A Novel Insulin Oral Delivery System Assisted by Cationic β-Cyclodextrin Polymers

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ABSTRACT: This work describes a new oral pharmaceutical formulation of insulin that is complexed with cationic β-cyclodextrin polymers (CPβCDs), and then encapsulated into alginate/chitosan microspheres, which are prepared by ionotropic pregelation/polyelectrolyte method. CPβCDs were synthesized through a one-step polymerization of β-cyclodextrin (βCD), epichlorohydrin, and choline chloride. CPβCDs have enhanced ability to complex with insulin due to the assistance of their polymeric chains, as well as the electrostatic interactions between insulin (negatively charged while pH>5.3) and quaternary ammonium groups of CPβCDs. The noncovalent inclusion complex formed between CPβCDs and insulin was analyzed by Fourier transform infrared and fluorescence emission spectra. With the increase of zeta potential of CPβCDs from 1.8 to 14.2 mV, the insulin association efficiency (AE) of current system was increased from 55.2 to 71.8%, whereas the AE of insulin-loaded microspheres at the same condition was only 50.7%. The cumulative insulin release in simulated intestinal fluid was also higher than that of the insulin-loaded microspheres and βCD-insulin encapsulated microspheres. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1371–1379, 2010

Key words: cationic β-cyclodextrin polymer; alginate/chitosan microsphere; insulin; oral delivery system

INTRODUCTION

The development of suitable carrier systems for oral administration of insulin remains a major challenge because its bioavailability is limited by the epithelial barriers of the gastrointestinal tract and gastrointestinal degradation by digestive enzymes. An ideal drug carrier for insulin should provide a stable and biocompatible environment to ensure that the main fraction of the therapeutic protein will be biologically active during both particle processing and insulin release.[1]

Alginate and chitosan have been described as biocompatible, biodegradable, and mucoadhesive, which are suitable for designing drug-controlled release devices.[2–6] They can form polyelectrolyte complex which are often used to protect proteins from the aggressive environment of the stomach.[7,8] Moreover, chitosan could enhance paracellular permeability for peptides such as insulin by charge-mediated polymer binding to epithelia, resulting in a structural reorganization of tight junction-associated proteins.[9] It has been reported that the association efficiency (AE) of the insulin-loaded alginate/chitosan nanoparticles could achieve 90% or higher, but a significant amount of insulin was associated to the surface of the nanoparticles. In this case, up to 50% insulin was released in a burst effect in simulated gastric fluid before it could make therapeutic sense in simulated intestinal fluid (SIF).[7,10]

Among the additives of absorption enhancers or protease inhibitors used in insulin oral pharmaceutical formulation, the polysaccharides play an important role to help circumvent these inconveniences: chemical and enzymatic instability, poor absorption through biological membranes, and rapid plasma clearance.[11–13] Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six to eight glucose units linked by α-1,4-glucosidic bonds. CDs contain internal hydrophobic cavities and external hydrophilic surface so that they could act as host molecules to form inclusion complexes with a number of guest molecules. The internal hydrophobic cavities in CDs facilitate the inclusion of a number of guest molecules.[14] It has been suggested that CDs and their derivatives might undergo degradation in the colon since there has vast microflora, which break CDs and their derivatives into small saccharides to be absorbed in the large intestine.[15,16] Thus, CDs and their derivatives have been widely used in the drug delivery applications.[17-21]

Proteins such as insulin are too bulky to be wholly included into CDs' cavities. Their hydrophobic side

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chains partially penetrate into the CDs' cavity leading to the formation of noncovalent inclusion complexes.[11] 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.[13] However, parent CDs usually have limited pharmaceutical applications due to their low water solubility and cytotoxicity.[22] Therefore, various CDs derivatives have been developed to overcome these drawbacks, such as sulfobutylether β-cyclodextrin (SBE-βCD) and hydroxypropyl β-cyclodextrin (HP-βCD).[13] In our previous work, cationic β-cyclodextrin polymers (CPβCDs) with various degrees of polymerization and different cationic charge densities were synthesized by a one-step polycondensation. CPβCDs exhibited excellent water solubility, better hemocompatibility, and drug delivery performance than that of βCD.[23,24] Cationic cyclodextrin polymers have also been reported to improve antimicrobial activities of antibiotics and play the role of nonviral gene vectors in recent researches.[25–28]

The aim of this work was to develop a new oral pharmaceutical formulation of insulin: prepare CPβCDs-insulin complexes utilizing CPβCDs' polymeric chain and cationic charge, and then encapsulated into alginate/chitosan microspheres. The influence of charge densities of CPβCDs on the AE and the *in vitro* release profile of insulin were also investigated.

