Calcium Pectinate Gel Bead Intended for Oral Protein Delivery: Preparation Improvement and Formulation Development

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Calcium pectinate gel (CPG) micrometer-sized beads (microbeads) containing insulin, as a model amphoteric protein, were prepared by ionotropic gelation technique together with an air compressor. The influences of phosphate buffer, pH as well as calcium and pectin concentrations of cross-linking solution on the characteristics and release profiles of microbeads were investigated. With the aid of compressed air flow, the mean diameters of beads were successfully decreased to micron-sized. The results showed that all the factors investigated greatly affected the entrapment efficiencies and release profiles of the microbeads. Suitable formulation concentrations should be considered and great care should be taken to maintain the pH of working solutions at or close to isoelectric point of protein loaded during the whole preparation process. Hence, CPG microbeads of perfect spherical shape, uniform sizes, enhanced mechanical strength, good entrapment efficiencies and delayed release profiles were prepared for a load of amphoteric protein and peptide drugs, without any use of organic solvents or harsh ingredients. Therefore, CPG microbeads could be a promising carrier for oral controlled-release systems of amphoteric protein and peptide drugs.

Key words oral protein delivery; pectin; calcium pectinate gel; insulin; pH; isoelectric point

With recent advances in biotechnology, protein and peptide drugs have become a focus on current formulation research. Most of protein pharmaceuticals are mostly administered parenterally,[1] with the limitations included that oscillating blood drug concentrations caused by repeated doses and patient compliance resulted from frequent injections.[2] Oral delivery, which allows for a more varied load to be released, is the most convenient method of administration. However, it still remains a challenge especially since the labile structures of proteins are prone to hydrolization and enzymatic degradation.[3]

Natural hydrogel systems, especially polysaccharides such as pectin and chitosan, have been researched extensively in the development of protein and peptide drugs delivery systems in recent years.[2,4—6] Pectin is an anionic, soluble heterogeneous polysaccharide containing linear chains of α-(1→4)-D-galacturonic acid residues.[7,8] Low-methoxy pectin with degree of esterification less than 50% can form rigid gels by the action of calcium ions or multivalent cations and cross-link the galacturonic acid chains. The main drawback of calcium pectinate gel (CPG) beads is their macroporous structures, which may cause low entrapment efficiency[4,9] and fast release[9—11] of incorporated drugs, especially for those of low molecular weight and water-solubility. In recent investigation, emulsion-gelation method[12] or a multi-particulate system[13] could also exhibit a high drug entrapment efficiency and a slow release profile. However, to some proteins, this method does not work since the proteins have to be exposed to organic solvent systems which lead to denaturation. Therefore, it is of great importance to develop a method capable to increase drug entrapment efficiencies and stable for protein and peptide drugs loaded.

Isoelectric point is of great significance in protein properties because it is the pH at which proteins have minimal solubility and most stable property against denaturation.[14—16] We hypothesized that minimization of the protein dissolution and diffusion during their preparation by maintaining the pH of all working solutions at or close to its isoelectric point could enhance the entrapment efficiency of the microbeads. It was of interest to see whether microbeads containing proteins such as insulin could be prepared by this strategy and exhibited better profiles.

In this study, the CPG microbeads were prepared by ionotropic gelation technique coupled with working solution pH design for potential use of oral delivery system of protein and peptide drugs. Insulin, one of the most important drugs for diabetes, was chosen as a model amphoteric protein due to its comparative stability and crystallizing under different conditions. In addition, an air compressor was employed to reduce the particle size. This work also focused on the influences of some factors, such as phosphate buffer concentration, pH value of $CaCl_2$ solution, calcium concentration and pectin concentration, on the CPG microbeads properties and release behaviors of the model protein.

Experimental

Materials Amidated low methoxy (LM) pectins with degree of esterification (DE) of 28% and degree of amidation (DA) of 20% (GENU pectin type LM-104 AS-FS) were the generous gifts of Copenhagen Pectin (Denmark). Chitosan was supplied by Qingdao Heppe Biotechnology Co., Ltd. (China, Batch No: 070811). Recombinant human insulin was purchased from Tonghuadongbao Medicines Co., Ltd. (China, Batch No: 20070623). Calcium chloride, sodium dihydrogen phosphate, secondary sodium phosphate and acetic acid were obtained from China National Chemicals (China). All reagents were of analytical grade.

