSTRACT

In an alginate/chitosan nanoparticle system, insulin was protected by forming complexes with cationic β-cyclodextrin polymers (CPβCDs), which were synthesized from β-cyclodextrin (β-CD), epichlorohydrin (EP) and choline chloride (CC) through a one-step polycondensation. Due to the electrostatic attraction between insulin and CPβCDs, as well as the assistance of its polymeric chains, CPβCDs could effectively protect insulin under simulated gastrointestinal conditions. The nanoparticles have their mean size lower than 350 nm and can load insulin with the association efficiency (AE) up to 87%. It is notable that the cumulative insulin release in simulated intestinal fluid was significantly higher (40%) than that without CPβCDs (18%) because insulin was mainly retained in the core of the nanoparticles and well protected against degradation in simulated gastric fluid. Far-UV circular dichroism analysis also corroborated the preservation of insulin structure during the nanoparticle preparation and release process.

1. Introduction

Parenteral administration of insulin has many disadvantages such as common infections, the pain caused by subcutaneous injections and the stress in the long-term use. In recent years, oral insulin delivery system has been widely studied. However, most methods have very low bioavailability due to enzymatic degradation in the gastrointestinal tract, low stability at gastric pH and the physical barrier of the intestinal epithelium. Thus an ideal oral insulin delivery system should provide a stable environment to ensure that the main fraction of the therapeutic protein will be biologically active during both particle processing and insulin release (Sarmento et al., 2006; Reis et al., 2007).

The polyelectrolyte complex formed by alginate and chitosan is one of such carrier systems (Sinha et al., 2004; George and Abraham, 2007; Chen et al., 2008; Liouni et al., 2008). Alginate and chitosan are both natural polymers that are mucoadhesive, biodegradable and biocompatible, enabling numerous pharmaceutical and biomedical applications such as drug delivery and cell encapsulation (Shilpa et al., 2003). Alginate can ionically crosslink with multivalent cations such as Ca^{2+} to form a reversible gel which can make the drug retention within the gel matrix (Sarmento et al., 2007a). Meanwhile, chitosan could enhance the paracellular permeability of peptides such as insulin by charge-mediated polymer binding to epithelia, resulting in a structural reorganization of tight junction-associated proteins (Jung et al., 2000).

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six to eight glucose units linked by α-1,4-glucosidic bonds, resulting in the formation of toroidal molecules with internal hydrophobic cavities and external hydrophilic surface. The internal hydrophobic cavities in CDs can facilitate the inclusion of a number of guest molecules stabilized by non-covalent interactions (Denadai et al., 2007). It has been suggested that CDs and their derivatives might undergo degradation in the colon since there has vast microflora, which can break CDs and their derivatives into small saccharides to be absorbed in the large intestine (Shao et al., 1994; Denadai et al., 2007). Therefore, CDs and their derivatives are extensively studied for oral administration of peptide drugs (Moses et al., 2000; Sajeesh and Sharma, 2006; Carrier et al., 2007; Krauland and Alonso, 2007; Li and Loh, 2008). However, proteins such as insulin are too bulky to be wholly included into CDs' cavities. Their hydrophobic side chains can only partially penetrate into the CDs' cavity to form non-covalent inclusion complexes (Irie and Uekama, 1999). CDs–insulin complex could stabilize insulin against aggregation, thermal denaturation and degradation. It could also enhance the absorption of insulin across the biological barriers by perturbing the membrane fluidity to lower the barrier function (Uekama, 2004).

However, the unmodified CDs have cytotoxicity and low water solubility, which limit their further pharmaceutical applications (Szente and Szejtli, 1999). So various CDs derivatives have been developed to overcome these drawbacks, such as sulfobutylether β-cyclodextrin (SBE-βCD) and hydroxypropyl β-cyclodextrin (HP-βCD). In our previous work, a series of cationic β-cyclodextrin

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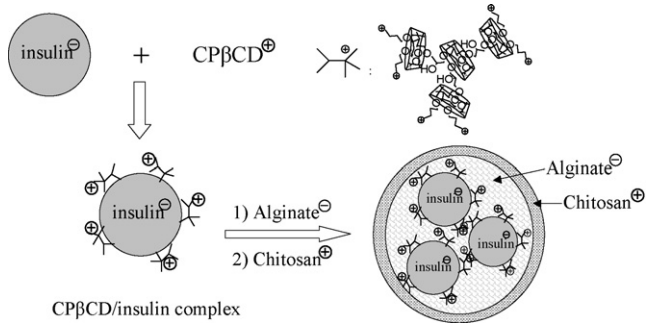

Fig. 1. Schematic representation of the complexation and protection of insulin by CPβCDs in alginate/chitosan nanoparticles.

polymers (CPβCDs) were synthesized by a one-step polycondensation and were found to have excellent water solubility, better hemocompatibility and drug delivery performance than that of βCD (Li et al., 2004; Gil et al., 2009; Xin et al., 2010). Meanwhile, some other cationic cyclodextrin polymers have also been reported to be safe enough to play the role of non-viral gene vectors in recent researches (Li et al., 2006; Yang and Li, 2009; Yang et al., 2009; Li et al., 2010).

