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Research paper

Preparation and evaluation of alginate–chitosan microspheres for oral delivery of insulin

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ABSTRACT

The alginate–chitosan microspheres with narrow size distribution were prepared by membrane emulsification technique in combination with ion (Ca^{2+}) and polymer (chitosan) solidification. The preparation procedure was observed, and the physical properties (particle size distribution, surface morphology, chitosan distribution, zeta potential) of the microspheres were characterized. Subsequently, the microspheres were employed to load model peptide of insulin. The effect of loading ways on the loading efficiency and immunological activity of insulin were investigated. It was shown that the higher loading efficiency (56.7%) and remarkable activity maintenance (99.4%) were obtained when the insulin was loaded during the chitosan solidification process (Method B). Afterward, the release profile *in vitro* for the optimal insulin-loaded microspheres was investigated. Under the pH conditions of gastrointestinal environment, only 32% of insulin released during the simulated transit time of drug (2 h in the stomach and 4 h in the intestinal). While under the pH condition of blood environment, insulin release was stable and sustained for a long time (14 days). Furthermore, the chemical stability of insulin released from the microspheres was well preserved after they were treated with the simulated gastric fluid containing pepsin for 2 h. Finally, the blood glucose level of diabetic rats could be effectively reduced and stably kept for a long time (~60 h) after oral administration of the insulin-loaded alginate–chitosan microspheres. Therefore, the alginate–chitosan microspheres were found to be promising vectors showing a good efficiency in oral administration of protein or peptide drugs.

1. Introduction

Pharmaceutical proteins or peptides are becoming an important class of therapeutic drugs with lower side effects and specific treatment effects [1]. However, due to their large molecular weight and size, they show poor permeability characteristics through various mucosal surfaces and biological membranes. Moreover, rapid clearance in liver and other body tissues by proteolytic enzymes as well as inherently chemical and physical instability are also factors which result in the low bioavailability associated with the oral delivery [2]. Among several attempts proposed to improve oral bioavailability, microencapsulation represents a promising strategy [3].

Recently, the natural polysaccharide, alginate, is used as a carrier material for protein and peptide drugs and has attracted increasing attentions due to its excellent biocompatibility, mucoadhesive biodegradability and mild gelation conditions [4]. It possesses a unique property of mild gel-formation in the presence of multivalent cations such as calcium ions in aqueous media, which takes place at junctions in the G–G sequence rich chain region known as the "egg-box junctions" [5,6]. However, the loose network of bead results in a major limitation of drug-leakage through the pores during alginate-Ca bead preparation [7]. Fortunately, the mechanical properties and permeability of alginate-Ca bead can be effectively improved by a polycation, such as chitosan. Chitosan is a naturally occurring polysaccharide comprising D-glucosamine and N-acetyl-glucosamine with unique polycation characteristics [8,9]. Upon mixing with the alginate, the strong electrostatic interaction of amino groups of chitosan with the carboxyl groups of alginate leads to the formation of chitosan–alginate complex. The complexation reduces the porosity of alginate beads and decreases the leakage of the encapsulated drugs [10,11]. On the other hand, this complex exhibits pH-sensitivity that release of macromolecules from alginate beads in low pH solutions is significantly reduced, thereby being used as an oral delivery vehicle [12].

To date, several methods have been performed for the preparation of alginate–chitosan microspheres, such as spray drying [13], coacervation technique [14] and emulsification/solidification

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technique [15]. However, limitations are found in the practical application. For example, the size of particles prepared by the spray drying and coacervation technique is too large to be effectively absorbed in the intestinal tract [16]. Although smaller particles can be obtained by emulsification/solidification technique, the emulsion is usually prepared by high-energy instruments or methods with high shear force, which may result in deactivation of polypeptide drugs. In addition, the microspheres prepared by spray drying and emulsification/solidification technique have broad size distribution, leading to possible side effects and poor repeatability [17] during the practical applications.

To overcome the disadvantages mentioned above, the membrane emulsification technique was applied to prepare alginate–chitosan microspheres with narrow size distribution. It is a unique method to mildly provide emulsion with uniform droplet size. Then, the droplets can be solidified to form microspheres or microcapsules by various solidification techniques. Nevertheless, in the case of alginate microsphere, the solidification process of alginate droplets in W/O emulsion is a big challenge because it is totally different from the direct solidification by $CaCl_2$ solution. In this study, we developed a two-step solidification process with $CaCl_2$ mini-emulsion in W/O system followed by chitosan in aqueous phase. The microspheres properties of particle size, surface morphology, chitosan distribution and zeta potential were characterized in detail. As a model peptide, insulin was loaded in the microsphere by three methods. Consequently, an effective insulin loading method with higher activity retention was developed. Further evaluations of the insulin-loaded alginate–chitosan microsphere were performed *in vitro* and *in vivo* (diabetes rats).

2. Materials and methods

2.1. Materials

Sodium alginate was purchased from Acros Organics (New Jersey, USA). According to the manufacturer, the alginate contained 65–75% guluronic acid (G) subunits and 25–35% mannuronic acid (M) subunits and had a molecular weight of 450–550 kDa, specifically with a viscosity of 485 cP for a 1 wt% aqueous solution. Chitosan was ordered from Yuhuan Ocean Biochemical Co., Ltd. (Zhejiang, China), and the degree of deacetylation is 89% and MW (viscosity-average molecular weight) is 150 kDa. A fast acting insulin analog (IA) (25.8 IU/mg), which is identical to human insulin except for inversion of ProB28–LysB29, was kindly provided by Gan & Li Biotechnological Co., Ltd. (China). Alex Fluoresce-488 was from Invitrogen reagent corporation (USA), and Streptozotocin (STZ) from Alexis Biochemicals (USA). PO-500 (Hexaglycerin penta ester) was supplied by Sakamoto Yakuhin Kogyo Co., Ltd. (Japan). KP-18C (C_{18} silane coupling agent) was provided by Shin-Etsu Chemical Co., (Japan). Other reagents were of analytical grade.

