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Preparation and improvement of release behavior of chitosan microspheres containing insulin

Lian-Yan Wang [a], Yong-Hong Gu [b], Zhi-Guo Su [a], Guang-Hui Ma [a,*]

[a] *State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China*
[b] *College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China*

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Abstract

Chitosan microsphere has potential applications in orally and other mucosally administration of protein and peptide drug, because it shows excellent mucoadhesive and permeation enhancing effect across the biological surfaces. The control of the size and size distribution of chitosan microsphere is necessary in order to improve its reproducibility, bioavailability and repeatable release behavior. Furthermore, it is a big challenge how to maintain the chemical stability of protein drug and improve its release behavior in the preparation of chitosan microspheres, because conventional crosslinking method by glutaraldehyde cannot be used in encapsulation of protein drug containing amino group. In this study, we established a method to prepare uniform-sized microsphere, and solve above problems by combining a special membrane emulsification technique and a step-wise crosslinking method. The preparative condition was optimized, and the chemical stability of protein, encapsulation efficiency, and release behavior were compared with conventional preparative method of drug-loaded chitosan microspheres. As a result, fairly uniform chitosan microspheres were obtained with a coefficient of variation (C.V.) value less than 11%, and the step-wise crosslinking method developed specially for membrane emulsification method provided the microspheres with higher encapsulation efficiency (80%), higher chemical stability of insulin (>95%), lower burst release and steady release behavior.

Keywords: Chitosan microspheres; Uniform size; Membrane emulsification; Step-wise crosslinking; Insulin

1. Introduction

Bioactive proteins and peptides are a rapidly growing class of therapeutic agents. Though only a few are currently marketed, there are hundreds under clinical test. Most of them, however, are administrated by veinally or parenterally injection because their bioavailabilities via orally administration are generally very low. They are easily degraded by proteolytic enzymes in the gastrointestinal (GI) tract and are impermeable to the intestinal mucosa due to their hydrophilic characteristics and large molecular size. Injection administration of proteins have to be given frequently because their half-life times in vivo are generally no more than several hours. Therefore, the sustained delivery system for proteins and peptides is necessary not only for injection administration but also for developing an orally administration system (Takahiro et al., 1997; Pan et al., 2002; Mahasen and Hassan, 2001; Rosa et al., 2000; Andreas and Kast, 2001; Paolo et al., 2000).

Chitosan is a kind of hydrophilic polysaccharides, which not only is degradable and nontoxic, but also shows excellent mucoadhesive and permeation enhancing effect across biological surfaces. Chitosan microspheres have arisen as a promising candidate in orally or other mucosally administrations for improving the transport of bio-macromolecules such as peptides, proteins, oligonucleotides and plasmids across biological surfaces. This is because chitosan microspheres can improve the drug adsorption of paracellular route (Thanou et al., 2001a,b). Borchard et al. (1996) showed that chitosan has the significant potential of reducing transepithelial electrical resistance and transiently opening tight conjunction between epithelial cells. By addition of chitosan microspheres containing drug on Caco-2 cell monolayers, Lueßen et al. (1997) and Kotzé et al. (1997) achieved a strongly increasing transport of buserelin, insulin and a vasopressin derivative. In addition, the mucoadhesive property of chitosan is another advantage for enhancing drug adsorption because the cationic polymer (chitosan) can combine with

* Corresponding author. Tel.: +86 10 82627072; fax: +86 10 82627072.
E-mail address: ghma@home.ipe.ac.cn (G.-H. Ma).

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anionic substructures such as the mucosa gel layer with sialic acid moieties. The adhesion of microspheres at the site of drug absorption offers various advantages for an improved uptake of therapeutic peptides (Thanou et al., 2001a,b). Firstly, in the case of orally or mucosally delivery, the released peptides or proteins will be degraded by luminally secreted proteases on the way between the dosage form and the absorption membrane. Such degradable metabolism is strongly reduced by mucoadhesive formulations, because it provides an intimate contact with the intestinal mucosa. Secondly, the adhesion of microspheres containing drug on mucosal membrane provides a high concentration gradient of the drug towards the absorption membrane. This provides high driving force for the passive paracellular uptake. Thirdly, a prolonged residence time of microspheres on mucosal tissues such as the buccal, colonic or nasal mucosa leads to an extended time period of drug absorption and subsequently to an improved bioavailability. Therefore, chitosan microspheres, as drug delivery system, not only protect protein and peptide from degradation in vivo, but also improve the uptake and bioavailability of the drug.

However, chitosan microspheres is usually prepared by mechanical stirring method or ultra-sonification technique, the size of microspheres is difficult to control and the size distribution is very broad. This disadvantage will bring some limitations in applications (Simon and Gun, 2000): (1) the reproducibility of microspheres is poor among batches, which will result in poor repeatability of the release behavior and efficacy of drug among doses, and furthermore, it is difficult to investigate the relationship between doses and treatment effects; (2) because the accumulated location of the microspheres containing drug depends on the size of the particles, the bioavailability of drug will be low if the size distribution of microsphere is broad. And, the side-effects of the drug will be increased, especially in the case of anti-cancer agents. Therefore, it is necessary to prepare uniform-sized microspheres and control the size of microspheres for their applications in drug delivery system.

For the encapsulation of protein drug in chitosan microsphere, on the other hand, it is a big challenge how to maintain the chemical stability of protein, and release the protein steadily. Usually, the W/O emulsion was prepared at the first, then the chitosan droplet containing protein drug was solidified by glutaraldehyde. This method has two obvious disadvantages as follows (Lin et al., 2004): (1) glutaraldehyde could react with amino group of protein or peptide to induce denaturation of drug and also cause crosslinking between proteins to form aggregates of proteins; (2) glutaraldehyde also could crosslink the protein into microspheres. This not only brings the denaturation of drug but also makes it difficult to release drug from microspheres. Therefore, people usually prepared blank microsphere crosslinked by glutaraldehyde at the first, then loaded the protein into microsphere by adsorption. However, loading efficiency is lower and the burst effect is higher by the adsorption method.