MATERIAL AND METHODS

Materials

Insulin powder was purchased from Xuzhou Wanbang Biological Pharmaceutical Enterprise (Jiangsu, China). Sodium alginate and β-cyclodextrin (βCD) were procured from Kelong Reagent Co. (Chengdu, China). Chitosan with approximate molecular weight 10,000 and 90% deacetylated was obtained from Golden-shell Biochemical Co. (Zhejiang, China). All the other reagents and solvents were of AR grade.

Synthesis and characterization of CPβCDs

Synthesis of CPβCDs

CPβCDs were synthesized by one-step polycondensation of βCD, epichlorohydrin (EP) and choline chloride (CC) as described in Li's report.[23] The feeding molar ratios of the reactants (βCD/EP/CC) were 1/15/2, 1/15/4, and 1/15/6 in this work. For example, a typical synthetic procedure of CPβCD1/15/2 is: 1 g of NaOH was dissolved in 20 mL of water, and then 5.675 g of βCD were dissolved in the sodium hydroxide solution. The solution was electromagnetically stirred at 25°C for 24 h in a water bath. CC of 1.396 g were then fed into the solution rapidly, and 6.940 g of EP were added at a flow rate of about 0.1 mL/min. After the completion of EP feeding, the mixture was heated to 60°C and kept for 2 h, the polymerization was stopped by neutralized with an aqueous hydrochloride acid solution (3*N*). The solution obtained was dialyzed for 24 h with a dialysis membrane of molecular weight cutoff 3500. The solution obtained was evaporated, and the solid was pulverized to powder.

Analysis of molecular weight by GPC

Gel permeation chromatography (pump: Waters 600E System Controller; detector: Waters 410 Differential Refractometer) was carried out with Ultrahydrogel 120 and Ultrahydrogel 250 columns at 40°C and the flow rate at 0.7 mL/min. Water was used as an eluent. Calibration was made using standard Pulluan samples from P-5 to P-50 (P-82 from Shodex), HP-βCD (M_n = 1402), and β-CD (M_n = 1135). Samples were used with concentration of 0.2–0.4% (w/v) and filtrated with a 0.45 μm Nylon Cameo filter-syringe prior to the use.

NMR study of CPβCD

Nuclear magnetic resonance (NMR) spectra were conducted in D_2O using a Bruker AV II-400 spectrometer operated at 400 MHz for ^{1}H-NMR. The β-CD contents of the polymers were calculated according to the integral area.

Preparation and characterization of CPβCDs-insulin complexes

Preparation of CPβCDs-insulin complexes

Insulin of 10 mg was dissolved in 10 mL of HCl (pH 3). Then the pH of the insulin solution was adjusted to 6.3 ± 0.1 using 0.1 *N* NaOH. CPβCDs-insulin complexes were prepared by mixing 100 mg CPβCDs with 5 mL insulin solution (equivalent to 150 IU) and then stirred for 1 h at room temperature. Residual uncomplexed free insulin was not separated due to its negligible amount.

FTIR studies

Having followed above procedures, CPβCD1/15/6-insulin complex was allowed to remain for another hour after stirring 1 h at room temperature. Resultant complex was lyophilized to obtain solid complex for FTIR measurement. Infrared absorption spectra of CPβCD1/15/6 and CPβCD1/15/6-insulin complex were obtained by using a Nexus 670 FTIR

(Thermo Nicolet Company), running from 4000 to 500 cm^{-1}. The samples were pressed with KBr before measuring.

Fluorescence spectroscopic studies

Fluorescence spectroscopic analysis was done using Shimadzu RF-5301 spectrophotometer (Japan). Different amounts of CPβCD1/15/6 (20 and 40 mg) were added to the insulin solution containing 10 mg insulin (pH was adjusted to 6.3) and stirred for 1 h before measurements. Fluorescence emission spectra were recorded (290–410 nm) at an excitation wavelength of 280 nm.

Zeta potential measurements

The zeta potential of CPβCDs and CPβCDs-insulin complexes was measured by Malvern zetasizer and Particle Analyzer (Malvern Instruments). The CPβCDs were dissolved in deionized water before measurement. 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 adjustation. The value was recorded as the average of five measurements.

Microsphere preparation

Sodium alginate was dissolved in deionized water by magnetic stirring overnight, and chitosan was dissolved in 1% acetic acid solution followed by filtering.

Alginate/chitosan microspheres were prepared in a two-step procedure based on the ionotropic pergelation. Insulin solution of 1 ml (equivalent to 30 IU) was mixed with 20 mg of CPβCDs and then gently stirred for 60 min to form complexes. Then the solution containing complexes was mixed with 2 mL of 2% alginate solution. The alginate solution containing CPβCDs-insulin was added dropwise for 15 min under gentle stirring (300 rpm) into a beaker containing 10 mL of 5% calcium chloride solution. The microspheres formed were allowed to harden for 10 min. They were washed by water and then dried. Then 10 mL of 0.5% chitosan solution was added into the beaker containing the dried microspheres. It was stirred for another 15 min to improve curing and then washed again by water and dried.