2.2. Preparation of blank alginate–chitosan microspheres

The experimental set-up in details for membrane emulsification process is referred to our previous work [17]. The schematic preparation process of alginate–chitosan microspheres is shown in Fig. 1. Briefly, SPG (Shirasu Porous Glass) membrane with a specific pore size (7.0 μm) was premodified to be hydrophobic with KP-18C (C_{18} silane coupling agent). Five milliliters of 1.0 wt% alginate in acetic acid buffer solution (pH = 4.2) was used as the water phase. Under the pressure of nitrogen, the water phase was pressed through the membrane into 50 ml of oil phase (liquid paraffin and petroleum ether in a volume ratio of 7:5; emulsifier: 4 wt% PO-500) to form uniform-sized droplets. Then, the mini-emulsion of $CaCl_2$ prepared by dispersing 1.5 ml $CaCl_2$ solution

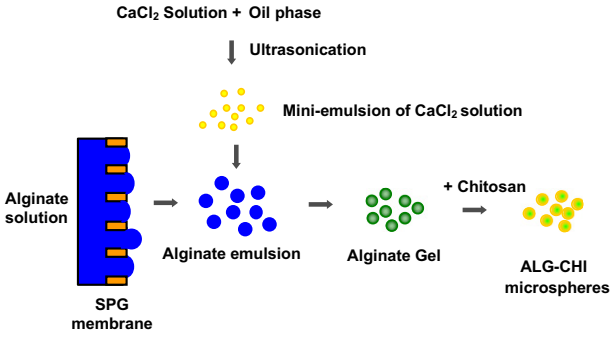

Fig. 1. A schematic presentation of the preparation process of blank alginate–chitosan microsphere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(0.5 mol/l) into 10 ml oil phase (the same recipe as mentioned above) by ultrasonication (S450D, Branson Ultrasonics Corporation, USA) was added into the sodium alginate emulsion as the first-step solidification. The solidification process was continued for 5 h under stirring of 300 rpm. The solidified alginate gels were collected and washed two times with petroleum ether and four times with distilled water by centrifugation (1000*g*, 5 min, 25 °C) and then dispersed in 5 ml of 1.6 wt% chitosan acetic acid buffer solution (pH = 4.2) for 1 h under stirring of 300 rpm as the second-step solidification. Finally, the alginate–chitosan microspheres were washed twice with 1 wt% aqueous acetic acid to remove the residual chitosan on the surface and then dried by lyophilization (Labconco Freezone Plus 6, USA) at 0 °C for 48 h.

2.3. Sample characterization

An optical microscope (XSZ-H_3, ChongQing Opitical & Electrical Instrument Co., Ltd., PR China) installed with picture capturer (wv-CP230, Panasonic Co., Ltd., Japan) was used to monitor the droplets before and after solidification. The surface morphology of the dried samples was observed by a scanning electron microscopy (SEM, JEM-6700F, Japan). The size distribution was analyzed by a Mastersizer 2000 laser particle analyzer. Polydispersity was determined by the SPAN factor expressed as

$$\mathrm{SPAN} = [\mathrm{D}(v,90) - \mathrm{D}(v,10)]/\mathrm{D}(v,50) \quad (1)$$

where D(v, 90), D(v, 10) and D(v, 50) are volume size diameters at 90%, 10% and 50% of the cumulative volume, respectively.

The ξ-potential was measured by ZetaPlus 21421 (Brookhaven Instruments Corporation, USA). The average pore size of microspheres was measured by BET method (ASAP 2020, Micromeritics, USA). It was found that the compound of chitosan–glutaraldehyde exhibited fluorescence [18]. TCS SP2 Laser Scanning Cofocal Microscopy (LSCM, Leica) was used to observe the chitosan distribution in the blank alginate–chitosan microsphere. In detail, 1 ml of 50 wt% glutaraldehyde solution was slowly dropped into as-prepared alginate–chitosan microspheres dispersed in 5 ml distilled water at a stirring speed of 300 rpm for 1 h, and then the glutaraldehyde-crosslinked microspheres were washed twice with distilled water. Different content of chitosan led to varied fluorescence intensity of microspheres, so the chitosan in samples prepared by different conditions in orthodox experiments were also quantified by LSR flow cytometer (Becton Dickinson). The results are shown in Supplementary material 2.

2.4. Insulin loading

Insulin (pI = 5.35–5.45) was positive on the condition of microsphere preparation, while alginate was negative. If mixing the

insulin and alginate in the solution directly, the aggregation would form immediately because of electrostatic interactions, which could not pass the membrane pores to prepare insulin-loaded microspheres. Therefore, the insulin was loaded in the alginate–chitosan microspheres by other ways. The Method A was dissolving the insulin (1.5 mg) in the $CaCl_2$ solution (1.5 ml), then the solution was formed the first solidification liquid ($CaCl_2$ mini-emulsion) as mentioned in Section 2.2. The Method B was dissolving the insulin (1.5 mg) in the second solidification liquid (1.5 ml chitosan solution). The Method C was the combination of Method A and Method B: 3.0 mg insulin was added in the preparation process (1.5 mg in the first solidification and 1.5 mg in the second solidification).