In this study, we established a method to prepare uniform-sized microsphere, and solve above problems by combining a special membrane emulsification technique and step-wise crosslinking method. That is, the chitosan aqueous solution containing insulin was permeated through the membrane pores into the oil phase to form the W/O emulsions with uniform size under adequate pressure, finally the droplets of emulsions were solidified by a drop-wise crosslinking method. The preparative condition was optimized, and the chemical stability of protein, encapsulation efficiency and release behavior were compared with conventional methods of drug-loaded chitosan microspheres. It was found that this method showed higher drug encapsulation efficiency, less loss of drug activity and more stable drug release profile. The uniform size of protein-loaded microspheres was expected to increase bioavailability of protein drug, and repeatability of release behavior, and it is also become possible to investigate the relationship between the size of microsphere and bioavailability.

2. Materials and methods

2.1. Materials

Chitosan (DD: 89%, $M_v = 780{,}000$) was purchased from Putian Zhongsheng Weiye Co. Ltd. (Fujian, China). Recombant insulin analog was provided by Gan & Li Biotechnological Co. Ltd. (Beijing, China). The Shirasu porous glass (SPG) membrane was bought from SPG Technology Co. (Japan). TPP was a gift from Dalian Zhencheng Chemical Engineering Co. Ltd. (Liaoning, China). Glutaradehyde was obtained from Sigma–Aldrich Inc. (Germany). PO-500 (hexaglycerin penta ester) was purchased from Sakamoto Yakuhin Kogyo Co. Ltd. (Japan). KP-18C, a silane coupling agent with C_{18} hydrophobic chain, was provided by Shin-Etsu Chemical Co. (Japan). Trifluoroacetic acid (TFA) and HPLC grade acetonitrile were supplied by Dikma Co. Ltd. (USA). Other reagents were analytical grade.

2.2. Measurement of chitosan solubility in different pH values

A definite weight of chitosan was dissolved into acetic acid–sodium acetate (HAC–NaAC) buffers with different pH values to form 1.5 wt% solutions. Spectrophotometer was used to measure transmittance (T%) at 420 nm of chitosan solutions. The transmittance is in propotion to chitosan solubility. When chitosan dissolves completely, the solution is clear and transparent and the transmittance is almost 100%. With the decent of chitosan solubility, the insoluble chitosan particles suspended in suspension, which holds back the tranverse of the light and results in the decreasing of the transmittance. The more insoluble chitosan particles, the smaller transmittance. The solubility of chitosan solutions were expressed by the value of $(100 - T)$%. A smaller value of $(100 - T)$% expresses a better solubility of chitosan in the solution.

2.3. Measurement of TPP amount on precipitation efficacy

A definite amount of TPP solution was dropped into 1.5 wt% chitosan solutions. The T% at 420 nm was measured by spectrophotometer. The precipitation efficacy of TPP on chitosan solutions were expressed by the value of $(100 - T)$%. A larger value of $(100 - T)$% indicates a higher precipitation efficacy of TPP.

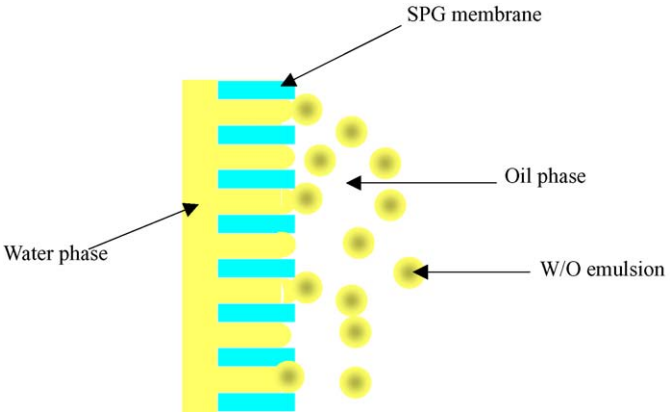

Fig. 1. Principle of membrane emulsification (W/O emulsion).

2.4. Preparation of insulin-loaded microspheres with uniform size

2.4.1. Preparation of uniform droplet by membrane emulsification technique

The principle of membrane emulsification process is shown in Fig. 1. A definite volume of chitosan aqueous solution was used as a dispersed phase (aqueous phase), then it was pressed through the pores of the membrane into a continuous phase (oil phase) containing oil-soluble emulsifier under nitrogen gas pressure to form W/O emulsion with uniform size, then the emulsifier will be adsorbed on the surface of the droplets to stabilize them. The standard recipe was as follows if it was not specified: the pore size of diameter was 4.7 μm, the C.V. value of pore size of the membrane was always below 10%, chitosan was dissolved in HAC–NaAC buffer (pH 4.03) containing 0.9% NaCl (also containing insulin, if necessary) and the concentration of chitosan was 1.5 wt%, the oil phase was a mixture of liquid paraffin and petroleum ether 7:5 (v/v) containing 4 wt% PO-500 emulsifier, volume ratio of water and oil phase was 1:10.

2.4.2. Solidification of uniform droplet

The obtained droplets were solidified by two methods, step-wise method developed in this study and conventional glutaraldehyde crosslinking method.