Morphology observation

The morphology of microspheres has been 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.

Insulin association efficiency

The AE was calculated by the difference between the total amount of insulin used to prepare the microspheres and the amount of residual unassociated insulin present in the supernatant.

$$AE = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100\%$$

Insulin *in vitro* release study

The microspheres were placed into test tube with simulated gastric fluid (SGF) containing 20 mL of HCl (pH 1.2) for 2 h, washed by distilled water and then were put into the simulated intestinal fluid (SIF) containing 20 mL of phosphate (pH 7.4) for 4 h. At appropriate time intervals, a definite volume of the release medium were taken and replaced by fresh medium. The amount of insulin released was estimated by the method of Coomassie Brilliant Blue staining.[29] All the experiments were carried out in triplicate, and the data presented are the average of the three measurements.

RESULTS AND DISCUSSION

Physicochemical properties of CPβCDs

In this study, three types of CPβCDs, i.e., CPβCD1/15/2, CPβCD1/15/4, and CPβCD1/15/6, were synthesized according to previous method,[23] in which the number following CPβCD means the feeding molar ratio of βCD/EP/CC in synthesis. The number average molecular weight (M_n) of CPβCD1/15/2, 1/15/4, and 1/15/6 are 4830, 4680, and 4840 g mol^{-1}, respectively (Table I). The M_n depends on the feeding ratio of βCD/EP/CC and reactivity between EP and CC, and EP and βCD. When CC/βCD

TABLE I
Molecular Weight and Zeta Potential Measurements ($n = 5$)

Samples	M_n (g mol^{-1})	Zeta potential (mv)[a]
Insulin		−28.0 ± 1.3
CPβCD1/15/2	4830	1.8 ± 2.3
CPβCD1/15/4	4680	6.0 ± 2.9
CPβCD1/15/6	4840	14.0 ± 3.8
βCD-insulin complex		−26.5 ± 2.2
CPβCD1/15/2-insulin complex		−21.6 ± 3.1
CPβCD1/15/4-insulin complex		−14.8 ± 1.7
CPβCD1/15/6-insulin complex		−9.7 ± 1.3

[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 adjustation.

feeding ratio is increased from 2 to 4, relatively more EP reacts with CC so that less crosslinked βCDs induced by EP exist, resulting in lower molar mass of CPβCD as explained previously.[24] When CC/βCD ratio is further increased to 6, the positive effect of increased amount of CC incorporated into the CPβCD exceeds the negative effect of decreased amount of EP reacting with βCD on molar mass. Thus, the molar mass of CPβCD1/15/6 is higher than that of CPβCD1/15/4. As for the three CPβCDs investigated in this work, the contents of βCD in CPβCD1/15/2, 1/15/4, and 1/15/6 were 77.74%, 77.79%, and 47.99%, respectively, whereas their CC contents were 1.78%, 2.30%, and 10.69%, respectively(calculated according to ^{1}H NMR data).

Since this study proposes to utilize the cationic charge of CPβCDs to form electrostatic interactions with insulin (negatively charged while pH>5.3) before preparing CPβCDs-insulin complexes included alginate/chitosan microspheres with high insulin association efficiency, the surface charge of CPβCDs plays an important role in CPβCDs-insulin interactions. Thus, we also measured the zeta potential values of the CPβCDs (Table I). The zeta potential values of 1/15/2, 1/15/4, and 1/15/6 are 1.8, 6.0, and 14.0 mV, respectively. It is noteworthy that CPβCDs have positive zeta potential values, and the values are increased with the increase of the feeding molar ratio of CC to βCD. In general, parent βCD has intrinsic negative charge property. Therefore, the cationic charge effect of quaternary ammonium groups of CC outperforms the intrinsic negative charge effect on the charge property of the CPβCDs.

The *in vitro* cytotoxicity, hemolytic property, and drug inculsion performance of CPβCDs have been discussed elsewhere.[23–25]

CPβCDs-insulin complexes

Since FTIR is a useful technique to confirm the formation of cyclodextrin-drug complex, FTIR spectra of CPβCD1/15/6 and CPβCD1/15/6-insulin complex are shown in Figure 1(a,b), respectively. Insulin has typical absorption peaks at 1654 and 1541 cm^{-1} corresponding to amides I and II (spectrum not shown). As for CPβCD1/15/6 [Fig. 1(a)], the absorption bands were observed at 3402 and 1642 cm^{-1} due to the presence of OH groups and bending vibration of the OH groups. The peaks at 1032 and 1078 cm^{-1} are due to the coupled C—C and C—O stretching vibrations, whereas the band at 1158 cm^{-1} is contributed by the antisymmetric stretching vibration of the C—O—C glycosidic bridge. In the case of CPβCD-insulin complex [Fig. 1(b)], the insulin peak moves to 1646 cm^{-1}, and an additional peak at 1535 cm^{-1} are observed, which confirms the formation of the CPβCD1/15/6-insulin complex.