The M-cells of the Peyer's patches represent a type of lymphatic island within the intestinal tract. Particles smaller than 10 μm can be taken by the M-cells and transported into the Peyer's patches by mucosal adhesion. Among them, most microparticles larger than 5 μm remain in the Peyer's patches but those smaller than 5 μm will transport through the efferent lymphatics. So, nanoparticles, due to their even smaller size, can be directly uptaken and result in higher drug delivery efficiency (Fasano, 1998; Pan et al., 2002; Tiyaboonchai et al., 2003; Vauthier et al., 2003). Several insulin-loaded alginate/chitosan nanoparticles have been reported to have ideal insulin association efficiency, some of which could be higher than 90%. However, the insulin release efficiency was not high in simulated intestinal fluid (SIF) after being treated in simulated gastric fluid (SGF) because a significant amount of insulin was associated to the surface of the nanoparticles. As a result, up to 60% insulin was released in a burst effect in SGF before it could make therapeutic sense in SIF (Sarmento et al., 2007b,c).

In our previous work, we used CPβCDs to form complex with insulin and then encapsulated the complex into alginate/chitosan microspheres with improved insulin association efficiency (AE) (Huang et al., 2010). However, this system does not show significant improvement of insulin release efficiency in our following trials, which may due to the size of the microspheres (millimeters in diameter). In this work, we will report an oral insulin formulation of CPβCDs–insulin-loaded alginate/chitosan nanospheres as shown in Fig. 1. The polymeric chain and positive charge of CPβCDs may be helpful to form complex with insulin and retain it within the core of the alginate/chitosan nanoparticle, which may decrease insulin degradation in gastric environment. The AE, *in vitro* release profile and the ability to preserve insulin's intrinsic conformation of the system will be investigated.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD), epichlorohydrin (EP) and choline chloride (CC) were purchased from Bodi Chemical Co. (Tianjin, China). Sodium alginate was procured from Kelong Reagent Co. (Chengdu, China). Insulin powder was purchased from Xuzhou Wanbang Biological Pharmaceutical Enterprise (Jiangsu, China). Chitosan with approximate molecular weight 10,000 and 90% deacetylation degree was obtained from Golden-shell Biochemical Co. (Zhejiang, China). All the other reagents and solvents were of AR grade and used as received without further purification. Distilled water was used throughout.

2.2. Preparation and characterization of CPβCDs–insulin complexes

2.2.1. Syntheses of CPβCDs

CPβCDs were synthesized following the procedure reported in a previous work (Li et al., 2004). In this work, the molar feeding ratios of β-CD/EP/CC were 1/15/4, 1/15/6 and 1/15/10, respectively. A typical synthesis procedure of CPβCD1/15/4 is described as: 1.0 g NaOH was dissolved in 20 mL of water, and then 5.675 g β-CD were dissolved in the sodium hydroxide solution. The solution was electromagnetically stirred at 25 °C for 24 h in a water bath. After that, 2.792 g CC were fed into the solution rapidly then 6.940 g EP were added dropwise at a flow rate of 0.1 mL/min. After the completion of EP feeding, the mixture was heated to 60 °C and kept for 2 h. The reaction was quenched by neutralization with an aqueous hydrochloride acid solution (3N). The solution obtained was dialyzed for 24 h with a dialysis membrane of molecular weight cut-off 1000. The solution obtained was evaporated and the solid was pulverized into fine powder. The CD and CC content are 77.8% and 2.3%, 48.0% and 10.7%, and 52.4% and 19.3% for CPβCD1/15/4, 6 and 10, respectively, according to the ^{1}H NMR data.

2.2.2. Preparation of CPβCDs–insulin complexes

10 mg insulin was dissolved in 20 mL HCl solution (pH 3). Then the pH of the solution was adjusted to 6.3 ± 0.1 using 0.1N NaOH. CPβCDs–insulin complexes were prepared by mixing 3 mL CPβCDs solution (1 mg/mL, pH 6.3 ± 0.1) with 3 mL insulin solution (equivalent to 45 IU) and then gently stirred for 1 h at room temperature.

2.2.3. Characterization of CPβCDs–insulin complexes

The zeta potential of CPβCDs and CPβCDs–insulin complexes was measured by Malvern zetasizer and Particle Analyzer 5000 (Malvern Instruments, UK). The CPβCDs were dissolved in deionized water before measurement. The pH of insulin solution and CPβCDs solution were 6.3. The CPβCDs–insulin complexes were formed and measured without pH adjustation. The value was recorded as the average of five measurements. Circular dichroism spectroscopy (CD spectra) of insulin solution and CPβCD1/15/6–insulin complex solution (pH 6.3 ± 0.1) were performed at room temperature using a Jasco J-715 spectrophotometer (Japan).