For step-wise crosslinking method, the chitosan emulsion was solidified by TPP ionic gelation combined with glutaraldehyde crosslinking. Briefly, the chitosan droplets were gelated by dropping the same volume of TPP aqueous solution (1 mg/ml) gradually into uniform W/O emulsions by membrane emulsification method at 200 rpm for 1 h firstly (the dropping rate was 0.2 ml/min), then glutaraldehyde saturated toluene (GST) was dropped continuously at a speed of 300 rpm for 1 h, and the molar ratio of amino group of chitosan to aldehyde group of glutaraldehyde is 1:1. Finally the chitosan microspheres were collected and washed two times with petroleum ether by centrifugation and four times with distilled water by filtration under reduced pressure, and then the microspheres were lyophilized.

For the conventional crosslinking method, only glutaraldehyde was used, other condition was as the same as above. Briefly, GST was slowly dropped into the emulsion to crosslink chitosan droplets at a stirring speed of 300 rpm for 1 h, and the molar ratio of amino group of chitosan to aldehyde group of glutaraldehyde is 1:1. The processes for collection, washing and drying for chitosan microspheres were as the same as in the case of step-wise method.

2.4.3. Loading of insulin in chitosan microsphere

Two insulin-loading methods were used in order to compare their difference and show the advantages of the solidification method developed in this study, direct encapsulation method and adsorption method.

For direct encapsulation method, insulin was loaded in the chitosan microspheres directly during the preparation of microspheres. That is, insulin with concentration of 1 mg/ml was added in chitosan aqueous solution before membrane emulsification, then, the W/O chitosan emulsion containing insulin was prepared by membrane emulsification technique as described above. The chitosan emulsion containing insulin was solidified by above two methods, step-wise method and conventional method, which were referred as method A and method B as shown in Table 1.

For the adsorption method, insulin was loaded in the preformed blank chitosan microspheres by adsorption method. The blank chitosan microspheres were prepared by membrane emulsification technique, and also were solidified, respectively, by step-wise and conventional methods, which are referred as method C and method D as shown in Table 1.

After that, 5 ml of insulin aqueous solution (PBS with pH 8.09) with concentration of 1 mg/ml was mixed with 5 mg of blank chitosan microspheres prepared by method C and method D, respectively, and then the suspension was kept at 4 °C for 48 h under shaking to load insulin by adsorption.

2.5. Characterization of chitosan microspheres

2.5.1. SEM observation of microspheres

The shape and surface feature of chitosan microspheres after drying were observed by a JEM-6700F scanning electron microscopy (SEM, JEOL, Japan). Microspheres were re-

Table 1
The preparation methods of insulin-loaded microspheres

Methods	Insulin-loading methods	Solidification methods of chitosan microspheres
Method A	Direct encapsulation	TPP ionic gelation combined with glutaraldehyde crosslinking (step-wise)
Method B	Direct encapsulation	Crosslinked by glutaraldehyde (conventional)
Method C	Adsorption	TPP ionic gelation combined with glutaraldehyde crosslinking (step-wise)
Method D	Adsorption	Crosslinked by glutaraldehyde (conventional)

suspended in distilled water and the dispersion was dropped on aluminum foil and dried at ambient atmosphere. The sample was placed on a metal stub and coated with platinum under vacuum by an ion sputter (JFC-1600, JEOL).

2.5.2. Particle size distribution

The particle size distribution of chitosan microspheres after drying were measured by laser diffractometry. Freeze-dried microspheres were re-dispersed in distilled water and sized by laser diffractometry using Ls230 Coulter (Coulter Co., USA).

2.6. Measurement of insulin-loading efficiency

2.6.1. Insulin loaded directly during preparation of chitosan microspheres

Accurately weighted blank and insulin-loaded chitosan microspheres were immersed into 5 ml of 1 M HCl aqueous solution containing 1 mg/ml $NaNO_2$ to be completely degraded under 120 °C, which was referred as blank and unknown solutions, respectively. The same weight of blank chitosan microspheres was put into 5 ml of 1 M HCl aqueous solution containing 1 mg/ml $NaNO_2$ and 100 μg/ml insulin to be also completely degraded under 120 °C, which was diluted into different insulin concentration by blank solution to be used to make calibration curve. It was confirmed that the same adsorption value was got whether the same insulin solution was treated at 120 °C or not. The unknown degradable solution was determined by Lowry protein assay and the amount of insulin calculated from calibration curve. The LE was caculated by the equation as follows:

$$\mathrm{LE} = \frac{m}{m_0} \times 100\%$$

where m_0 is total mass of insulin added and m is the mass of insulin loaded in the chitosan microspheres.

2.6.2. Insulin loaded by adsorption in preformed chitosan microspheres

The insulin-loaded chitosan microspheres were separated from the medium and the residual drug in the medium was determined by Lowry protein assay. The loading efficiency (LE) for insulin was calculated according to the following equation:

$$\mathrm{LE} = \frac{m_0 - m}{m_0} \times 100\%$$

where m_0 and m are the insulin masses in the medium before and after adsorption by chitosan microspheres.

2.7. Measurement of insulin chemical stability during preparation and release

The insulin chemical stability during the release was analyzed by high-performance liquid chromatography (HPLC). Briefly, 5 mg of microspheres were suspended in PBS buffer (pH 7.4) containing 0.05% (w/v) sodium azide as preserving agent and 0.02% (v/v) polysorbate 80 as dispersing agent, and then were placed in a thermostatic shaker (37 °C, 120 rpm). After 7 days, when a detectable amount of insulin was released into the medium, the microspheres were separated from the medium and the supernatant was analyzed by HPLC. Twenty microliters 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 × 150 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% (20 min). The flow rate was 1.0 ml/min and the wavelength was set at 280 nm. The insulin stability was ensured by comparison between measured and fresh insulin solution.

