

## A protein delivery system: biodegradable alginate–chitosan–poly(lactic-co-glycolic acid) composite microspheres<sup>☆</sup>

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### Abstract

In the present study we developed alginate–chitosan–poly(lactic-co-glycolic acid) (PLGA) composite microspheres to elevate protein entrapment efficiency and decrease its burst release. Bovine serum albumin (BSA), which used as the model protein, was entrapped into the alginate microcapsules by a modified emulsification method in an isopropyl alcohol-washed way. The rapid drug releases were sustained by chitosan coating. To obtain the desired release properties, the alginate–chitosan microcapsules were further incorporated in the PLGA to form the composite microspheres. The average diameter of the composite microcapsules was  $31 \pm 9 \mu\text{m}$  and the encapsulation efficiency was 81–87%, while that of conventional PLGA microspheres was just 61–65%. Furthermore, the burst releases at 1 h of BSA entrapped in composite microspheres which containing PLGA (50:50) and PLGA (70:30) decreased to 24% and 8% in PBS, and further decreased to 5% and 2% in saline. On the contrary, the burst releases of conventional PLGA microspheres were 48% and 52% in PBS, respectively. Moreover, the release profiles could be manipulated by regulating the ratios of poly(lactic acid) to poly(glycolic acid) in the composite microspheres.

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**Keywords:** Microspheres; Poly(lactic-co-glycolic acid); Alginate; Chitosan; Bovine serum albumin

Biodegradable microspheres as drug delivery systems for proteins or peptides have been extensively investigated for decades, especially those based on poly(lactic-co-glycolic acid) (PLGA), which has excellent biocompatibility and changeable biodegradability [1–3]. The serial resistances of this hydrophobic polymeric

system are the low entrapment efficiency, the burst release, the instability of entrapped hydrophilic protein, and its incomplete release thereafter [4–6].

To overcome demerits of PLGA microspheres described above we constructed a novel composite microsphere, which composed of biodegradable polymers. Alginate and chitosan were used to form double-walled microcapsules [7–9]. This kind of microcapsules not only gives protein a proper microenvironment by dual hydrophilic protections but also makes the initial release be retarded. Moreover, the dual-walled microcapsules can be further incorporated in PLGA by W/O emulsification, and the release profiles including the burst release and sustained release can be accommodated with the parameters of alginate, chitosan, and ratios of poly(lactic acid)

<sup>☆</sup> Abbreviations: PLGA, poly(lactic-co-glycolic acid); BSA, bovine serum albumin; PLA, poly(lactic acid); PGA, poly(glycolic acid); PVA, poly(vinyl alcohol); BCA, bichinchonic acid; Span 80, sorbitan trioleate; Tween 80, polyoxyethylene sorbitan trioleate; SEM, scanning electron microscopy; ALM, alginate microcapsules; ACM, alginate–chitosan microcapsules; Co (50:50), alginate–chitosan–PLGA (50:50) microspheres; Co (70:30), alginate–chitosan–PLGA (70:30).

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(PLGA) to poly(glycolic acid) (PGA). In addition, the encapsulation efficiency of the composite microspheres is elevated obviously.

## Materials and methods

**Materials.** Chitosan ( $\geq 80\%$  deacetylation, MW 80,000) was obtained from Yuhuan Oceanic Biochemistry (China). Alginate (6 mps for 1% at 25 °C) and poly(vinyl alcohol) (PVA, average MW 30,000–70,000) were procured from Shanghai Chemical Reagent Company of Chinese Medicin (China). PLGA, lactic to glycolic acid molar ratios of 50:50 and 70:30, were purchased from Shandong Medical Instrumental Institute (China). Bovine serum albumin (BSA) was bought from Sino-American Biotechnology (China). Bicinchoninic acid (BCA) protein assay kits were supplied by Beyotime (China). Sorbitan trioleate (Span 80), polyoxyethylene sorbitan trioleate (Tween 80), iso-octane, isopropyl alcohol, calcium chloride, and all other reagents were of analytical grade supplied by Huadong Medical (China).

**Preparation of PLGA microspheres.** PLGA microspheres of BSA were fabricated by double-emulsion solvent extraction/evaporation method [10–14]. Briefly, 30 mg BSA was dissolved in 200  $\mu$ l distilled water or pH 7.4 PBS, and mixed with 4 ml methylene chloride containing 7.5% (w/v) PLGA. The emulsification was carried out by sonication for 10–30 s to produce w/o emulsion. The primary emulsion was then injected into a 50 ml of 0.5% poly(vinyl alcohol) (PVA) with stirring and further stirred at a high speed for 3 min to form the double w/o/w emulsion. After evaporation of the methylene chloride under the reduced pressure, the solidified microspheres were centrifuged for 10 min at 6000 rpm and then washed three times with distilled water. The resulting BSA-loaded microspheres were collected by lyophilization and stored at 4 °C.