Preparation of Insulin-Loaded CPG Microbeads CPG microbeads containing insulin were prepared by ionotropic gelation technique, which was improved from Sriamornsak's conventional method.[4,17] Low-methoxy (LM) pectin was dissolved by 5 ml of distilled water or phosphate buffer solution (pH 6.2) with agitation. The powder of $CaCl_2$ and chitosan was weighed separately and further dissolved in 1% acetic acid (w/v) under stirring to prepare 0.3% (w/v) chitosan solution containing $CaCl_2$ at a serial concentration. The desired formulation pH was obtained by adding 0.1 M HCl or 0.1 M NaOH to the $CaCl_2$–chitosan (cross-linking solution). Insulin powder (10 mg) was dispersed in pectin solution, and mixed by stirring gently. Applied an airflow from an air compressor (WM-6, Tianjin Medical Equipment Co., Ltd., China) downwards over the needle to reduce droplet size, the dispersion was spurted into a $CaCl_2$–chitosan solution at the rate of

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Table 1. Effect of Preparation of CPG Microbeads on Particle Size and Insulin EE

Concentration of phosphate (mM)	pH value of $CaCl_2$	Concentration of calcium (% w/v)	Concentration of pectin (% w/v)	Mean particle diameter (μm±S.D.[a)])	Encapsulation efficiency (%±S.D.[b)])
Concentration of phosphate (mM)					
0	5.4	1	5	174±13	6.55±2.39
100	5.4	1	5	186±10	60.81±2.55
300	5.4	1	5	210±26	63.70±2.98
pH value of cross-linking solution					
100	5.0	1	5	182±5	11.63±1.04
100	5.4	1	5	190±10	60.69±2.16
100	5.8	1	5	187±9	93.28±2.07
100	6.0	1	5	192±11	83.53±3.26
100	6.2	1	5	184±7	70.41±1.00
100	6.6	1	5	186±11	65.35±1.74
Concentration of calcium (%, w/v)					
100	5.8	0.5	5	218±14	97.28±2.81
100	5.8	1	5	185±9	91.42±1.52
100	5.8	2	5	184±11	21.73±1.30
100	5.8	5	5	176±8	18.71±2.00
Concentration of pectin (%, w/v)					
100	5.8	5	3	182±12	80.13±20.38
100	5.8	5	5	186±9	89.92±3.28
100	5.8	5	7	207±15	10.31±2.98

a) S.D. was calculated from fifty measurements. *b*) S.D. was calculated from three repeated measurements.

1 ml/min through a syringe needle of 0.45 mm inner diameter aided by a peristaltic pump at room temperature. The CPG microbeads formed were allowed to stand in the solution for a period of time, separated and washed two times with distilled water and consequently suspended in acetone solution for 20 min. Microbeads were stored in a desiccator under vacuum at 25 °C until constant weight. A schematic of the microbeads preparation is shown in Fig. 1. The effects of phosphate concentration, pH value of $CaCl_2$ solution, calcium concentration, pectin concentration on microbead properties and entrapment efficiencies were investigated and summarized in Table 1.

Characterization of Insulin-Loaded CPG Microbeads

1. Morphological and Particle Size Analysis: An optical microscope (Eclipse TS 100, Nikon, Japan) was used to study morphology and particle size of wet and dried microbeads. The microscope eyepiece was fitted with a micrometer by which the particle size of the microbeads could be determined. Morphological examination of the surface and internal structure of the dried CPG microbeads after gold coated under vacuum was performed using a scanning electron microscopy (SEM) (Quanta 200, FEI, Holland) at 20 kV.