2.5. Evaluation of the insulin loading efficiency and activity retention

Insulin content was determined by dispersing insulin-loaded alginate–chitosan microspheres (15 mg) in a phosphate buffer solution (pH = 8) for 48 h (37 °C, 300 rpm). The higher pH value and rotation speed were employed to prompt break of microspheres and release of insulin from the microspheres. As shown in Supplementary material 3, the structure of microspheres was destroyed and therefore entrapped insulin could be released. After centrifugation at 5000 rpm (Anke TGL-16G, Anting Co., China) for 5 min, insulin concentrations in the clear supernatant solution were determined by the Micro BCA Protein Assay (Pierce Inc., New York, USA) with BSA as the standard protein.

The loading efficiency (LE) was calculated according to

$$\text{Insulin LE} = X_t/X_i \times 100\% \quad (2)$$

where X_t is the total amount of insulin loaded in microspheres and X_i represents the initial amount of insulin added in the preparation process.

None of methods can be used to directly determine the activity of insulin *in vitro*. Alternatively, the ELISA kit was used to measure the immunological activity of insulin, which could indirectly reflect the activity retention of released insulin [19]. Briefly, the insulin-loaded alginate–chitosan microspheres (15 mg) were dispersed in a hydrochloric acid buffer solution (pH = 2) for 24 h with magnetic stirring (100 rpm) at 4 °C, which could prevent the insulin denaturizing after release. The insulin content was identified by the procedure mentioned above, and then the solution was diluted to the same concentration as the standard insulin solution (1 μg/ml). An iso insulin ELISA kit (Phoenix Biotech Co., Ltd.), which has broad cross-reactivity, was used to determine the insulin activity. The values of absorbance were changed to activity units (IU) by data processing.

The insulin activity retention (AR) was evaluated according to

$$\text{Insulin AR} = A_r/A_s \times 100\% \quad (3)$$

where A_r is the activity (IU) of insulin sample released from the microspheres and A_s is the activity (IU) of standard insulin.

2.6. Insulin distribution

The insulin was labeled by the method briefly described as follows: 2.5% (w/v) aqueous insulin solution (10 ml) was adjusted to pH 8 by sodium hydroxide solution (1 M). Alex Fluoresce-488 was dissolved in the same solution at a concentration of 1 mg/ml. Then, the dye solution (10 μl) was added into the insulin solution and stirred at 4 °C overnight. Free Alex Fluoresce-488 was subsequently removed by centrifugation (12,000 rpm, 1 h) through the centrifugal filter unit for several times. Finally, the Alex Fluoresce-488-insulin was dissolved in chitosan solution at pH 4.2 to be loaded in alginate-Ca microspheres as described above. Then, the distribution of insulin in the microsphere was observed by TCS SP2 Laser Scanning Cofocal Microscopy (LSCM, Leica).

2.7. Characterization of the insulin-loaded microspheres in vitro

2.7.1. Insulin release in the gastrointestinal environments

The release of insulin from microspheres under simulated pH conditions in gastric fluid followed by intestinal fluid was investigated. The detailed experiment was carried out by dipping the insulin-loaded alginate–chitosan microspheres (10 mg) into 10 ml simulated gastric fluid (hydrochloric acid buffer at pH 1.2). The fluid was incubated (37 °C, 100 rpm) for 2 h. At appropriate intervals, 0.2 ml of sample was taken and separated from the microspheres by centrifugation (5000*g*/5 min) to obtain the supernatant for insulin determination. Then, fresh medium was added to maintain a constant volume. To simulate the process of microspheres moving from the stomach into the intestine, after 2 h, the microspheres were transferred to 10 ml simulated intestinal fluid (phosphate buffer at pH 6.8). The fluid was incubated (37 °C, 100 rpm) for 4 h. At determined times, 0.2 ml of supernatant was taken, separated from microspheres by centrifugation (5000*g*/5 min) for insulin determination and replaced by fresh medium.

2.7.2. Insulin release in the blood environments

Insulin-loaded alginate–chitosan microspheres (10 mg) were dispersed into 10 ml PBS (pH = 7.4) at 37 °C in a shaking air bath (100 rpm). Samples (0.2 ml) at scheduled time intervals were taken and centrifuged (5000*g*/5 min) to obtain the supernatant. Fresh dissolution medium was added to maintain a constant volume.

2.7.3. Protection against the gastric juice

To assess the protective effect against gastric degradation, 10 mg of the insulin-loaded microspheres were incubated (37 °C) in 10 ml of simulated gastric fluid (pH 1.2) with 0.32 g pepsin (800 U/mg proteins) for 2 h in a water bath shaker (100 rpm). The enzyme was dissolved in the gastric juice immediately before starting the experiment to prevent loss of activity. Then, the microspheres were separated by centrifugation (5000*g*/5 min) and washed twice with distilled water. Insulin was released by dipping these microspheres in a hydrochloric acid buffer solution (pH = 2) for 24 h with magnetic stirring (100 rpm) at 4 °C, which could prevent the insulin denaturizing after release. Finally, the supernatant containing insulin was separated by centrifugation (5000*g*/5 min) for determination.

2.7.4. Insulin determination

The insulin concentrations in the supernatant were measured by the BCA Protein Assay, and the cumulative insulin release expressed as percentage of initial insulin loading.

The chemical stability of insulin after passing the simulated gastric juice was assayed by HPLC. As a control, the native insulin was also diluted to the same concentration and assayed. In detail, 20 μl of supernatant was injected in a chromatograph (Waters 2695XE, USA) which was equipped with a UV detector (Waters 2996) and a reversed phase column (Cosmosil 5C18-AR, 4.6 mm × 250 mm, Nakalai Tesque Co., Ltd., Tokyo). The following mobile phase systems were used: (A) 0.1% trifluoroacetic acid (TFA) in H_2O and (B) 0.1% TFA in acetonitrile. A linear gradient was used: the phase B from 20% to 50% (30 min). The flow rate was 1.0 ml/min and the wavelength was set at 280 nm.