The insulin stability and solubility in different pH values and with elapse of the time were also measured, in order to confirm the selected pH value of aqueous solution was adequate. The difference of insulin solubility and stability in different pH and with elapse of time was confirmed by the proportion of activity peak area between measured and standard insulin solutions (in 0.1 M HCl).

2.8. In vitro release studies

Five milligrams of microspheres were re-suspended in PBS buffer (pH 7.4) containing 0.05% (w/v) sodium azide as preserving agent and 0.02% (v/v) polysorbate 80 as dispersing agent, then were placed in a thermostatic shaker (37 °C, 120 rpm). At predetermined intervals, 0.5 ml of the suspension was taken, centrifuged (6000 rpm, 5 min) and the concentration of the supernatant was analyzed by the Lowry protein assay. The same volume of fresh PBS buffer was added into the release medium to top up to the original volume.

3. Results and discussion

3.1. Selection of pH value of chitosan aqueous phase

In order to select an optimum condition for preparation of insulin-loaded chitosan microspheres, the effect of pH value on chitosan solubility and insulin solubility and stability were investigated. The effect of pH value on chitosan solubility is shown in Fig. 2. Chitosan was insoluble in water when pH value was

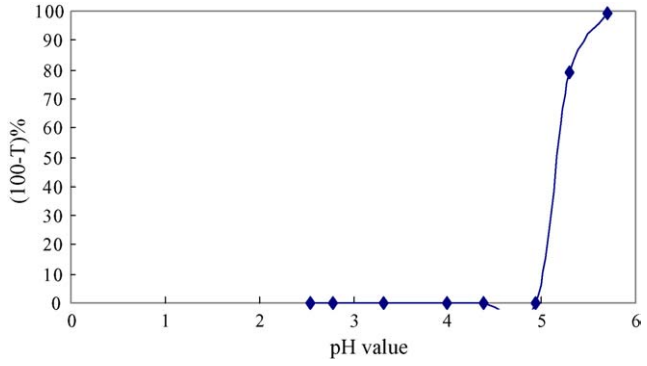

Fig. 2. Effect of pH value on chitosan solubility (measured at room temperature).

Table 2
The effect of pH value on insulin solubility ($n = 3$)

pH value	Proportion of active peak area between measured and standard insulin solutions (%)[a]
2.80	95.08 ± 0.88
3.57	95.26 ± 0.76
4.03	97.56 ± 0.92
4.43	82.04 ± 0.72
4.63	37.45 ± 0.93

[a] Measured within 3 h after insulin were added into solution.

larger than 5.0, therefore, the pH value of water phase should be less than 5.0. Insulin solubility was investigated below pH 5.0, the results are shown in Table 2. When pH value was below 4.03, the proportion of active peak area between measured and standard insulin solution was larger than 95%. It implied the better stability and solubility of insulin in these pH values. However, when pH was 4.43 and 4.63, the area proportion of two peaks decreased largely, it indicated that insulin became partly insoluble in these pH values. This is because the isoelectric point of insulin is about pH 5.8, insulin solubility will decrease when pH closed to 5.8. Therefore, pH 2.80, 3.57 and 4.03 were chosen to investigate the insulin stability with the elapse of time, and the results are shown in Table 3. As a rule, the preparation of microspheres was finished in 8 h, and the final product of microspheres was stored as powder at −20 °C which was favorable for insulin activity protection. Therefore, insulin activity in solutions was investigated in 24 h that was enough for microspheres preparation. When pH was 2.80, the activity of insulin decreased relatively rapidly with elapse of the time because insulin deamidated easily. It showed a better stability when pH was 3.57 and 4.03. Therefore, pH value of water phase was fixed at 4.0 in subsequent experiment, considering comprehensively the solubility of chitosan, the solubility and stability of insulin.

3.2. Preparation results of uniform chitosan microspheres

Figs. 3 and 4 show the typical SEM photographs and size distribution of chitosan microspheres prepared by membrane emulsification and solidified under adquate condition. It was

Table 3
Insulin stability with the elapse of time in different pH values ($n = 3$)

pH value	Time (h)	Proportion of active peak area between measured and fresh insulin solutions (%)
2.80	0	100 ± 0.56
	5	85.35 ± 0.68
	12	81.20 ± 0.59
	24	80.81 ± 0.66
3.57	0	100 ± 0.38
	5	97.64 ± 0.62
	12	93.18 ± 0.56
	24	92.90 ± 0.76
4.03	0	100 ± 0.28
	5	98.65 ± 0.53
	12	98.06 ± 0.61
	24	97.12 ± 0.55

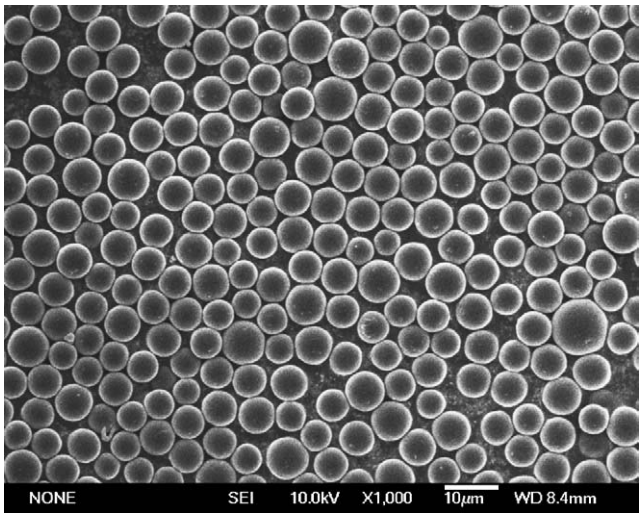

Fig. 3. The SEM photograph of chitosan microspheres prepared by membrane emulsification technique.

evident that the chitosan micospheres prepared by membrane emulsification were very uniform. The uniformity of the microspheres is very important for drug delivery system, the unform chitosan microspheres prepared by this novel membrane emulsification technology in this study are promising for realizing reproducibility, more repeatable release behavior and higher bioavailability and targetability.