2. Evaluation of Insulin Content and Entrapment Efficiency of CPG Microbeads: To determine the insulin content, 12 mg of dried insulin-loaded CPG microbeads were accurately weighed and incubated in 10 ml of sodium citrate solution (pH 8.0, 0.1 M), under magnetic stirring for 1 h. Samples withdrawn after incubation were centrifuged at 6000 rpm for 10 min and the supernatant was assayed. Insulin content (%, w/w) was defined as the amount of insulin (mg) in 100 mg microbeads. The entrapment efficiency (EE, %) of insulin was calculated from the ratio between the actual insulin content and the theoretical insulin content. The determinations were made in triplicate and the results were expressed as mean±S.D.

The concentration of insulin was determined by a HPLC apparatus consisted of a pump (Dionex P680, Dionex Co., Sunnyvale, U.S.A.) and a work station (Chromeleon Server Monitor, Dionex Co., Sunnyvale, U.S.A.), using a Thermo column (octadecyl silica (ODS)-2 hypersil, 4.6 mm×250 mm, 5 μm). Mobile phase consisted of 29% acetonitrile and 71% sodium sulfate buffer solution (pH 2.3, 0.2 M). The column was maintained at room temperature. Volumes of 20 μl were injected, the flow rate was 1.0 ml/min and the UV detector was set at 214 nm.

3. *In Vitro* Release Studies: Drug release kinetics from the CPG microbeads was evaluated using a shaking incubator (SPX-250B-D, Shanghai Boxun Medical Equipment Co., Ltd., China). Twenty milligrams CPG microbeads were re-suspended in 50 ml of phosphate buffer solution (pH 6.8), then were shaken at 50 rpm at 37 °C. At time intervals, 0.3 ml of samples were taken, centrifuged at 6000 rpm for 10 min and the supernatant was assayed for insulin content. The same volume of fresh buffer was added into the release medium to keep volume constant. The results measured in triplicate are expressed as a percentage of the drug released.

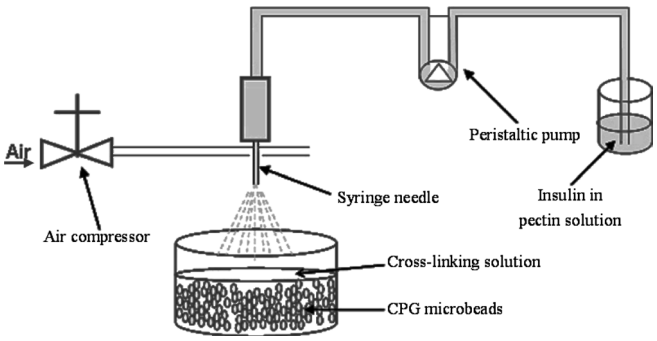

Fig. 1. Set-up for Preparation of Insulin-Loaded CPG Microbeads

Results and Discussion

Preparation of Microbeads Insulin-loaded CPG microbeads were prepared by a simple method. A phosphate buffer solution of pectin containing insulin was spurted into $CaCl_2$–chitosan solution and microbeads were formed instantaneously by ionotropic gelation in which intermolecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules. At the same time, the polycationic chitosan was entrapped in the microbeads and coupled to the pectin chains through electrostatic interaction to form polyelectrolyte complexes inside the microbeads and around the microbeads.

In addition, an air compressor was employed to improve the method. Airflow through a tube was applied downwards over the needle to reduce the droplet size. Flow rate of the compressed air can be changed based on the desired bead diameter and characteristics. Different from millimeter-sized CPG beads prepared by conventional ionotropic gelation method,[4)] smaller beads were prepared through an air compressor.

This method does not involve sophisticated equipment, harsh processing conditions, or organic solvent systems, which makes it a very attractive choice for the entrapment of

unstable biologicals, like labile insulin molecule.

Morphology of CPG Microbeads Typical scanning electron micrograph is shown in Fig. 2, illustrating the external and internal structure of the dried microbeads. Figures 2a and b show the appearance of insulin-loaded CPG microbeads. The microbeads had slight yellowish color, smooth surface, spherical shape and uniform size. As shown in Figs. 2b and c, the morphology of the external and internal structure was identical due to the homogeneous dispersion of insulin powder in pectin solution. Sponge-like structure could be observed in the cut surface of microbeads as proposed in Fig. 2d, which may correspond to the egg-box structure of calcium pectinate.