2.8. Oral administration and blood glucose concentration determination

The experiments on animals were carried out in accordance with the European Community Council Directive of November

24, 1986 (86/609/EEC) and approved by Experimental Animal Ethics Committee in Beijing. The animals used in this study were Male Sprague–Dawley rats, with weight of 200–250 g, which were provided by the animal service (experimental center of Peking University Health Science Center). The STZ-induced diabetic rats were prepared by injections of streptozotocin (70 mg/kg i.v.) in a citrate buffer at pH 4.5. The rats with the blood glucose level higher than 15 mM were selected as the diabetic model. These diabetic rats were fasted over 12 h before every blood sampling.

The following formulations were intragastrically administered to rats (six rats per group) by a single oral gavage: (1) distilled water (control group), (2) blank alginate–chitosan microspheres suspended in distilled water, (3) insulin-loaded alginate–chitosan microspheres (100 IU/kg) suspended in distilled water. The blood samples were taken from the eye-ground venous plexus at predetermined time points after the administration. The blood glucose concentration was measured by blood glucose assay kits (Glucose GOD-PAD kit, Biosino Bio-Technology and Science Inc., Beijing, China). Plasma glucose levels were plotted against time after administration, and the serum glucose concentrations of the control group were defined as 100%.

2.9. Statistical analysis

All analyses were carried out in triplicate ($n = 3$) and were presented as means ± SD. A one-way ANOVA (OriginPro®, Version 8.0) was used to determine statistical significance, and the difference is considered significant when $p < 0.05$.

3. Results and discussion

3.1. Preparation of alginate–chitosan microspheres

The alginate–chitosan microspheres were successfully prepared by SPG membrane technique resulting in a narrow size distribution. Fig. 2 displayed the optical micrographs of different preparation stages. First, the alginate droplets were produced as shown in Fig. 2a. Secondly, the droplets were surrounded by a huge amount of $CaCl_2$ mini-droplets (Fig. 2b). Gradually, the $CaCl_2$ droplets were absorbed by alginate droplets because of the different size, and then the Ca^{2+} was transferred into alginate droplets leading to solidified gels with good sphericity (Fig. 2c). The size of alginate-Ca microgels was far smaller than the size of alginate droplets. This was possibly because the water in droplets was squeezed out during the alginate-Ca network formation. Finally, the alginate-Ca microgels were further solidified by chitosan. The size of alginate–chitosan microspheres did not apparently change after the second solidification (Fig. 2d).

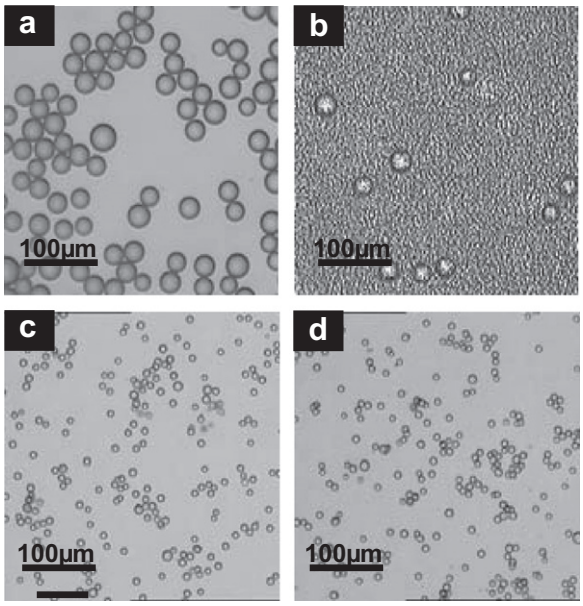

Fig. 2. The preparation process observed by the optical microscope. (a) W/O emulsion of alginate solution in oil phase. (b) The first-step solidification process after mixing alginate droplets and $CaCl_2$ mini-emulsion. (c) Alginate-Ca microspheres after the first-step solidification. (d) Alginate–chitosan microspheres in water after the second-step solidification.

The preparation conditions of alginate–chitosan microspheres were optimized by an orthogonal experiment with four factors and three levels, and the results were shown in Supplementary material 1. Eventually, the yield of alginate–chitosan microspheres was improved to 35.44% at the following conditions: alginate concentration: 1.0 wt%, chitosan concentration: 1.6 wt%, $CaCl_2$ amount: 1.5 ml (0.5 mol/l), pH value of solutions: 4.2. The subsequent experiments were conducted with the microspheres prepared by the optimized conditions.

3.2. Microspheres characterizations

The characterization results of alginate–chitosan microspheres are shown in Fig. 3. Fig. 3a illustrated that the microspheres was ~7.5 μm, and the SPAN value was 0.731. Compared with the microspheres or nanospheres prepared in other studies [20,21], the microspheres had narrow size distribution. The SEM observation (Fig. 3b) clearly demonstrated that these microspheres were spherical, and the diameter was consistent with the results from the particle size analyzer. Moreover, it revealed that the surface was rough and had many micropores (Fig. 3c).