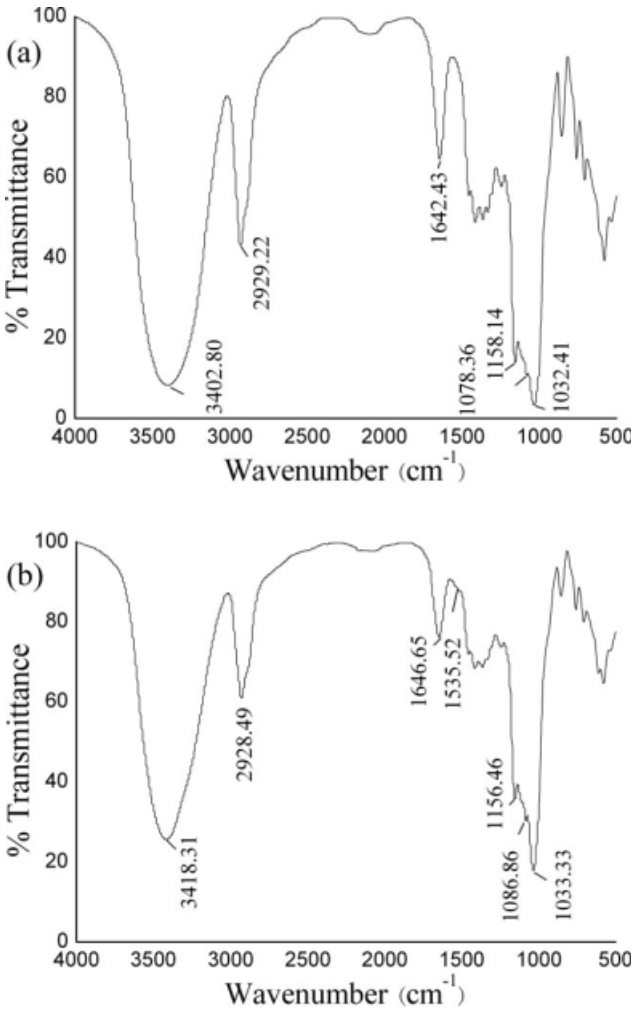

Figure 1 FTIR spectra of CPβCD1/15/6 (a) and CPβCD1/15/6-insulin complex (b).

Figure 2 exhibits the fluorescence emission intensity of insulin solutions with and without CPβCD1/15/6. It suggests that the addition of CPβCD1/15/6 to insulin solution has decreased the fluorescence emission intensity of insulin. As the concentration of CPβCD was increased, fluorescence intensity of insulin solution was also decreased significantly. The results were different from insulin complexed with neutral βCD derivatives, which could increase the fluorescence emission intensity of insulin solution due to the encapsulation of tyrosine into the cavity of CD and exclusion of water from the immediate tyrosine neighborhood.[18] The different phenomena in this work may be due to the electrostatic interactions formed between insulin and CPβCD in addition to normal inculsion complexation. The fluorescence emission of insulin at an excitation wavelength of 280 nm is mainly attributed to fluorescence of tyrosine. However, tyrosine was negatively charged while pH > 5.64, the electrostatic interaction between tyrosine and CPβCD1/15/6 leads to the

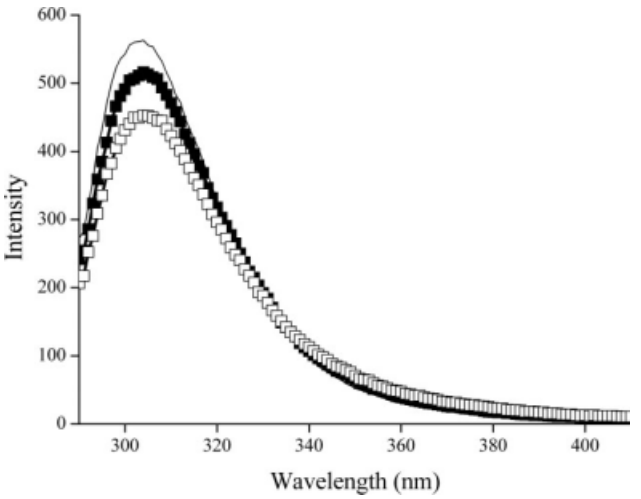

Figure 2 Fluorescence emission spectra of insulin (1 mg/mL, —) and its complexes formed by adding 20 mg (■) or 40 mg (□) CPβCD1/15/6 per milliliter.

observed decrease in fluorescence measurements. These studies confirm the complex formation between insulin and CPβCD.