Particle Size and Protein Entrapment The mean diameters of microbeads with different formulations are shown in Table 1, ranging between 180 and 220 μm. Compared to conventional CPG beads[17] (1.27 ± 0.08 mm), the diameter of CPG beads was significantly reduced to a micrometer level by using an air compressor.

Phosphate buffer solution used as a solvent of pectin instead of distilled water led to a great increase in the level of EE, probably because precipitation of amorphous calcium phosphate between Ca^{2+} and HPO_4^{2-} in the pectin droplets made hybrid microbeads with enhanced mechanical strength and reduced drug diffusion.[13,18] On the other hand, phosphate solution provided a buffer environment with a certain pH around isoelectric point of insulin, which also contributed to a better entrapment capability.

Comparatively, pH value of cross-linking solution was an important moderator on the EE of insulin-loaded CPG microbeads. The EE increased from 11.63 to 93.28% with increasing pH from 5.0 to 5.8. However, the EE decreased when the pH was higher than 5.8. The result is consistent with insulin solubility variation caused by solution pH, since solubility of protein is often minimal at its isoelectric point. The lower solubility of insulin in cross-linking solution, the less insulin diffusion from microbeads, thus, a good efficiency of drug entrapment was achieved. Figure 3 provided an intuitional evidence for this change of EE. In Figs. 3b and c, crystal-like powder of insulin was easily observed in CPG microbeads while that in other pictures (Figs. 3a, d—f) was less clear because the latter, most of insulin was dissolved in cross-linking solution at pH away from isoelectric point of insulin. However, the maximum EE value was observed at pH 5.8 and pH value tested after cross-linking was 5.4, which was between the insulin isoelectric point range 5.35—5.45.[19] This observation can be explained by the fact that pectin makes an acidic solution when dissolved in water (pH of 3% pectin solution is 4.41). Therefore, pH value of cross-linking solution decreased when pectin solution was spurted in.

The level of EE decreased dramatically when $CaCl_2$ concentration rose from 0.5 to 5%. The value of EE determined for 0.5% $CaCl_2$ was about 5.2 times higher than that of 5% $CaCl_2$. This is probably because the squeezing pressure induced by pectin cross-linking was higher in the presence of a larger amount of calcium ions, forcing more entrapped proteins to leach out during the formation of microbeads.[20]

The EE of microbeads rose a bit when pectin concentration increased from 3 to 5%, but that value went down suddenly when the concentration reached to 7%. This may be

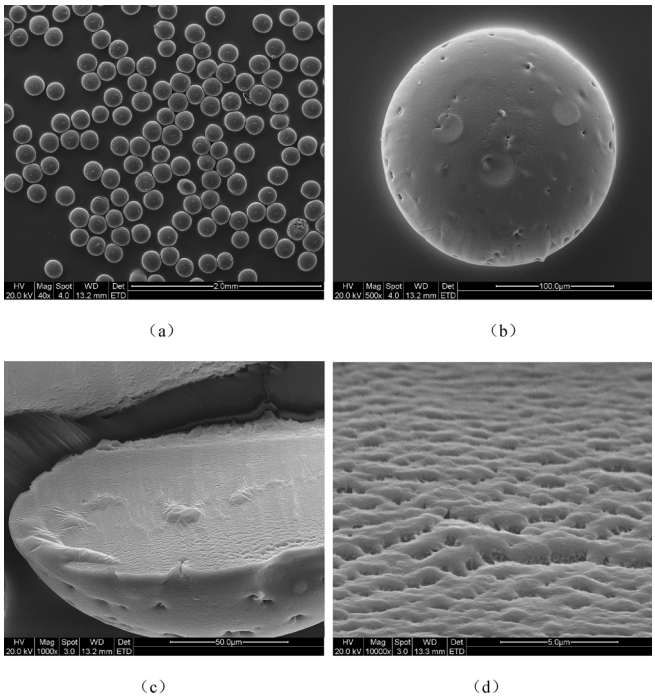

Fig. 2. SEM of External (a, b) and Internal (c, d) Structure of Dried CPG Microbeads Containing Insulin

The scale bars are shown on the individual photographs.