Although, according to the literature, alginate also could react with glutaraldehyde, the reaction happens in methanol containing 1% glutaraldehyde and 1% of 1 N HCl [22], which is impossible in the mild environment of our study. Therefore, alginate–chitosan microspheres further crosslinked with glutaraldehyde could be used to probe the distribution of chitosan. A previous report speculated that chitosan was only coated on the outer shell of alginate-Ca microspheres after they were solidified in chitosan solution [21]. However, as shown in Fig. 3d, the fluorescence indicated that chitosan was homogeneously distributed inside the microsphere, which reflected that chitosan could permeate into the alginate-Ca microspheres. This phenomenon could be ascribed to the mild solidification in the first-step by the $CaCl_2$ mini-emulsion, which resulted in a loose structure with good permeability. In the preparation of reported alginate–chitosan microspheres [21], alginate droplets contacted with Ca^{2+} directly, which caused a rapid solidification and then led to a compact surface of alginate-Ca. As a result, it prevented chitosan molecules penetrating into the alginate-Ca membrane successively.

The charge properties were important to the function of drug carrier, so it was necessary to examine the surface charge of alginate-Ca and alginate–chitosan microspheres. As expected, alginate-Ca microspheres showed a negative value (−11.6 mV) because of the carboxyl groups on the surface. After the reaction with chitosan, the negative charges were neutralized and excessive amino groups made the surface exhibit positive (+16.7 mV). As reported in literatures, the introduction of chitosan could improve the bioadhesive of microsphere to specific regions of the gastrointestinal tract like the stomach [23,24], small intestine, ileum, colon [25,26] and buccal mucosa [27,28]. Herein, as a designed oral drug delivery system, it is promising to prolong residence time at the site of application and absorption [29].

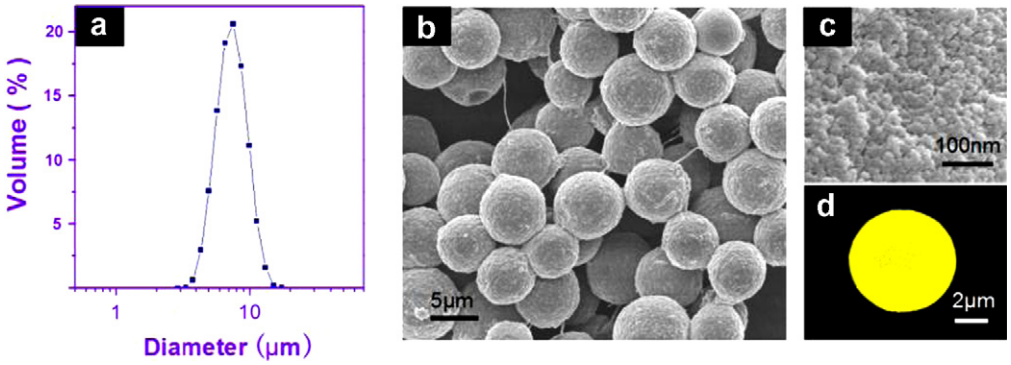

Fig. 3. Characterization results of blank alginate–chitosan microsphere. (a) Size distribution. (b) SEM image. (c) Surface morphology. (d) Confocal image showing chitosan distribution after cross-linking with glutaraldehyde. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Optimization of insulin loading methods

Insulin was entrapped in alginate–chitosan microspheres by three methods. The corresponding loading efficiency and immunological activity retention were investigated. The results in Fig. 4 showed Method B had the highest loading efficiency (LE) of 56.7% and immunological activity retention of 99.4%. In general, insulin was oppositely charged to alginate droplets or alginate-Ca microspheres on the condition of optimized pH value (4.2), so it was loaded in the microspheres by electrostatic attraction regardless of Methods A, B or C, which made it easily enter into the microspheres. However, when insulin was loading by Method A, it would leak out during the washing process after the first-step solidification because of high porosity of alginate-Ca microspheres. While in Method B, the network formation between chitosan and alginate-Ca microspheres greatly reduced the porosity and decreased the leakage of insulin. In the case of activity retention, the denaturation of the insulin in Method A was probably caused by the ultrasonic oscillation during the preparation of mini-emulsion. To Method B, insulin was loaded in the spheres with chitosan solution, which is a mild process and could effectively maintain the activity of insulin. Method C was the combination of Method A and Method B; hence, it was not surprising that the loading efficiency and activity retention decreased to 42.6% and 87.4%, respectively.

The results demonstrated when the insulin was loaded during the second solidification process, not only the loading efficiency was improved, but also the immunological activity was well preserved. Accordingly, Method B was the best way for insulin loading for the further investigation.

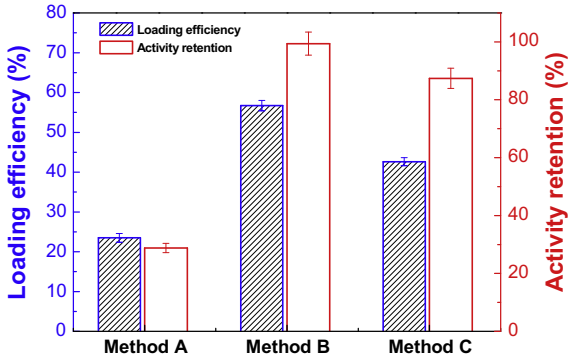

Fig. 4. Insulin loading efficiency and activity retention obtained with different insulin loading procedures (A, B, C). Results are expressed as mean ± SD ($n = 3$). Statistical significant differences ($p < 0.05$) exist between all the series. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Insulin distribution in the optimized microsphere

Insulin labeled with Alex Fluoresce-488 was used to visualize its distribution in alginate–chitosan microsphere. As shown in Fig. 5a, the main fluorescence emission came from the whole microsphere, indicating the insulin could enter the inside. Notably, as shown in Fig. 5b, the fluorescent intensity decreased slightly at the core section. That is to say, a little more insulin distributed at the edge of the microsphere.