2.3. Preparation of CPβCDs–insulin-loaded alginate/chitosan nanoparticles

Sodium alginate was dissolved in deionized water under magnetic stirring overnight. Chitosan was dissolved in 1% acetic acid solution followed by filtering. 3 mL insulin solution (0.5 mg/mL, pH 6.3 ± 0.1, equivalent to 45 IU) was mixed with 3 mL CPβCDs solution (1 mg/mL, pH 6.3 ± 0.1), and then gently stirred for 1 h at room temperature to form complexes. The complex solution was then mixed with 23.5 mL of 0.063% alginate solution under magnetic stirring for 10 min. After that, 1.5 mL of 20 mM calcium chloride solution was added dropwise into the alginate solution containing insulin within 15 min under gentle stirring (300 rpm) to provide an alginate pre-gel. Finally, 5 mL of chitosan solution of different concentrations (fix the mass ratio of Alg:Chit at 6:1, 5:1 and 4.3:1, respectively) was dropped into the pre-gel in 30 min. The pH of alginate and chitosan solutions was initially set to 4.9 and 4.7, respectively. After chitosan addition, nanoparticles were maintained under stirring for additional 30 min to improve curing. The nanoparticles were separated by centrifugation at 18,000 × *g* for 45 min.

The control sample of insulin-loaded alginate/chitosan nanoparticles were prepared by the same procedure except that using 3 mL of insulin solution (45 IU) plus 3 mL water instead of the 6 mL CPβCDs–insulin complex solution.

2.4. Nanoparticle size analysis

To measure the size of the nanoparticle, 5 mL of sample was gently shaken and placed into the analyzer chamber. Readings were performed at 25 °C with a detected angle of 90° using a Malvern Zetasizer and Particle Analyzer 5000 (Malvern Instruments, UK). The morphology of nanospheres was studied by scanning electronic microscopy (SEM) using Hitachi S-450 (20 kV, Japan). The samples were lyophilized and then mounted on metal stubs, gold coated under vacuum before examination.

2.5. Insulin association efficiency (AE) and loading capacity (LC)

The AE was determined after separation of nanoparticles from the solution containing non-associated insulin. The amount of insulin associated with the particles was calculated by the difference between the total amount used to prepare the particles and the amount of insulin present in the aqueous phase after centrifugation.

$$\mathrm{AE} = \frac{\text{total amount of insulin} - \text{free insulin in supernatant}}{\text{total amount of insulin}} \times 100\%$$

The difference between the total amount of insulin and the amount of residual unassociated insulin as a percent of total nanoparticle dry mass is determined as LC. Dry mass was obtained by freeze-drying an aliquot of hydrated nanoparticle.

$$\mathrm{LC} = \frac{\text{total amount of insulin} - \text{free insulin in supernatant}}{\text{total dry weight of nanoparticles}} \times 100\%$$

2.6. In vitro release of insulin under simulated gastrointestinal conditions

Nanoparticles were placed into simulated gastric fluid (SGF) containing 20 mL HCl (pH 1.2) for 2 h, and then put into simulated intestinal fluid (SIF) containing 20 mL phosphate (pH 6.8) for 4 h, under continuous magnetic stirring (100 rpm) at 37 °C. At appropriate time intervals, aliquots were taken and replaced by fresh medium. The amount of insulin released from the nanoparticles was estimated by the method of Coomassie Brilliant Blue staining (Kruger, 1994). All the experiments were carried out in triplicate and the data presented are the average of three measurements.

2.7. Stability studies in pepsin solution

1.5 mL pepsin solution (0.05 mg/mL, Tris–HCl buffer, pH 2) was added into 1.5 mL of insulin-loaded nanoparticles dispersion, CPβCD1/15/6–insulin complexes loaded nanoparticles dispersion and insulin solution, respectively. The mixture was immediately incubated at 37 °C. Aliquots were taken at appropriate time intervals and the pepsin digestion reaction was stopped by adding 100 μL 0.05 mol/L NaOH (Zhang et al., 2006). The remaining concentrations of insulin in the samples were determined by HPLC, which was running with a Waters 600 Pump and a Waters 996 photodiode Array Detector (Waters, USA). The column was Symmetry C18 with 5 μm particle size, 4.6 mm id × 150 mm length (Waters, USA). The mobile phase was composed of acetonitrile (ACT) and 0.1% trifluoroacetic acid (TFA) aqueous solution (30:70) operated at a flow rate of 1 mL/min. Protein identification was made by UV detection at 214 nm.