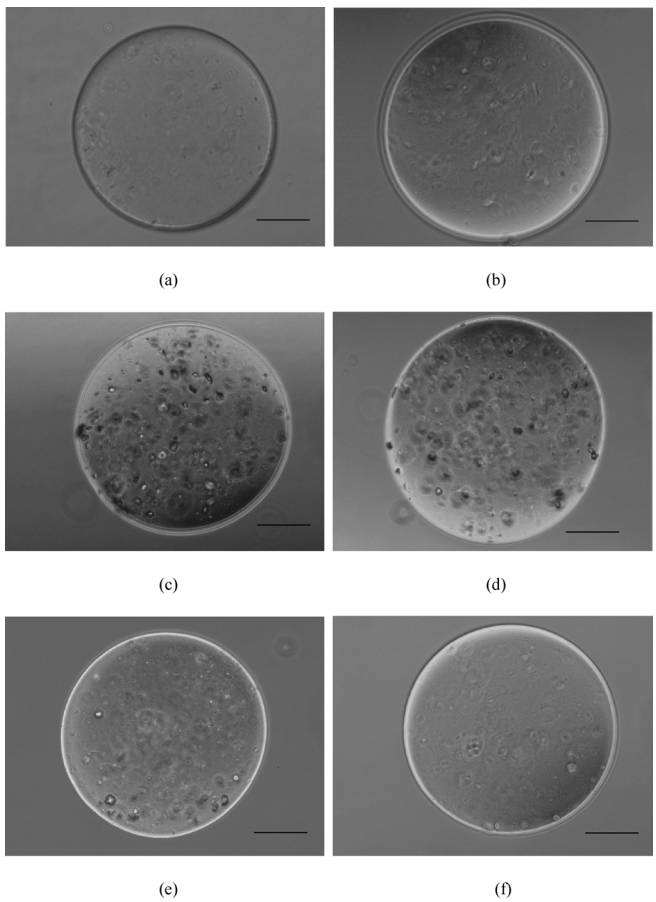

Fig. 3. Microscope Pictures of Wet CPG Microbeads Containing Insulin Prepared at (a) pH 5.0, (b) pH 5.4, (c) pH 5.8, (d) pH 6.0, (e) pH 6.2, (f) pH 6.6 Show the Effect of Cross-Linker pH on Insulin Loading Capacity

Bar=25 μm.

caused by the acidity of pectin in cross-linking solution, thus, the higher pectin concentration, the more decrease in pH of cross-linking solution. In the experiment of 7% pectin solution, pH value of $CaCl_2$–chitosan solution tested after cross-linking was below 5.0 while the others were above 5.2. At pH 5.0, most of insulin dissolved and diffused to cross-linking solution, which resulted in low EE of microbeads.

Protein Release The release profiles of insulin-loaded CPG microbeads are shown in Fig. 4.

Figure 4a demonstrated that CPG microbeads prepared from 300 mM phosphate showed the quickest drug release, almost 50% of insulin was released in the first 2 h. The acceleration of the insulin release from microbead in the presence of 300 mM phosphate was caused by its bigger size and less spherical shape. Besides, the displacement of some Ca^{2+} by Na^+ from Na_2HPO_4 may also worsen the release behavior because sodium pectinate and other monovalent salts of pectin were in the form of a non-viscous liquid and did not form gel at neutral environment.[21)]

Effect of pH value of cross-linking solution on the release profiles was shown in Fig. 4b. The release profiles were similar, suggesting that pH value may not be a primary factor affecting the insulin release when the microbeads were prepared in the pH range of 5.0—6.6.

As shown in Figs. 4c and d, insulin release from microbeads was decreased with an increase concentration of $CaCl_2$ and pectin because more intermolecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules. These cross-links result in a slower swell process, a more difficult diffusion of insulin from the swollen microbeads and a slower rate of protein release.