Zeta potential measurements could provide further information about the formation of electrostatic interactions between insulin and CPβCD (Table I). The zeta potential values of insulin (at pH 6.3) and the complexes of βCD-insulin, CPβCD1/15/2-insulin, CPβCD1/15/4-insulin, and CPβCD1/15/6-insulin are −28.0, −26.5, −21.6, −14.8, and −9.7 mV, respectively. It is noted that although the surface charge of βCD is negative, the βCD-insulin complex exhibits a little higher zeta potential than that of insulin alone. This might because part of the insulin's side chains had been included in the hydrophobic cavities of βCD. Meanwhile, with the increase of zeta potential of CPβCDs, a similar increasing trend of the zeta potential of CPβCDs-insulin complex was observed due to the cationic property of CPβCDs. It indicates that the electrostatic interactions had formed between insulin and CPβCDs besides noncovalent inclusion interaction.

In a word, the CPβCD-insulin complex is formed by both inclusion and electrostatic interaction as shown in Figure 3, which would be helpful to provide stronger protection for insulin.

Preparation and characterization of microspheres

The CPβCDs-insulin complexes loaded alginate/chitosan microspheres were prepared via the ionotropic gelation method. Since this method utilizes the ionic interaction between the positively-charged chitosan and the negatively-charged alginate, as well as the ability of chitosan to form a gel after contact with polyanions by forming inter- and intramolecular linkages,[30] it is very important to control the ionic gelation process. Several factors had been investigated during the preparation. For example, when the concentrations of calcium chloride solution were 4, 5, and 6%, the AE of insulin-loaded microspheres were found to be 40.6, 52.0, and 53.2%, respectively. It seems that the AE could be improved with the increase of the concentrations of calcium chloride solution. Since the AE of microspheres with 4% of calcium chloride solution was relatively low, and it has been reported that protein retention in acid could be decreased with the increase of the concentrations of calcium chloride solution,[31] we fixed the concentrations of $CaCl_2$ at 5% for all the investigations.

Surface morphology and cross section information for CPβCD1/15/6-insulin complex loaded alginate/chitosan microspheres have been obtained by SEM analysis and are shown in Figure 4. As presented in Figure 4(a), the spherical shape of microspheres was lost after lyophilizing, and the irregular shape had a relatively smooth surface with some wrinkles. This is because of the rapid evaporation of water in surface of microspheres while the freeze-drying process. Figure 4(b,c) show the cross section and internal structure of the same microsphere. It seemed to be less porous than that of insulin-loaded alginate/chitosan microspheres reported elsewhere.[32] It might be due to the tight junction between CPβCDs-insulin complex and alginate/chitosan microsphere caused by electrostatic interactions.

Influence of CPβCDs-insulin complexes on insulin association efficiency and release profile

Complexation time

Figure 5 exhibits the AE and insulin *in vitro* release performance from the systems loaded with the same amount of CPβCD1/15/6-insulin but complexed for

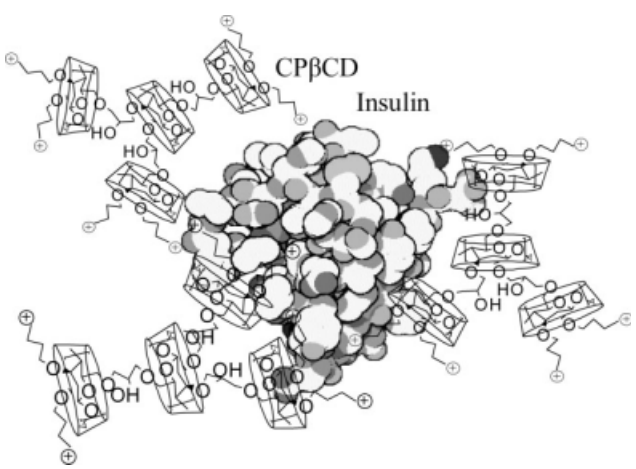

Figure 3 Schematic drawing of the CPβCD-insulin complex formed by both inclusion and electrostatic interaction.