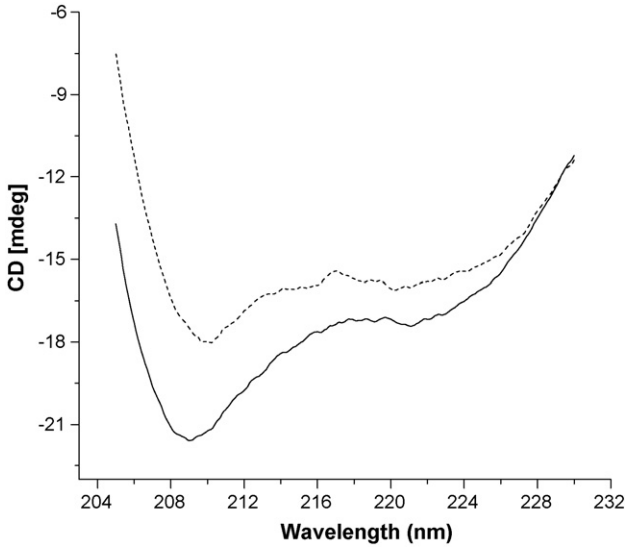

Fig. 2. CD spectra of insulin (—) and CPβCD1/15/4–insulin complex (- - -) at pH 6.3.

2.8. Far-UV circular dichroism analysis (Far-UV CD)

CD spectra of insulin after release in both simulated gastric and intestinal environments were collected to investigate potential changes in insulin structure. It was obtained at room temperature on a Jasco J-715 Spectrophotometer (Japan). In the far-UV region CD spectra were recorded in a 0.01 cm cell from 230 to 200 nm, using a step size of 0.5 nm, a bandwidth of 1.5 nm, and an averaging time of 5 s, with the lamp housing purged with nitrogen to remove oxygen. For all spectra, an average of 5 scans was obtained.

3. Results and discussion

3.1. CPβCDs–insulin complexes

Circular dichroism spectroscopy (CD spectra) was used to clarify the existence of the CPβCDs–insulin complexes. CD spectra of insulin in the presence or absence of CPβCD1/15/4, at pH 6.3, are presented in Fig. 2. A negative CD band was observed at 209 nm for insulin alone. In the presence of CPβCD1/15/4, this band decreases its intensity as a result of perturbation of the electronic transition of insulin, which is caused by the asymmetric cavity of CPβCD1/15/4 after complexation (Hirayama and Uekama, 1987; Ventura et al., 1998). The complexation has also been proved in our previous work by FTIR and fluorescence spectroscopic methods (Huang et al., 2010).

As shown in Table 1, the zeta potential of insulin alone is around −28 mV, which could be increased to −0.8, −0.7 and −0.4 mV after being complexed with CPβCD1/15/4, 6 and 10, respectively.

Table 1
Zeta potential of CPβCDs–insulin complexes.

Samples	Zeta potential (mV)[a]
CPβCD1/15/4	6.8 ± 1.6
CPβCD1/15/6	13.2 ± 3.1
CPβCD1/15/10	16.6 ± 1.8
Insulin	−28.0 ± 1.3
CPβCD1/15/4–insulin complex[b]	−0.8 ± 0.2
CPβCD1/15/6–insulin complex[b]	−0.7 ± 0.1
CPβCD1/15/10–insulin complex[b]	−0.4 ± 0.1

[a] The pH of insulin solution and CPβCDs solution were 6.3 and 6.8, respectively. The CPβCD–insulin complexes were formed and measured without pH adjustment (n = 5, ±S.D.).
[b] CPβCDs:insulin = 2:1.

This is because some of the insulin's side chains are included in the hydrophobic cavities of CPβCDs, as well as the electrostatic attraction between the insulin (negatively charged while pH > 5.3) and quaternary ammonium groups of CPβCDs. There is a similar increasing trend of the zeta potential of CPβCDs–insulin complex with the increase of zeta potential of CPβCDs. This is due to the increasing amount of cationic groups (CC) in CPβCDs. It is notable that the preparation procedure of the CPβCDs–insulin complexes has been optimized. In our previous work, the CPβCDs–insulin complex was formed at a mass ratio of 20:1, resulting in the zeta potential to be −14.8 and −9.7 mV for CPβCD1/15/4–insulin and CPβCD1/15/6–insulin complexes, respectively (Huang et al., 2010). In the current work, by mixing 3 mL CPβCDs solution (1 mg/mL) with 3 mL insulin solution (0.5 mg/mL) instead of adding solid CPβCDs into insulin solution at a higher concentration (1 mg/mL), a significantly improved electrostatic attraction between insulin and CPβCDs was observed, at a much lower mass ratio of CPβCDs:insulin of 2:1. This might due to the saturated concentration of insulin solution is around 1 mg/mL, at which insulin may aggregate together, thus the electrostatic attraction between insulin and CPβCDs could not reach the highest level. The optimum complexation condition revealed here is helpful to use fewer additives in formulations.

The CPβCDs–insulin complexes could provide effective protection to insulin due to the strong connection by both inclusion and electrostatic attraction.