3.3. Effect of TPP concentration on precipitation efficacy and morphology of microspheres

The effect of TPP concentration on precipitation efficacy is shown in Fig. 5. The precipitation of chitosan occurred only when TPP concentration in water phase was more than 0.2 mg/ml. Therefore, in the solidification of W/O chitosan droplets, the concentration of TPP was changed from 0.2 to 1.0 mg/ml, to gelate chitosan droplets (W/O) and then it was solidified by glutaraldehyde. The SEM photographs of microspheres are shown in Fig. 6. With the increase of TPP con-

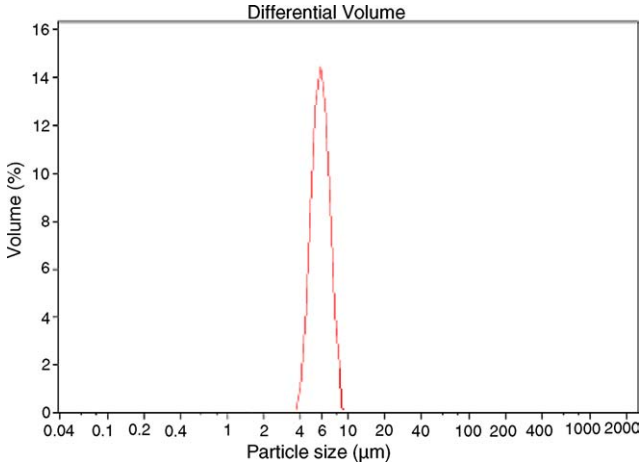

Fig. 4. Size distribution of chitosan microspheres prepared by membrane emulsification technique.

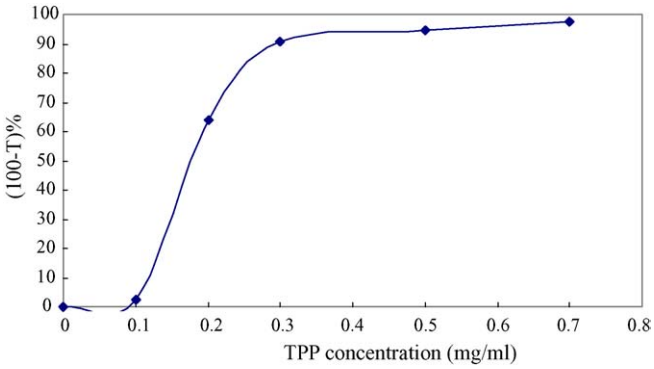

Fig. 5. Effect of TPP concentration on precipitation of chitosan (measured at room temperature).

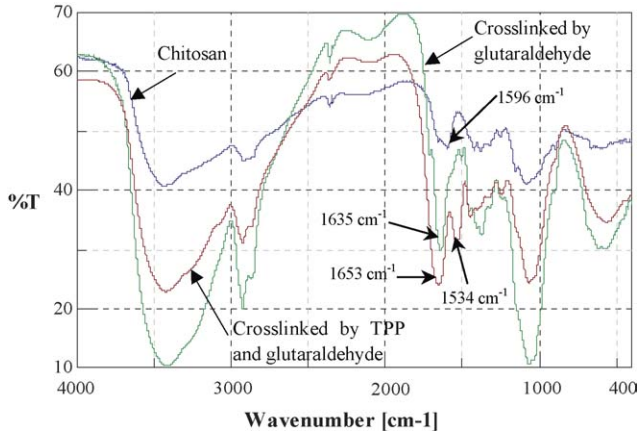

Fig. 7. FTIR spectrum chitosan and chitosan microspheres with different crosslinking methods.

centration, the smoothness and spherality of the microspheres became worse. There was only a few or no microsphere to be formed when TPP concentration reached to above 0.8 mg/ml. This was because that the reaction degree between TPP and chitosan increased with increase of TPP amount, which resulted in more water existed in gel network formed by TPP and chitosan. At the same time, the reaction degree between chitosan and glutaraldehyde was greatly reduced because a large part of $–NH_2$ on chitosan had been consumed by TPP. Therefore, the microspheres were more easily deformed in the washing and drying processes. Therefore, it is necessary to select an adequate TPP concentration, and the subsequent glutaraldehyde crosslinking is needed to obtain the spherical microspheres. Here, 0.5 mg/ml TPP concentration was chosen in order to maintain the spherality of chitosan microspheres.