Conclusion

In this paper, the preparation process of CPG microbeads by an ionotropic gelation technique was successfully improved for protein and peptide drugs delivery. Compared to conventional method, the presented work have three improvements as follows: (1) micron-sized bead was achieved by the introduction of an air compressor, which was used as a cell of preparation device to reduce particle size; (2) phosphate buffer solution in place of distilled water used as a solvent of pectin has resulted in an increase of mechanical strength as well as drug EE and at an appropriate concentration, slowed down the release of a model protein (insulin); (3) great care was taken to keep the pH of all working solutions at or close to isoelectric point of protein, which contributed to good entrapment of amphoteric protein. Hence, microbeads with enhanced mechanical strength, uniform sizes, good entrapment efficiencies and delayed release profiles for amphoteric protein and peptide were prepared successfully. All investigated formulation variables have significantly influenced the entrapment efficiency and release properties of insulin-loaded CPG microbeads. The study demonstrated that CPG microbeads could be a promising carrier used in oral controlled-release systems for amphoteric protein and peptide drugs. Further studies are underway to assess *in vivo* feasibility of this system for protein and peptide drugs.

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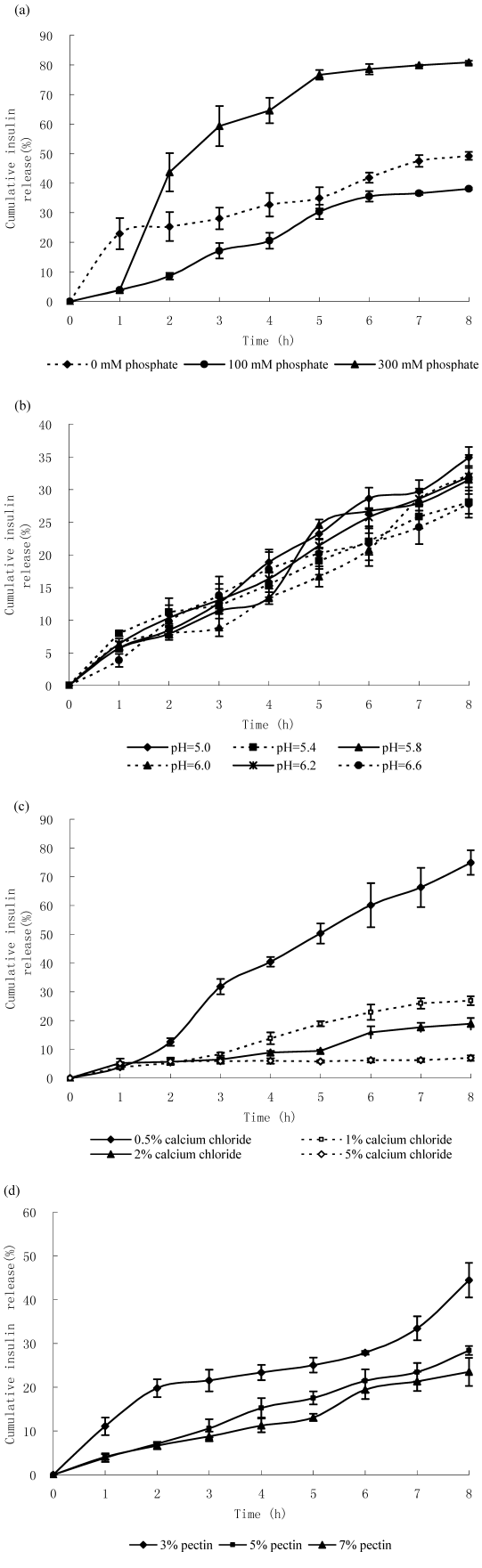

Fig. 4. Release Profiles of Insulin from CPG Microbeads; (a) Effect of Phosphate Concentration, (b) Effect of pH Value of Cross-Linking Solution, (c) Effect of Calcium Concentration, (d) Effect of Pectin Concentration

Each point represents as the mean±S.D.

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