We speculated the microstructure of insulin-loaded alginate–chitosan microspheres as Fig. 6 to explain the results of LSCM observation from chitosan (Section 3.2) and insulin. It could be understood by associating the chitosan solidification process with the insulin loading process. At the beginning, alginate and chitosan gradually formed homogenous network from core to edge, which was just like many 'cages'. Insulin could easily enter the inside because of the loose structure outside microspheres. With the solidification process proceeded, the insulin was hindered because the structure of microspheres became compact due to the matrix formation between alginate and chitosan. Until the negative charges were exhausted, insulin still could be attracted but only stayed on the edge of the microsphere. Accordingly, the chitosan distribution in the microsphere was more homogenous than insulin distribution.

3.5. Insulin release in vitro

As a designed carrier for protein or peptides delivery, the alginate–chitosan microspheres have to experience the gastrointestinal tract when they are orally administrated. Hence, the insulin release behavior *in vitro* in the low pH (1.2) environment of the stomach, and then in the near neutral pH (6.8) of the small intestine was captured. The results are showed in Fig. 7a, which displayed that only 5% insulin released from the microspheres after 2 h incubation in the simulating gastric condition. Microspheres were then transferred to simulating intestinal conditions and after 4 h incubation, 32% insulin of the initial amount was released. The observed little amount release of insulin at pH 1.2 could be attributed to a tight alginate network that formed at low pH [13,15]. On the other hand, insulin was loaded under pH 4.2 as mentioned in Section 2.4; when the microspheres experienced lower pH value, the electrostatic attraction between insulin and alginate was became stronger because of more positive charges of insulin. Consequently, the insulin release was significantly prevented. At pH 6.8, although the alginic acid formed during acid incubation was converted to a soluble salt of sodium alginate, rapid or entire release of insulin did not happened which indicated the microsphere structure still preserved. This was mainly because chitosan in the

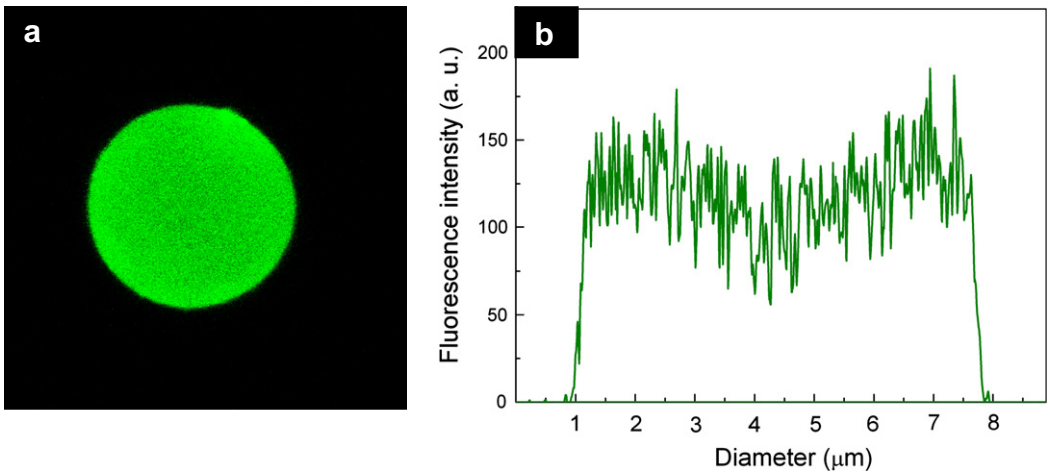

Fig. 5. Confocal image of Alex Fluoresce-488-insulin distributed in alginate–chitosan microspheres (a) and corresponding fluorescence intensity along the diameter (b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

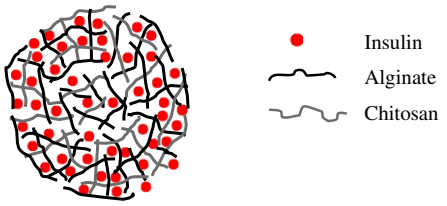

Fig. 6. Schematic microstructure of insulin-loaded alginate–chitosan microsphere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microspheres was insoluble under the condition of weakly alkaline which provided an additional physical barrier. However, the release rate of insulin was slightly increased. We thought it was due to the insulin charges alternation which produced forces of repulsion to alginate. Moreover, the reduction in the gel strength after acidic treatment was also helpful to insulin release [20].

The insulin activity retention after passing through the stomach is a key factor to evaluate an oral carrier system. Nevertheless, it was found that free insulin was found to be intact in the simulated gastric fluid without pepsin [30] and completely degraded during incubation with pepsin [30]. Thus, to estimate the protective effect of microsphere on insulin, the chemical stability of insulin was evaluated after it released from the microspheres, which were treated with simulated gastric fluid containing pepsin. The result showed in Fig. 7b and c demonstrated that the chemical stability of insulin released from the microspheres was well preserved. This suggested that insulin in the microsphere had no opportunity to contact with the pepsin in the medium during the incubation. It was consistent with the results in Fig. 7a that only a little insulin was release under the condition of pH 1.2. Furthermore, although the molecular weight of insulin is only 5800, it was hindered to release because of the shrinkage of the microsphere in acidic environment [31]. Then, the much higher molecular weight of pepsin (35,000) would necessarily prevent it from permeating into the microspheres. As a result, most of the insulin was isolated in the microsphere and well protected against the simulated gastric fluid.