Fig. 7 shows the FTIR spectrum of chitosan microspheres, solidified, respectively, by step-wise and conventional glutaraldehyde crosslinking method. In glutaraldehyde crosslinking

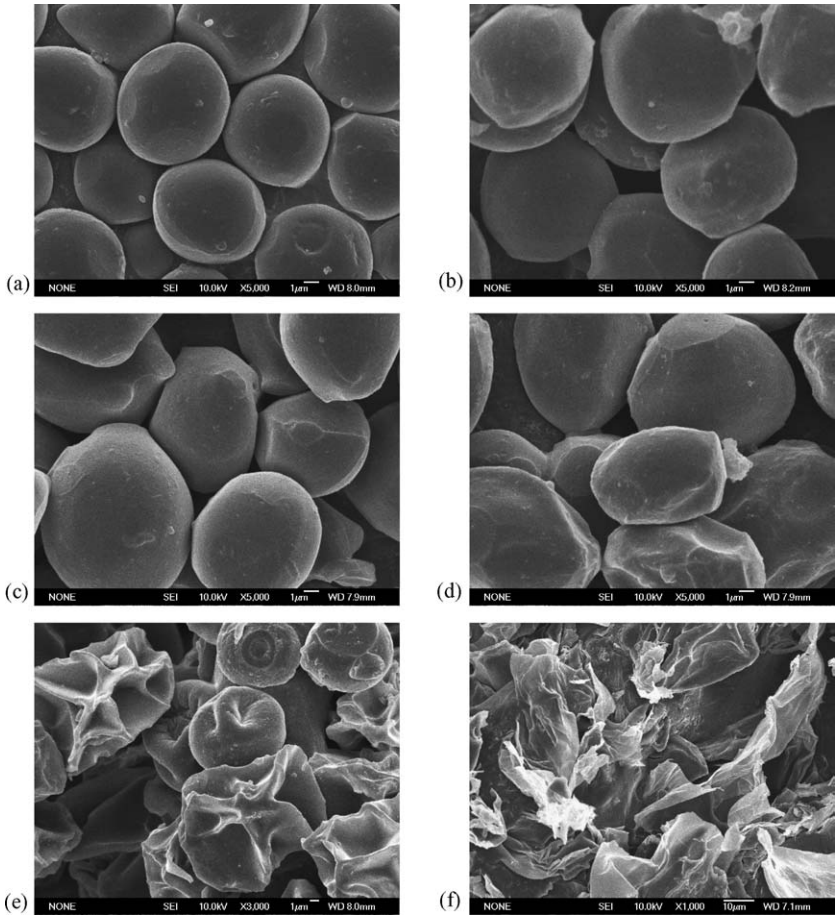

Fig. 6. Effect of TPP concentration on morphology of chitosan microspheres TPP concentration (mg/ml): (a) 0; (b) 0.2; (c) 0.3; (d) 0.5; (e) 0.8; (f) 1.0.

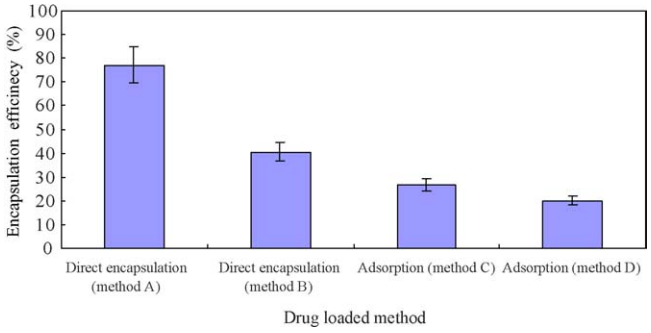

Fig. 8. Effect of insulin-loading method on loading efficiency of insulin. Each column represents the mean ± S.D. ($n = 6$).

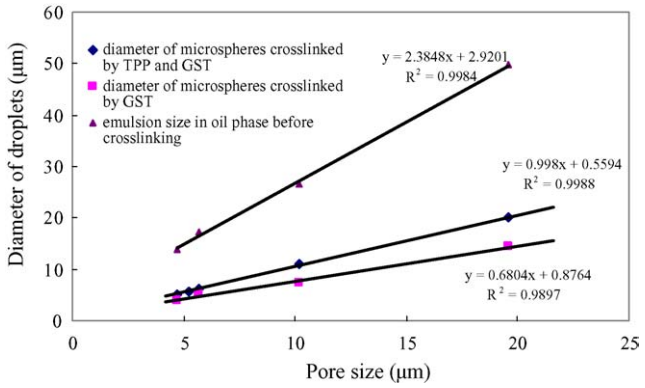

Fig. 9. Relationship between membrane pore size and diameters of droplets and solidified microspheres.

method, the peak of 1635 cm^{-1} stands for stretch vibration of C=N in Schiff base. In TPP combination with glutaraldehyde crosslinking method, there were two peaks in 1653 cm^{-1} (σ_{asN-H}) and 1534 cm^{-1} (σ_{sN-H}) to sand for chitosan salt formation, indicating ionic crosslinking occurred.

3.4. Effect of insulin-loading methods on loading efficiency

The encapsulation efficiency of insulin by different loading methods is shown in Fig. 8. The loading efficiency of insulin by adsorption (method C and method D) was the lowest. In these two cases, the insulin was loaded into the microspheres mainly by surface adsorption and diffusion into the microspheres during the swelling of microspheres. Most of insulin was adsorbed near the surface of microspheres. Because the swelling degree of crosslinked microspheres was limited, there existed saturated adsorption, depending on crosslinking degree (Mi et al., 2000). The loading efficiency of insulin by adsorption with method C was higher than that with method D, because the swelling degree of microspheres by method C was 22.36%, and that in the method D was 17.26%. The adsorption of insulin in the microspheres is a reversible process, a dynamic balance between adsorption and desorption existed. Therefore, the loading efficiency is lower than direct encapsulation methods (method A and method B). In the case of direct encapsulation, the loading efficiency of insulin by method B was lower than 50%. A lot of water was squeezed out of the droplets during crosslinking by glutaraldehyde because chitosan concentration was very low (1.5 wt%), therefore, shrinkage occurred from droplets to microspheres as shown in Fig. 9. This would result in drug leakage. However, drug leakage could be effectively avoided by method A developed in this study, because drug was interlaced in gel network formed by TPP and chitosan during TPP ionic gelation process and drug leakage was prevented apparently in the subsequent glutaraldehyde crosslinking process. A schematic explanation is shown in Fig. 10. At the same time, less shrinkage occurred from emulsion to microspheres by method A as shown in Fig. 9. Therefore, the loading efficiency was the highest and exceeded 70% by method A, it is a prospective preparation method of chitosan microspheres containing protein and peptide drugs.