3.2. Preparation and characterization of CPβCDs–insulin-loaded Alg/Chit nanoparticles

CPβCDs–insulin-loaded alginate/chitosan nanoparticles were prepared in a modified three-step procedure based on the ionotropic pre-gelation of polyanion with calcium chloride followed by polycationic crosslinking as described in previous reports (Rajaonarivony et al., 1993; Sarmento et al., 2007b), except for preparing CPβCDs–insulin complex first instead of using insulin alone. There are several factors that may affect the properties of these CPβCDs–insulin-loaded Alg/Chit nanoparticles. The first factor is the concentration of Ca^{2+} because the production of alginate pre-gel is a key step of this process. If the concentration of Ca^{2+} is too low, the pre-gel will be of poor quality. On the contrary, an overdoes of Ca^{2+} will led to the formation of a continuous gel because Ca^{2+} starts to ionically crosslink between alginate coiled structures in a form of intermolecular crosslinking. So when the concentration of alginate is set to 0.063%, 1.5 mL 20 mM Ca^{2+} will be dropped into the alginate solution to give a final concentration of 0.9 mM (Sarmento et al., 2007a). Second, the time of pre-gel formation and the stirring speed could also affect the association efficiency and nanoparticle size, which will finally lead to different insulin release profiles. After a series of experiments (data not shown), the time of pre-gel is fixed at 15 min while the stirring speed is adjusted to 300 rpm. Finally, the most important factor that could affect the AE, LC and size of above nanoparticles is the mass ratio of alginate:chitosan, which determines the quantity of stabilizing polycationic needed to entrap the maximum amount of insulin during formulation. As can been seen in Table 2, no matter what kind of CPβCDs, the adding of CPβCDs significantly enhance the insulin AE at a same Alg/Chit mass ratio. This result demonstrates that the formation of CPβCDs–insulin complexes could greatly promote the encapsulation of insulin into the alginate/chitosan nanoparticles due to the electrostatic attractions among CPβCDs, insulin, surrounding alginate and the chitosan shell as elucidated in Fig. 1. Also, a decrease of the Alg/Chit mass ratio leads to an increase in insulin AE while using a same CPβCDs–insulin complex, which is because chitosan is the outmost shell of the nanoparticles and dominantly affect the insulin encapsulation efficiency. However, the insulin

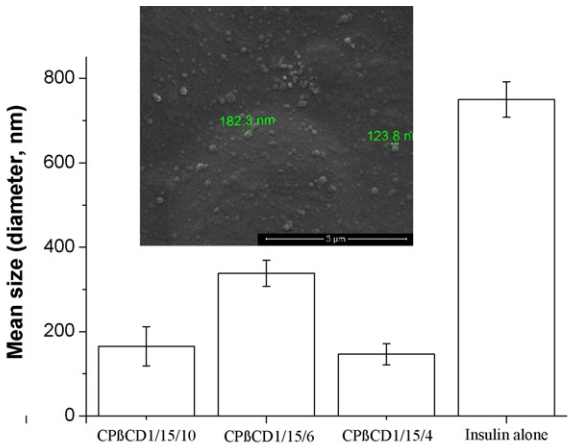

Fig. 3. Mean size of CPβCDs–insulin-loaded Alg/Chit nanoparticles. Inset is the SEM morphology of CPβCD1/15/4–insulin-loaded sample.

loading capacity (LC) of CPβCDs–insulin-loaded alginate/chitosan nanoparticles are somewhat lower than that of insulin-loaded one. This is reasonable because the addition of CPβCDs into nanoparticles increased the total polymers/insulin ratio. Although insulin is entrapped with higher AE when the alginate:chitosan mass ratio decreases as shown in Table 2, a further low ratio will lead to aggregation of nanoparticles such as 4.3:1 for CPβCD1/15/6 and CPβCD1/15/10–insulin-loaded samples. Also, we found that with the increase of the positive charge of CPβCDs, the aggregation phenomenon happened earlier in the nanoparticulate system when we gradually decreased the Alg/Chit ratio. It might because that both CPβCDs and chitosan are positively charged, the system would be close to charge balance when they are increased to some extent. At that point, the electrostatic repulsion between nanoparticles is minimized thus they tend to form aggregates. In the scope of our investigation, we found CPβCD1/15/4–insulin-loaded nanoparticle achieved the highest AE of 87% at an Alg/Chit mass ratio of 4.3:1, which may due to both the CPβCD1/15/4–insulin interaction and the highest chitosan content in this formulation.

The mean size of CPβCDs–insulin-loaded nanoparticles could be affected by the Alg/Chit mass ratio and the CPβCDs content as shown in Fig. 3. The particle sizes are 146, 338, 165 and 750 nm for CPβCD1/15/4–insulin, CPβCD1/15/6–insulin, CPβCD1/15/10–insulin and insulin-loaded nanoparticles, respectively. It seems that CPβCDs exert a significant influence on the particle size, as all the systems loaded with CPβCDs–insulin complex are much smaller that loaded with insulin alone, which may because that the strong electrostatic attraction can condense the nanoparticle into a much more compact system. However, the size of nanoparticles loaded with different CPβCDs–insulin complex do not decrease with the increase of the charge density of CPβCDs, which may due to the different Alg/Chit mass ratio in particle preparation. Morphology study observed by SEM provides further information about the nanoparticle size as shown in Fig. 3 (inset). Most particles are of irregular shape (may due to the sample preparation process) and have a size in the range of 100–200 nm. It should be noted that the particle size measured in solution may be larger than that shown in SEM, which is under dry state. Particle size of current system is lower than the critical size necessary to enable GI absorption by M-cells on Peyer's patches. Some studies cited 5 μm while others described particles well under 10 μm as critical for absorption.