Wei et al. [32] found that the chitosan microsphere with the size of 7.2 μm could transfer from the gastrointestinal tract to the systemic circulation. While the microspheres prepared in this study were modified by chitosan and with a size of 7.5 μm, therefore, they similarly behaved as the chitosan microspheres which made it essential to investigate the insulin release profile from the alginate–chitosan microspheres under the conditions of the blood environment. Fig. 8 shows the cumulative release curve of insulin in PBS (pH 7.4), and the fluorescence images of corresponding stages were also observed. Approximately, 91% of the total insulin was released after 14 days. In detail, the release process was comprised of two parts. First, about 40% of insulin was released in 1 day with a faster rate and the fluorescence became homogeneous compared with original stage (Fig. 5a). This revealed

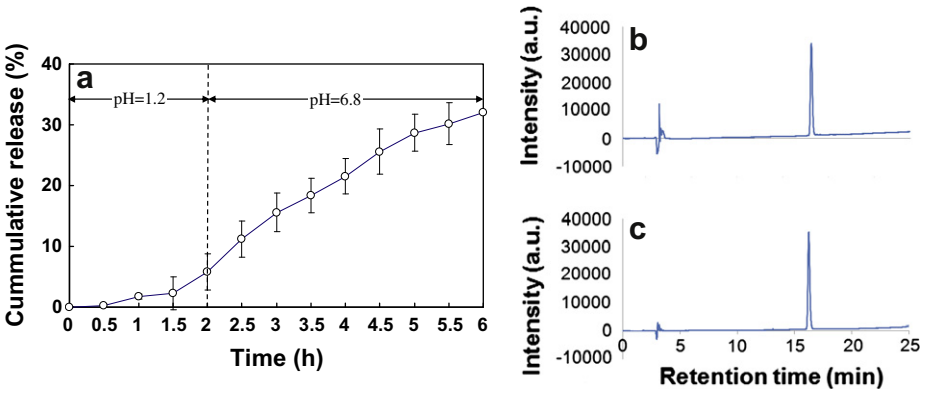

Fig. 7. The *in vitro* release profile of insulin in simulated gastric fluid and intestinal fluid (a) and HPLC chromatograms of insulin solutions; (b) non-loaded insulin, (c) insulin released from the microsphere treated with the simulated gastric fluid containing pepsin for 2 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

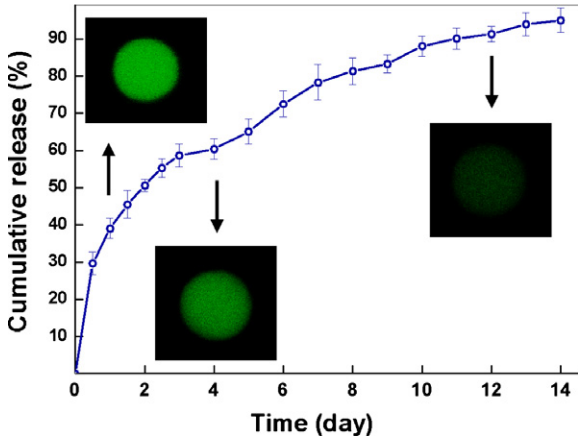

Fig. 8. Cumulative release of insulin and corresponding LSCM images of insulin release from alginate–chitosan microspheres prepared by the Method B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that most of the insulin in the outside was released at the first stage. Then, a decreased release rate followed, and about 60% insulin was released in the later 3 days. The fluorescence was weaker which suggested insulin was released from the framework of the whole microsphere. When 90% of insulin was released, the fluorescence almost disappeared.

The release at the first stage was resulted from the charge transformation of insulin. The loaded insulin was positively charged at pH 4.2 as mentioned above, thus strongly attracted with the negative alginate-Ca microsphere. The insulin charge became negative at pH 7.4 and preferentially moves into the solution from the microspheres due to the electrostatic repulsion, leading to a rapid release. However, because of the barrier of chitosan–alginate matrix as we speculated in Fig. 6, the insulin inside the microsphere was difficult to diffuse out through the pores. As a result, the release rate was significantly reduced. The approximate linearity of release rate in the second stage could be ascribed to the homogeneous distribution of insulin in uniform-sized microspheres.

In addition, it could be clearly found from the LSCM images that the microspheres preserved spherical in the whole release process, which revealed that the matrix of microspheres was intact and the drug diffused out through the pores. This release profile of insulin in our study was described as the most suitable approach for protein delivery because the controlled release or delayed release was possibly realized in this way [31]. We also thought this release behavior was the main reason to result in the much longer release period of insulin compared with other insulin carrier [33,34].

On the whole, the *in vitro* release of insulin under different environment could be summarized that (1) small amount of insulin (32%) was released under the simulated gastric fluid and intestinal fluid after incubation at simulated transit time of drug (2 h in stomach and 4 h in intestinal); (2) the chemical stability of insulin was protected well by the microspheres after passing the simulated stomach environment; (3) the release of insulin in the simulated blood environment was stable and sustained for a long time.

3.6. Evaluation of insulin-loaded alginate–chitosan microspheres in vivo

The insulin-loaded and insulin-free alginate–chitosan microspheres were orally administrated to the STZ-induced diabetic rats, and changes in plasma glucose are shown in Fig. 9. A significant difference between insulin-loaded and empty microspheres was observed. The blood glucose concentration of the diabetic rats in the treatment group was decreased evidently after oral administration. While hyperglycemia was sustained in the group administrated of insulin-free microspheres, which confirmed that the hypoglycemia effect was exclusively due to the intact insulin in the microspheres.