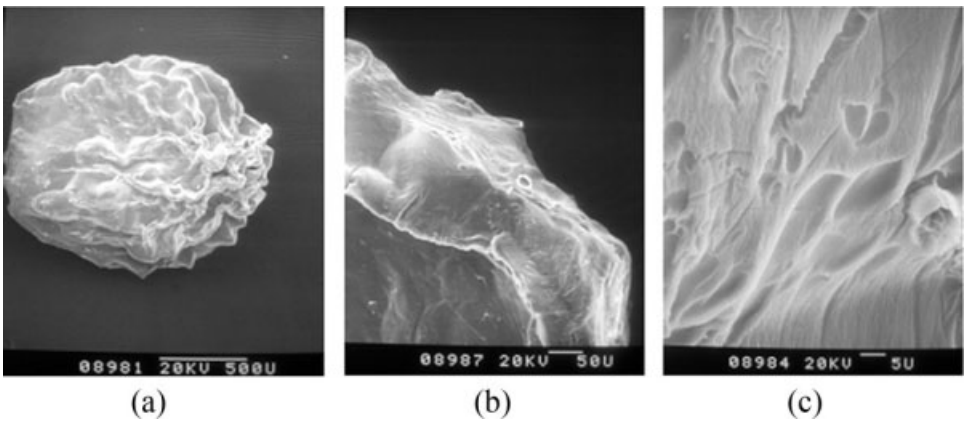

Figure 4 SEM micrographs of CPβCD1/15/6-insulin complex loaded alginate/chitosan microsphere (×50, a), cross section (×200, b) and internal portion (×1500, c) of the same structure.

different periods of time. It is found that the insulin AE of systems with CPβCDs-insulin complexation time of 40, 60, and 120 min are 60.1, 71.8 and 71.7%, respectively. The result suggests that the insulin AE increases with the elongation of complexation time but is almost stabled after 60 min.

On the other hand, the complexation time could also affect the cumulative insulin release profile in SGF and SIF as shown in Figure 5. In SGF where pH equals 1.2, part of chitosan in the surface of microspheres was dissolved. However, the electrostatic interactions between the negative carboxylic groups of alginate and the positive amine groups of chitosan prevented the chitosan to be further dissolved. Additional gastric protection against the insulin release may be due to the more effective retention by a tight alginate network that forms at low pH.[33] In SIF where pH equals 7.4, alginate swells as it forms an ionic state so that a higher amount of insulin is released. For the system of 120 min complexation time, a faster rate of insulin release was observed. This could be attributed to the smaller size of the nonaggregated molecules of insulin on complexation with CPβCD.[34] Although the 120 min complexation time leads to the highest cumulative insulin release amount, the corresponding system also lose the highest percent of insulin in SGF, which is unwanted for insulin oral delivery system. Besides, the cumulative insulin release in SIF of the 60 min-system was higher than that of the 40 min-system because of its higher AE. Therefore, a suitable complexation time of 60 min could help to obtain high insulin AE and desirable insulin release profile.

Amount of CPβCD in complex

CPβCD-insulin complexes are formed by both noncovalent inclusion interaction and electrostatic interactions between the two components as addressed previously. Thus, the amount of CPβCD used to form the complex might play an important role to affect the insulin AE and release profile of resulted microsphere system. Figure 6 shows the insulin *in vitro* release profile of microspheres containing the same amount of insulin (30 IU) complexed with different amount of CPβCD1/15/6 with 60 min of complexation time. The AE of the systems with 10, 20, 30, and 40 mg of CPβCD1/15/6 were 67.7, 71.8, 71.8, and 72.5%, respectively. It seems that the AE of the systems with the amount of 40 mg (1/15/6-40 system) and 30 mg (1/15/6-30 system) CPβCD 1/15/6 did not have significant improvement compared with that of the system with 20 mg CPβCD1/15/6 (1/15/6-20 system). It may be due to the presence of excessive CPβCD1/15/6, which acts as filler and

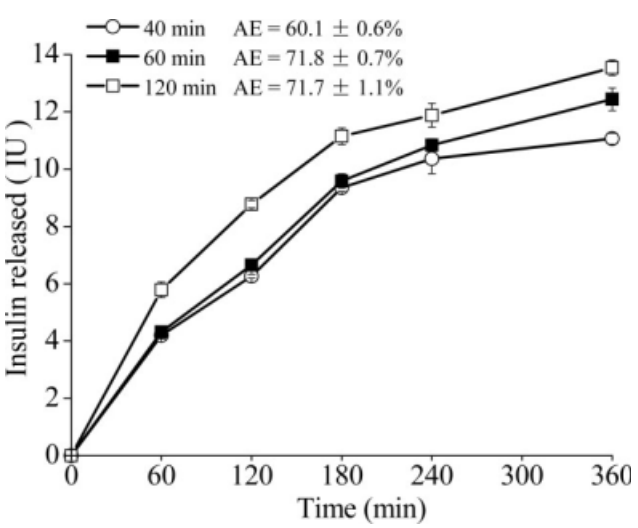

Figure 5 The insulin AE and release profile of microspheres in SGF for 2 h followed by in SIF for 4 h at 37°C. ($n = 4$, mean ± SD). The microspheres are loaded with the same amount of CPβCD1/15/6-insulin but complexed for different time.