To investigate the capability of the designed system for oral insulin delivery, we measured the cumulative release profile of insulin from them in SGF for 2 h followed by in SIF for 4 h as shown

Table 2
Characterization of CPβCDs–insulin-loaded Alg/Chit nanoparticles in terms of insulin AE and LC ($n = 4$, mean ± S.D.).

Alg/Chit nanoparticles loaded with	AE (%)			LC (%)[a]
	Alg:Chit = 6:1 (w/w)	Alg:Chit = 5:1 (w/w)	Alg:Chit = 4.3:1 (w/w)	
Insulin alone	58 ± 1	67 ± 1	**80 ± 3**	9.6 ± 0.6
CPβCD1/15/4–insulin	67 ± 2	78 ± 1	**87 ± 1**	9.5 ± 0.7
CPβCD1/15/6–insulin	72 ± 1	**82 ± 2**	N/A[b]	9.5 ± 0.6
CPβCD1/15/10–insulin	71 ± 2	**75 ± 1**	N/A[b]	7.5 ± 0.4

[a] Measured on formulations with the highest AE of each sample as pointed out with bold.
[b] Could not form stable nanoparticle system due to aggregation.

in Fig. 4. It is to be noted that all of them were measured on formulations with the highest AE of each sample as pointed out with bold in Table 2, i.e., the Alg/Chit mass ratio were 4.3:1, 4.3:1, 5:1 and 5:1 for nanoparticles loaded with insulin, CPβCD1/15/4–insulin, CPβCD1/15/6–insulin and CPβCD1/15/10–insulin, respectively. As it can be seen, insulin is released in a burst effect in SGF while lacking of the protection from CPβCDs, that is, up to 60% of the loaded insulin was released from nanoparticles in SGF (pH 1.2) in the initial 2 h. As a result, most of the encapsulated insulin had been lost before it could reach intestine to make any therapeutic sense: only 18% of the loaded insulin was released in SIF (pH 6.8) in the next 4 h. This result is in close agreement with what has been reported by others (Sarmento et al., 2007b). Considering that insulin bioavailability *in vivo* is usually much lower than the value of that release in SIF *in vitro*, the insulin-loaded Alg/Chit nanoparticle system does not look promising for clinical trial.

On the other hand, CPβCDs–insulin complex loaded nanoparticles exhibit much better oral insulin delivery profile (Fig. 4): the cumulative release of insulin in SGF in the initial 2 h is significantly decreased while the percentage of insulin released in SIF is obviously increased. For example, CPβCD1/15/4–insulin-loaded nanoparticles with an alginate:chitosan mass ratio of 4.3:1 released 48% of insulin in SGF in the initial 2 h and achieved a total release amount of 88% in the following 4 h in SIF, that is, 40% of insulin was successfully released in SIF. This result strongly demonstrates the effective protection and controlled release ability of CPβCDs on insulin in the Alg/Chit nanoparticles system. The principle here is close to that of non-viral gene vector field, in which cationic polymers are used to condense plasmid DNA (negatively charged) into a much smaller size thus can provide better protection and easier cell internalization (Li et al., 2006, 2010). CPβCDs form a compact complex with insulin (negatively charged while pH > 5.3

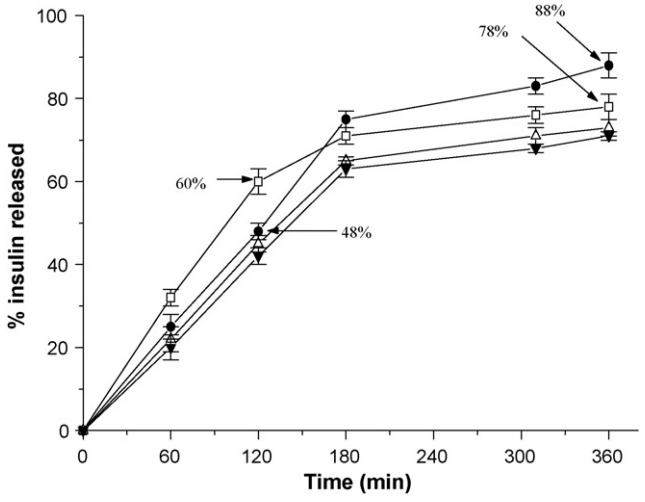

Fig. 4. Cumulative insulin release in SGF for 2 h followed by in SIF for 4 h at 37 °C. Alginate/chitosan nanoparticles were loaded with insulin (□), CPβCD1/15/4–insulin (●), CPβCD1/15/6–insulin (△) and CPβCD1/15/10–insulin (▼).

in the complexation process) by both electrostatic attraction and inclusion. The complexed insulin is smaller in size and its surface is partially covered by the cationic polymer (CPβCDs), which could make it to be better entrapped in the anionic alginate than that of insulin alone. Thus, the insulin is dominantly retained in the core of above nanoparticles in the form of CPβCDs–insulin complex. This specific composition and drug dispersion character make insulin to be better protected in the acidic environment of SGF, in which the electrostatic attraction between the negative carboxylic groups of alginate and the positive amine groups of chitosan could also prevent the nanoparticles to be further dissolved. When the nanoparticles are transferred into SIF where pH equals 6.8, alginate swells as it formed an ionic state so that the encapsulated insulin is released out gradually.