3.5. Effect of insulin-loading methods on insulin integrity

The chemical stability of insulin during loading and release should be maintained. The integrity of insulin released from chitosan microspheres by different insulin-loading methods are shown in Fig. 11. There was no loss of insulin activity in the case of adsorption method. However, there caused a lot of active loss of insulin in the method B (crosslinking by glutaraldehyde). The main reason was that glutaraldehyde produced crosslinking reaction between insulin and between insulin and chitosan. In addition, dehydration of the chitosan droplets occurred during crosslinking of the droplets, it induced the increase of insulin concentration inside of the microspheres. Therefore, insulin

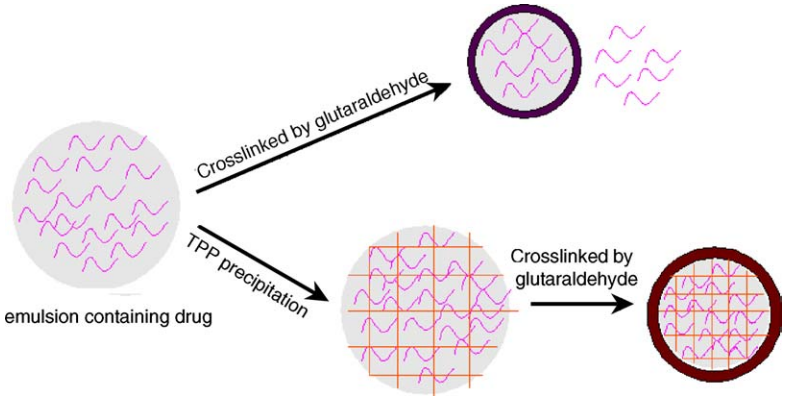

Fig. 10. The schematic explanation for chitosan microspheres formation by different crosslinking methods.

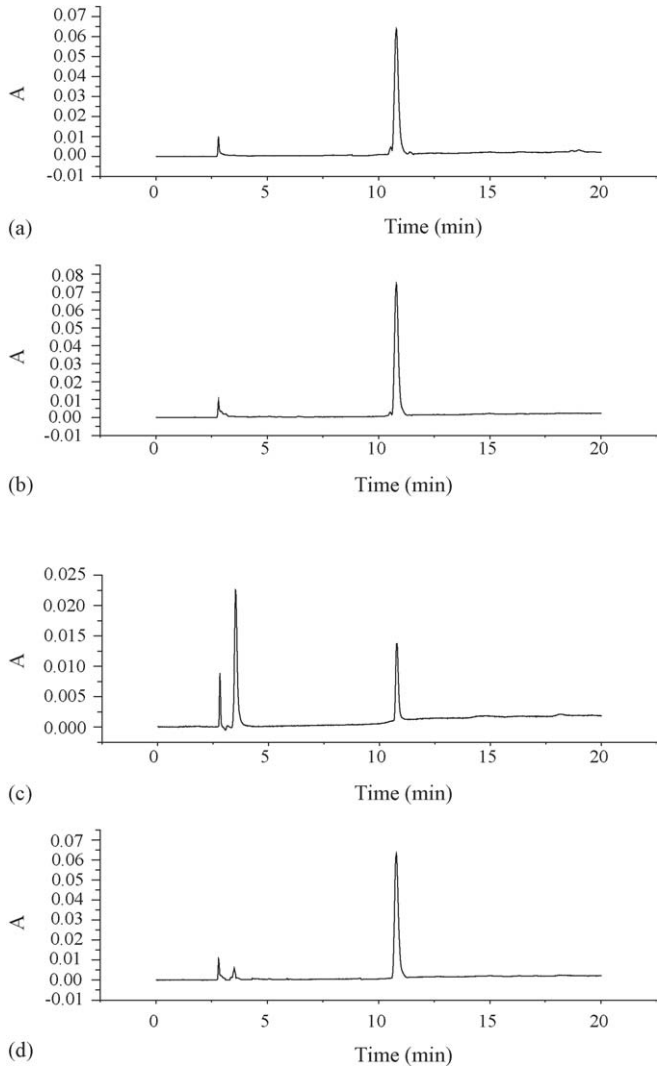

Fig. 11. The integrity of insulin released from chitosan microspheres by different insulin-loading methods. (a) Standard insulin, (b) method C or method D, (c) method B and (d) method A.