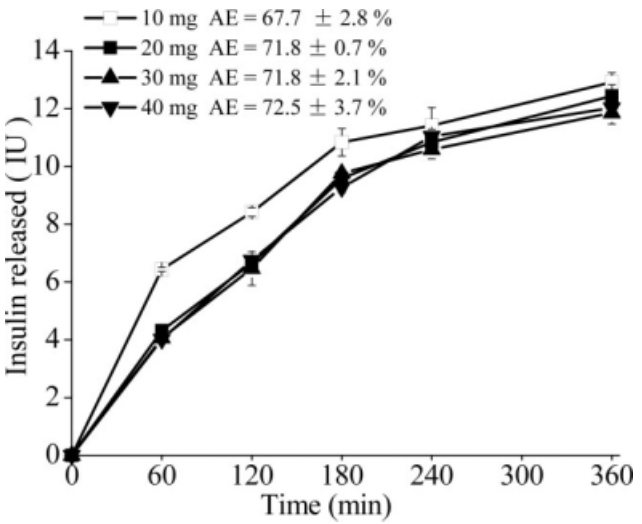

Figure 6 The insulin release profile from microspheres containing the same amount of insulin complexed with different amounts of CPβCD1/15/6 in SGF for 2 h followed by in SIF for 4 h at 37°C ($n = 4$, mean ± SD). The amount of CPβCD 1/15/6 are 10 mg (□), 20 mg (■), 30 mg (▲) and 40 mg (▼).

reduces the crossliking between alginate and chitosan, and the decreasing amount of crosslinking may reduce the retention of the drug.[17]

The release of insulin from the system with 10 mg CPβCD (1/15/6-10 system) was much faster than that from the other systems. This should because of the weak interaction between insulin and CPβCDs caused by the deficiency of CPβCDs. However, with the amount of CPβCD1/15/6 further increased from 20 to 40, 1/15/6-20 system insulin release in a similar trend to that of 1/15/6-30 system and 1/15/6-40 system both in SGF and SIF. Therefore, an appropriate ratio of CPβCD/insulin at 20 mg/30 IU in complex is critical for obtaining high AE and good release performance.

Different CPβCDs in complex

The values of the AE of microspheres containing insulin complexed with the same amount (20 mg) of different CPβCDs for fixed complexation time (60 min) are shown in Table II. The AE of microspheres containing βCD-insulin or CPβCDs-insulin complexes are higher than that of insulin-loaded microspheres (control system). It may be due to the formation of noncovalent inclusion complexes between βCD (or CPβCDs) and insulin,[18] as well as additional electrostatic interactions between CPβCDs and insulin as indicated by FTIR spectra and zeta potential measurements discussed above. Meanwhile, the AE of microspheres containing CPβCD1/15/6-insulin complex (1/15/6 system) and the one containing CPβCD1/15/4-insulin complex (1/15/4 system) were higher than that containing βCD-insulin complex (βCD system). It is probably attributed to the polymeric chains and positive charge of CPβCDs, which could enhance the complexation with insulin. First, the polymeric chains of CPβCDs could assist the inclusion of drugs, especially those with large molecular structure such as insulin.[35] Second, insulin is mainly negatively charged, while pH>5.3 and CPβCDs are positively charged at this condition. As shown in Table I and II, with the increase of zeta potential of CPβCDs-insulin complexes, a similar increasing trend of the AE was observed. We also investigated CPβCDs with even higher positive charge, e.g., the AE of microspheres containing CPβCD1/15/8-insulin complex was 76.3%, which is higher than the AE of 1/15/6 system (71.8%). However, the cumulative insulin release of CPβCD1/15/8-insulin system in SIF was only 5.3 IU, which was lower than that of 1/15/6 system (5.8 IU). This means CPβCD1/15/8 system does not have better oral insulin delivery performance than that of CPβCD1/15/6 system. The electrostatic interactions between CPβCD1/15/8 and insulin might be too strong for insulin to be effectively released in SIF. Moreover, a higher cationic charge density might induce higher cytotoxicity,[36] thus CPβCDs with higher surface charge are not discussed in this article.