**Preparation of alginate microcapsules.** The preparation method of alginate microcapsules was adopted from an emulsification method described by Lemoine et al. [15]. In order to prepare much smaller microcapsules, surfactant kind and some procedures were modified. The operation factors including the stirring rate and the rate of the addition of  $\text{CaCl}_2$  were investigated. BSA was dissolved in the alginate solution at the BSA/alginate ratio of 1:10, 2:10, and 3:10 (w/w), respectively. Span 80, a lipophilic surfactant, was dispersed in iso-octane at the concentration of 5% (w/v), and then 40 ml of the oil phase (iso-octane) was poured into 20 ml of the alginate aqueous solution. The mixture was emulsified for 3 min using a mechanical stirrer (FJ-200 homogenizer, Shanghai, China) at 12,000 rpm, 3.0 ml Tween 80 aqueous solution (30%, w/w) was added as the second emulsifier for attaining a proper HLB value, and the mixture was further stirred at the same speed for 3 min. Then 8 ml calcium chloride solution (8%, w/v) was added dropwise. The distance from the dropper tip to the liquid surface was about 4 cm. This cross-linking process lasted for 3 min. Then 40 ml isopropyl alcohol was added to harden the solidified microcapsules and to separate the microcapsules from the organic phase. After the mixture was stirred for another 3 min, the alginate microcapsules were collected by centrifugation and could be coated with chitosan described in the next step. If not, the alginate microcapsules were washed twice with isopropyl alcohol and dried under vacuum.

**Preparation of chitosan-coated alginate microcapsules.** The alginate microcapsules prepared according to the above-mentioned procedures were dispersed into chitosan solution at different concentrations from 0.1% to 1.0% (w/v) with pH 4 or 6. Then the mixture was shaken gently for 30 min to form the alginate–chitosan complex membrane. The microcapsules were centrifuged at 2000 rpm for 10 min and collected, then washed once in the same chitosan solution, and twice in distilled water, and finally lyophilized.

**Preparation of alginate–chitosan–PLGA microspheres.** About 0.5 g PLGA was dissolved in 3 ml acetonitrile. The alginate–chitosan

microcapsules were suspended in the PLGA solution (the ratios of microcapsules to PLGA in weight were between 1:4 and 1:10) and sonicated for 10–30 s at 200 W, the peanut oil containing 6% (w/v) Span 80 was used as oil phase. The suspension was added into the oil dropwise at 600 rpm, and the emulsion was further stirred for 1–2 min at 800 rpm. After evaporation of the acetonitrile under reduced pressure, the solidified microspheres were centrifuged for 10 min at 1500 rpm and then washed three times with petroleum ether. The resulting BSA-loaded composite microspheres were collected by lyophilization and stored at 4 °C.

**Morphology observation.** The morphologies and approximate sizes of the single, double microcapsules and the composite microspheres were studied with an optical microscopy and a scanning electron microscopy (SEM, XL30, and PHILIPS). The size and distribution of the single and double microcapsules were determined with a laser light scattering size analyzer (Zetasizer 3000 HAS, Malvern Instrument, England).

**Determination of BSA content.** The respective encapsulation efficiency of BSA was determined by the following methods: (i) PLGA microspheres: a combined extraction and hydrolysis method [16] was used to determine the BSA content in PLGA microspheres using a BCA protein assay kit according to the instructions of manufacturer (Beyotime Biotechnology, China). (ii) Alginate microcapsules coated by chitosan or not: an extraction method was used. Briefly, the microcapsules were dispersed in 3 ml PBS (pH 7.4, 0.05 mol L<sup>-1</sup>) and incubated in a shaking water bath at 37 °C, 100 rpm for 2 h. Then the sample was centrifuged at 2000 rpm and the supernatant was collected. This extraction step was repeated three times before BSA concentration was determined. (iii) Composite microspheres: 50 mg of composite microspheres was dissolved in 2 ml acetonitrile and the supernatant containing polymer was discarded after centrifugation of 16,000 rpm for 10 min. The pellet was vacuum dried and extracted according to the methods used in PLGA microspheres and alginate–chitosan microcapsules. From these results, the encapsulation efficiency (actual BSA content/theoretical BSA content) was calculated.