It is noted that the increase of the positive charge of CPβCDs could also lead to an increase of the protection efficiency. As can been seen in Fig. 4, the accumulated release of insulin is 48, 45 and 42% for nanoparticles carrying insulin complexed with CPβCD1/15/4, 1/15/6 and 1/15/10, respectively, in which the last number stands for the cationic group's ratio (CC). Considering that the latter two samples are prepared with less chitosan (Alg:Chit = 5:1 while it is 4.3:1 for CPβCD1/15/4, all ratios are chosen at the highest AE point) and it is well known that a higher chitosan ratio would result in a better protection due to the formation of an alginate/chitosan complex film on the surface of the nanoparticles (Sarmento et al., 2007b), the effect of cationic groups on the insulin protection efficiency might be even underestimated. Thus we could conclude that a higher cationic group ratio in CPβCDs could lead to a better protection in acidic condition. However, the amount of insulin release in SIF in the following 4 h did not increase with the increase of CPβCDs' positive charge ratio as the latter two samples only released around 29% insulin in SIF. This might because a stronger electrostatic attraction between CPβCDs and insulin will make the complex to be too firm to release insulin. After 24 h of release study, almost 100% of encapsulated insulin was released from all the tested samples due to the totally disruption of nanoparticles (data not shown).

In short, CPβCDs–insulin-loaded Alg/Chit nanoparticles can effectively protect insulin in SGF and the release profile in SGF/SIF could be controlled by varying the molecular structure of CPβCDs besides the Alg/Chit mass ratio.

3.3. Stability studies and structural analysis of insulin

It is known that in the hydrolysis of crystalline insulin with pepsin the destruction of the physiological activity progresses at a much greater rate than does the hydrolysis of the protein. Also, the insulin digested with pepsin could not be reactivated. Thus, the stability study against peptic digestion is essential to evaluate a novel oral insulin delivery system. Fig. 5 shows the remaining percentage of insulin after incubation of insulin-loaded nanoparticles or CPβCD1/15/4–insulin complexes loaded nanoparticles in pepsin solution. Protective effect is observed in the following sequence: CPβCD1/15/4–insulin complexes loaded nanoparticles > insulin-

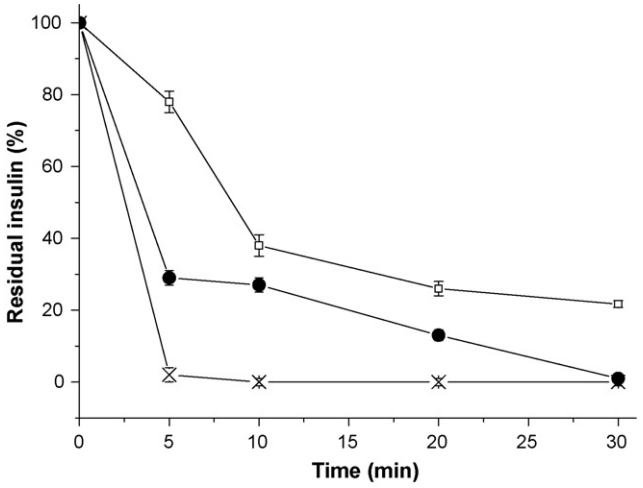

Fig. 5. Residual percentage of insulin after incubation of insulin solution (×), insulin-loaded nanoparticles dispersion (●) and CPβCD1/15/4–insulin complex loaded nanoparticles dispersion (□) in pepsin solution ($n = 3$, mean ± S.D.).

loaded nanoparticles ≫ insulin alone. It is not surprising to see that almost all insulin was destructed within 5 min when it was incubated with pepsin solution. Usually, the loss in physiological activity is accompanied by a decrease in the tyrosine content of insulin. However, the intact protein structure is essential for the physiological activity. This is also the reason why directly oral administration of most peptide drugs would come with very low bioavailability. The Alg/Chit nanoparticle system could provide moderate protection to insulin against peptic digestion because it could keep part of insulin in the core of the nanoparticle. However, the protective efficiency is not high because quite a few amount of insulin are adsorbed on the surface of the particle (Sarmento et al., 2007b,c). In the case of CPβCD1/15/4–insulin-loaded nanoparticles, a better protection is observed because most insulin is retained in the core of the nanoparticle as described in Fig. 1. This system constructs a much tighter barrier to keep pepsin away from insulin in the center.