The cumulative insulin *in vitro* release profile from these microspheres was carried out, and the results are shown in Figure 7. In SGF at pH 1.2, more than

TABLE II
The AE of Alginate/Chitosan Microspheres Containing Insulin Alone (Control System), βCD-Insulin Complex and Insulin Complexed with Different CPβCDs ($n = 4$, Mean ± SD)

Alginate/chitosan microspheres	Added insulin (IU)	Loaded insulin (IU)	AE
Containing insulin alone	30	15.2 ± 0.2	50.7% ± 0.8%
Containing βCD-insulin complex	30	16.9 ± 0.4	56.3% ± 1.3%
Containing CPβCD1/15/2-insulin complex	30	16.7 ± 0.2	55.2% ± 0.8%
Containing CPβCD1/15/4-insulin complex	30	18.9 ± 0.3	62.9% ± 1.1%
Containing CPβCD1/15/6-insulin complex	30	21.3 ± 0.2	71.8% ± 0.7%

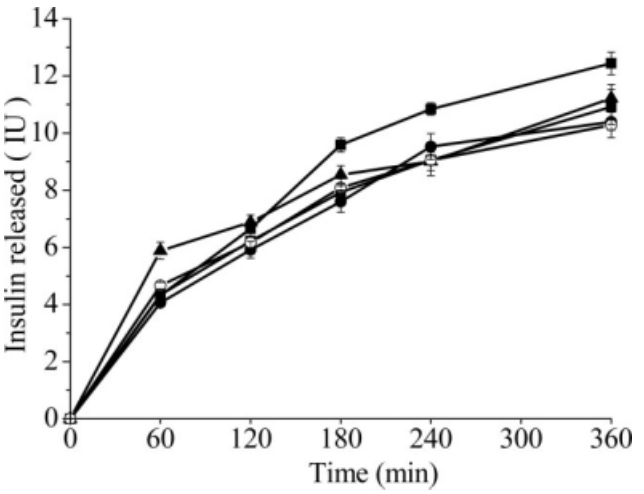

Figure 7 The insulin *in vitro* release profile from microspheres containing insulin complexed with CPβCD1/15/6 (■), CPβCD1/15/4 (●), CPβCD1/15/2 (▲), βCD (○) and insulin alone (□) in SGF for 2 h followed by in SIF for 4 h at 37°C ($n = 4$, mean ± SD).

30% of loaded insulin was released from the control system and the βCD system in the first hour. However, for the 1/15/6 system and the 1/15/4 system, only around 20% of loaded insulin was released. It is shown that the control system and βCD-insulin system released 40.6 and 40.7% of loaded insulin in SGF, respectively, both approximately 8% higher than that of 1/15/6 system. The lower insulin release in SGF from the microspheres containing CPβCDs-insulin complexes is attributed to the strong interactions between insulin and CPβCDs. Nevertheless, the release patterns of microspheres containing different CPβCDs-insulin complexes do not exhibit obvious difference in SGF.

However, after release for 4 h in SIF, the percentages of cumulative insulin released from βCD system and control system are 66.4 and 67.6%, respectively, which are higher than that of CPβCDs-insulin complex systems (62.8, 57.9, and 57.8% for 1/15/2, 1/15/4, and 1/15/6 systems, respectively). It can be noted that with the increase of zeta potential of CPβCDs, the percentage of cumulative insulin released from microspheres decreased in the same condition. It might because that the electrostatic interaction between insulin and CPβCDs becomes stronger with the increase of zeta potential of CPβCDs, which might hinder the release process of insulin. Even so, the cumulative amounts of insulin released from microspheres containing the three types of CPβCDs-insulin complexes in SIF are still higher than that of other systems due to their higher values of AE. The cumulative insulin released in SIF from the control system, and the systems that complexed with CPβCD1/15/6, 1/15/4, 1/15/2, and βCD are 4.1 IU, 5.8 IU, 4.7 IU, 4.5 IU, and 4.3 IU, respectively. In a word, the system containing CPβCDs-insulin complexes are superior to βCD-insulin system and the control system, especially the one loaded with CPβCD1/15/6-insulin complex, in terms of AE and release profile.

CONCLUSION

In this work, a novel insulin oral delivery system composed of CPβCDs-insulin complexes encapsulated alginate/chitosan microspheres has been described. A series of CPβCDs with different charge densities were synthesized. The characteristics of CPβCDs, e.g., the molecular weight and surface charge, are controllable in polymerization process. Zeta potential measurements suggest that the charge densities of CPβCDs are increased with the increase of the feeding molar ratio of CC to βCD. It is found that the zeta potential of CPβCDs, complexation time and the ratio of CPβCD/insulin in complexes could affect both the insulin association efficiency and insulin *in vitro* release profile. In conclusion, the complex of CPβCD1/15/6 (zeta potential = 14.2 mv) with insulin at a ratio of 20 mg to 30 IU with complexation time of 60 min exhibited the highest AE, good gastric protection and highest intestinal release amount of insulin. Thus, the alginate/chitosan microsphere containing CPβCD1/15/6-insulin complex is a promising system for improving insulin oral delivery efficiency.

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