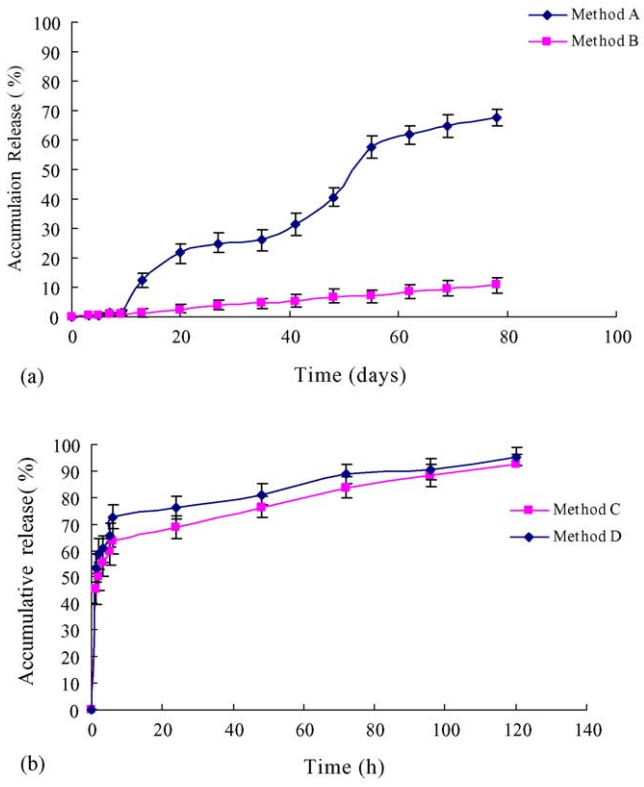

Fig. 12. The effect of insulin-loading methods on in vitro release profile of insulin (a) direct encapsulation in chitosan microspheres and (b) adsorption. Each point represents the mean ± S.D. ($n = 6$).

aggregation might be formed to also bring the activity loss of insulin. In the method A, TPP reacted with chitosan to form gel to keep water inside of the microsphere, which weakened the dehydration in the subsequent crosslinking by glutaraldehyde. Moreover, the gel formed between TPP and chitosan would reduce the contact between glutaraldehyde and insulin, so the crosslinking reaction between insulin and between insulin and chitosan was greatly avoided. As a result, less loss of insulin activity was found in method A, which was developed in this study.

3.6. Effect of insulin-loading methods on in vitro release profile of insulin

The effect of insulin-loading methods on in vitro release profile of insulin is shown in Fig. 12. The obvious burst release attaining to more than 70% was observed in the case of adsorption method (method C and method D). This was because that most of insulin was adsorbed on the surface of the chitosan microspheres. Therefore, insulin diffused into the release medium rapidly because of the concentration gradient of insulin.

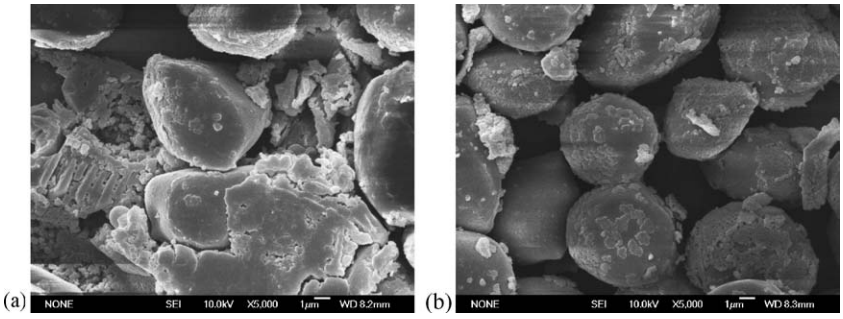

Fig. 13. The SEM photographs of chitosan microspheres collected after 80 days in vitro release (a) method A and (b) method B.

In the case of method B, the release rate was very slow, only 10% of insulin was released after 80 days. This was ascribed to that most of insulin was crosslinked into the microspheres as described above. In addition, the degradation rate of chitosan crosslinked by glutaraldehyde alone was slower. Fig. 13 shows the SEM photographs of chitosan microspheres after 80 days release in vitro. It was obvious that the microspheres crosslinked by glutaraldehyde alone (method B) hardly degrade, while apparent degradation was observed for microspheres solidified by TPP ionic gelation combined with glutaraldehyde crosslinking (method A) after 80 days. Therefore, the release profile was greatly improved when TPP ionic gelation was adopted before glutaraldehyde crosslinking. At the same time, no burst effect was observed, and the release rate was relatively stable within 80 days. This was because that the gel network between TPP and chitosan became a reservoir of drug, and allowed the insulin to diffuse steadily out of the microspheres. Furthermore, the addition of TPP could reduce the reaction degree of glutaraldehyde, so the structure of insulin-loaded microspheres was loose, this was also favorable for the diffusion of insulin.

Therefore, the method of membrane emulsification technique combined with step-wise solidification process developed in this study is a promise method to prepare chitosan microspheres containing active drug. The obtained uniform chitosan microspheres have potentiality in orally and other mucosally administration of protein and peptide drugs, because the uniformity of microspheres can grantee the reproducibility and increase the bioavailability, and the chitosan shows mucoadhesive and permeation enhancing effect across biological surfaces.

4. Conclusions

Uniform-sized chitosan microspheres with higher encapsulation efficiency, higher chemical stability lower burst effect and steady release behavior of insulin were successfully prepared by combining the membrane emulsification technique and step-wise solidification method, which was developed specifically for emulsification technique in this study. The C.V. value indicating the size distribution of chitosan microsphere was lower than 11%. In the step-wise solidification method, the chitosan droplets was solidified firstly by TPP, and then was crosslinked by glutaraldehyde, the preparation condition was optimized. By comparing with conventional drug loading methods, it was found that insulin activity was maintained, the loading efficiency of insulin reached to more than 70% and the insulin was released steadily with lower burst effect in vitro. It can be concluded that the membrane emulsification technique overcame the disadvantages of broad size distribution and poor reproducibility found in conventional mechanical stirring emulsification method, and the step-wise solidification method of chitosan droplets containing protein drug overcame the disadvantages of lower encapsulation efficiency and higher burst effect found in conventional adsorption method of protein drug in microsphere, and avoided the crosslinking between proteins and between protein and chitosan found in glutaraldehyde crosslinking method. The obtained uniform chitosan microspheres have potentiality in orally and other mucosally administration of protein and peptide drugs, because the uniformity of microspheres can grantee the reproducibility and increase the bioavailability, and the chitosan shows mucoadhesive and permeation enhancing effect across biological surfaces.

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