**In vitro release study.** The release study was carried out in PBS (pH 7.4, 0.05 mol L<sup>-1</sup>) or saline, both containing 0.01% sodium azide as the preservative. Accurately weighted amounts of chitosan-coated alginate microcapsules (about 30 mg) and composite microspheres (100–150 mg) were placed in Eppendorf tubes containing 3 ml of the release medium. The tubes were then incubated at 37 °C under the rotation speed of 70 rpm. At pre-determined time intervals, the tubes were centrifuged at 2000 rpm for 5 min. Supernatant (0.5 ml) was taken out and 0.5 ml fresh solution was added. The BSA concentration was determined by the BCA assay kit.

## Results

### Morphology observation

The PLGA microspheres were found to be spherical, smooth, non-aggregated, and with mean sizes  $3.21 \pm 0.85 \mu\text{m}$  by an optical microscopy (Fig. 1).

Small, smooth, and spherical alginate microcapsules coated with chitosan were observed by an optical microscopy or a SEM. Fig. 2 shows the SEM micrographs of the dried alginate–chitosan microcapsules prepared by 1% alginate and 1% chitosan. The mean size was  $1.15 \pm 0.21 \mu\text{m}$ .

The average diameter of the composite microspheres was  $30.96 \pm 9.08 \mu\text{m}$  when the alginate–chitosan microcapsules were further coated by PLGA. Some small

alginate–chitosan microcapsules can be seen scattering on the surface of the composite microspheres on the enlarged view (Fig. 3).

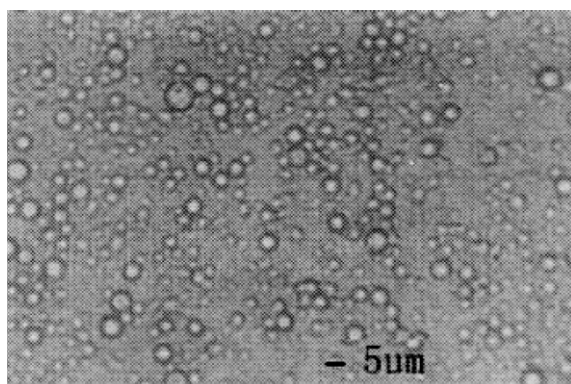


Fig. 1. The morphological picture of conventional PLGA microspheres fabricated by double-emulsion solvent extraction/evaporation method and taken by a reverse optical microscope. Scale bar, 5  $\mu$ m.

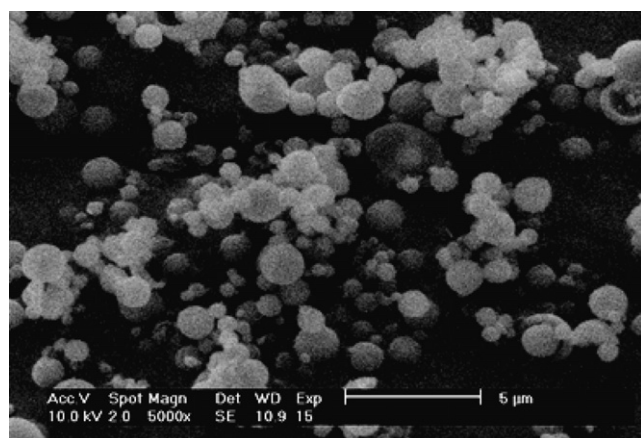


Fig. 2. Scanning electron micrographs of the dried alginate–chitosan microcapsules prepared with 1% alginate of low viscosity grade and 1% chitosan. Scale bar, 5  $\mu$ m.