In the group given insulin-loaded microspheres, a rapid reduction happened at 12 h to 56%, which was approximately consistent with the insulin release profile *in vitro* (Fig. 8). The rapid insulin release was probably due to the higher contribution of insulin in the outer part of a microsphere. Then, the glucose concentration underwent a plateau from 12 h to 72 h, which was consistent with the second stage *in vitro*. After that the concentration recovered to the original level at 8 days with a very slow rate. In particular,

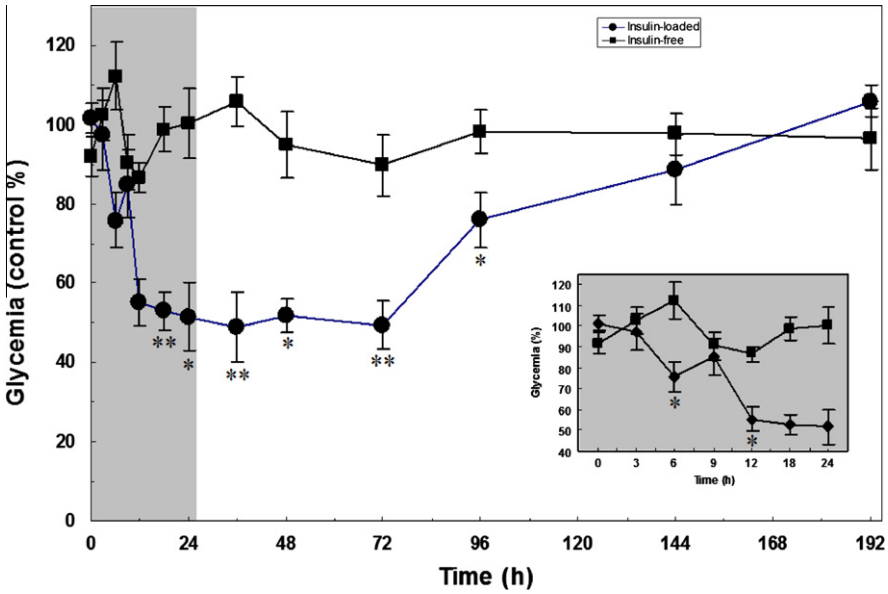

Fig. 9. The serum glucose concentration after oral administration of insulin-free and insulin-loaded (100 IU/kg) alginate–chitosan microspheres to STZ-induced diabetic rats. Statistically significant difference from insulin-free microspheres: $*p < 0.05$; $**p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

according to the enlargement in part, the reduction before 12 h experienced a slight fluctuation, which might be caused by a little burst release of the initial stage. In addition, insulin powder (200 IU/kg), which was twice as much as the dose in this study (100 IU/kg), was orally given to the rats in our previous work. The result was shown in Supplementary material 4, which indicated that most of the insulin powder was destroyed by the harsh environment of GI tract. However, 200 IU/kg was a high dose; the activity of a small part of insulin powder was still preserved and induced the decrease in blood glucose at 5 h. But the effect only lasted for 2 h. The comparison of the hypoglycemic effect between insulin powder and loaded insulin suggested that insulin loaded in microspheres was protected well in the stomach and intestinal, and then it could be absorbed and took effect *in vivo*.

The most interesting point in the blood glucose profile was a plateau that was kept for about 60 h, which implied that there was a long-period stable release with an appropriate amount of intact insulin from 12 h to 72 h. This result was far better than other correlative studies [21,35], and it could be explained by the correlation with the results from insulin release profile *in vitro*. First, only small amount of insulin was released in the gastrointestinal tract, which suggested that most of the insulin was maintained in the microspheres. It was helpful to protect insulin activity against the enzymatic attack in harsh environment of stomach and intestinal. Secondly, the modification of chitosan to the microspheres and suitable size (7.5 μm) of the microspheres made them easily adhere on the intestinal mucosa and transfer into the blood circulation [32]. Thirdly, the insulin release under the simulated pH of blood environment demonstrated that most of the insulin released from the microspheres in a steady rate and this process lasted for 14 days, which was much longer than other studies. Finally, the microspheres prepared with SPG membrane had narrow size distribution, which resulted in the same release rate, and helpful to maintain insulin concentration *in vivo*. In all, the above factors might bring about a stable plasma drug level which consequently induced the stable plasma glucose level.

The result suggested the microspheres could be orally administrated only once in 3–4 days, which will bring convenience to the diabetes. However, it was only a preliminary study for the application of alginate–chitosan microspheres to protein oral delivery. The prolonged release and insulin absorption mechanism would be investigated in detail in the future work.

4. Conclusions

Alginate–chitosan microspheres with narrow size distribution and good sphericity were successfully prepared by SPG membrane emulsification technique and a two-step solidification process. A series of characterization revealed that chitosan could permeate into the alginate-Ca microspheres through surface pores to form alginate–chitosan microspheres. Insulin as a model protein was then loaded in the microsphere by three methods. The comparison results showed higher loading efficiency and immunological activity retention achieved when insulin loaded in the second solidification step. According to the LSCM characterization of chitosan and insulin distribution in the microspheres, the microstructure of the insulin-loaded alginate–chitosan microsphere was reasonably speculated. Combined with the evaluation of chemical stability of insulin released from the microspheres treated with the simulated gastric fluid with pepsin, the results of insulin release behavior *in vitro* under simulated gastrointestinal environment and blood environment displayed that this system could protect insulin from protease digestion and then realized a sustained release. In the animal test with diabetic rates, an excellent hypoglycemic effect for a long time was obtained and possible reasons were discussed in this study. Consequently, the alginate–chitosan microspheres with narrow size distribution appear to be promising as an oral carrier of insulin for clinical therapy of diabetes and provide an oral administration system for other protein or peptide drugs.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.09.016.

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