The effect of nanoencapsulation process on the protein structure is an important issue because different types of forces are responsible for insulin physicochemical stability, such as hydrophobic and electrostatic interactions, covalent bonding, hydrogen bonding and van der Waals forces. The complex interaction between these forces places hydrophobic residues in the interior of insulin while directing hydrophilic residues to its outside, where they interact with the aqueous solvent. However, manipulation conditions of insulin might lead to some conformational changes, thus exposing hydrophobic areas to outside, resulting in reduced solubility and an increased tendency of aggregation. Meanwhile, physical forces such as stirring, filtration and centrifugation might expose insulin to air/liquid or liquid/solid interfaces resulting in similar undesired effects and loss of structural integrity. Insulin spectra after release in both SGF (pH 1.2) and SIF (pH 6.8) environments were collected to investigate potential changes in insulin structure as shown in Fig. 6. The CD spectra of insulin solution in SGF (Fig. 6A, —) and SIF (Fig. 6B, —) displays two minima at 209 and 222 nm, which is typical of predominant α-helix structure proteins. It indicates that in these conditions pH variation would not significantly change the a-helix/β-sheet structure of insulin. After being complexed with CPβCDs and encapsulated in Alg/Chit nanoparticles, there are only minor differences in the insulin's CD spectra. The slight shift might due to interference from the polymers on the nanoparticle. The CD spectra of insulin released from alginate/chitosan nanoparticles with CPβCDs in both SGF and SIF are found to be also coincident with the standard insulin solutions, except for minor differences

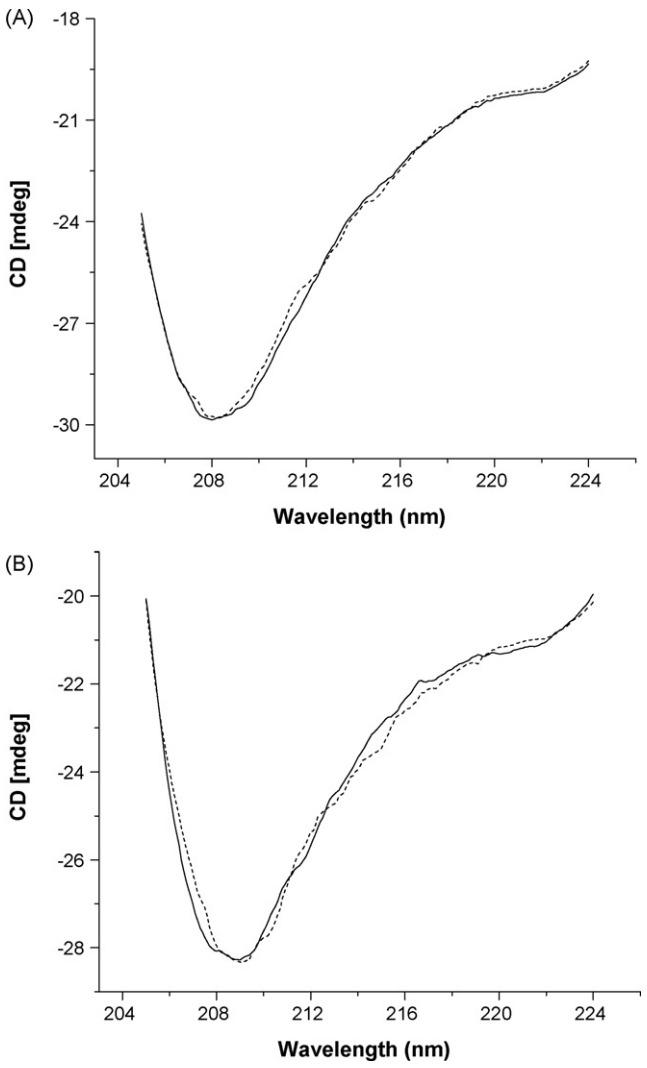

Fig. 6. Far-UV CD spectra of insulin (—) and insulin released from CPβCD1/15/4–insulin-loaded Alg/Chit nanoparticles (---) in SGF (pH 1.2, A) and SIF (pH 6.8, B), respectively.

which may caused by the ionic interaction between insulin and polyelectrolyte applied in the system (Sarmento et al., 2007b). This suggests that the CPβCDs–insulin-loaded alginate/chitosan nanoparticles system can carry and release insulin, in a manner of preserving the structure and potentially maintaining the activity of insulin.

4. Conclusions

In this work, insulin was complexed with CPβCDs through both electrostatic attraction and inclusion, which were proved by CD spectra and zeta potential analysis. The CPβCDs–insulin complex was then encapsulated into alginate/chitosan nanoparticles. The formation of Alg/Chit nanoparticles with above complex was dependent on the mass ratio of Alg:Chi, pH of production and the structure of CPβCDs. The nanoparticles have a size range from 146 to 338 nm and can load insulin with the association efficiency (AE) and loading capacity (LC) up to 87 and 9.5%, respectively. The cumulative insulin release in SIF was much higher (40%) than that without CPβCDs (18%) because insulin was mainly retained in the core of the nanoparticles and well protected against degradation in SGF. The insulin structure was also well preserved during the nanoparticle preparation and release process.

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