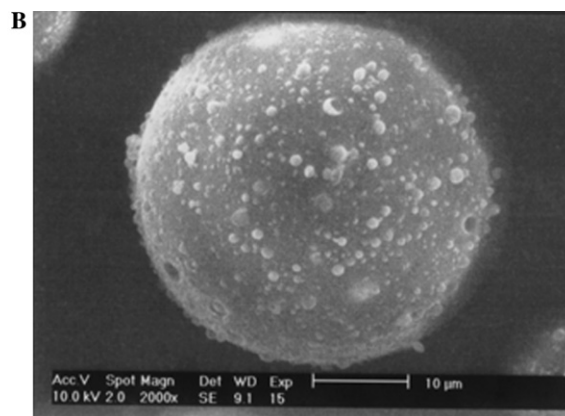
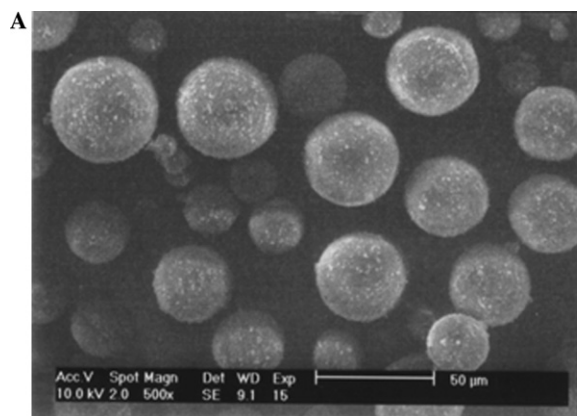


Fig. 3. Scanning electron micrographs of alginate–chitosan–PLGA composite microspheres in which alginate–chitosan microcapsules were further coated by PLGA. Some small alginate–chitosan microcapsules can be seen scattering on the surface of the composite microspheres on the enlarged view. (A) Scale bar, 50  $\mu$ m; (B) scale bar, 10  $\mu$ m.

#### Entrapment efficiency determination

BSA entrapment efficiency of conventional PLGA microspheres is between 61% and 65%, which is  $64.54\% \pm 0.18\%$  for PLGA (50:50) and  $60.81\% \pm 0.57\%$  for PLGA (70:30) (Fig. 4).

The encapsulation efficiency in the uncoated alginate microcapsules prepared by an emulsification method and washed with isopropyl alcohol was  $99.95\% \pm 0.06\%$ . When calcium alginate microcapsules were incubated into 1% (w/v) chitosan solution to form double-

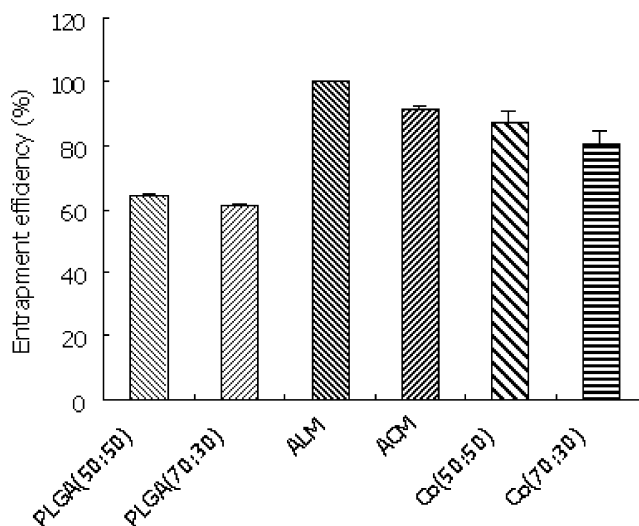


Fig. 4. The entrapment efficiencies of BSA in composite microspheres of PLGA (50:50) and PLGA (70:30) were presented compared with those in conventional microspheres of PLGA (50:50) and PLGA (70:30). The entrapment efficiency in uncoated alginate microcapsules prepared by an emulsification method and washed with isopropyl alcohol and that in alginate–chitosan (both 1%, w/v) double-walled microcapsules were also shown. ALM, calcium alginate microcapsules; ACM, alginate–chitosan microcapsules; Co (50:50), alginate–chitosan–PLGA (50:50) microspheres; and Co (70:30), alginate–chitosan–PLGA (70:30).

walled microcapsules, BSA entrapment efficiency was  $91.73\% \pm 0.45\%$ , and the alginate–chitosan capsules were further incorporated in PLGA to form the composite microspheres, the entrapment efficiency was  $87.29\% \pm 3.89\%$  for PLGA (50:50) and  $80.73\% \pm 3.59\%$  for PLGA (70:30), both were sharply elevated compared with conventional PLGA microspheres (Fig. 4).

*In vitro release test*

The initial burst releases were severe in conventional PLGA microspheres prepared by w/o/w emulsification method (Fig. 5). For PLGA (50:50) and PLGA (70:30) microspheres, their burst release rates were up to  $47.73\% \pm 8.93\%$  and  $51.53\% \pm 7.72\%$  at 1 h,  $52.83\% \pm 9.64\%$  and  $54.98\% \pm 7.88\%$  at 24 h, respectively.

Before the release characterization of the composite microspheres was evaluated, the release rates from the alginate microcapsules coated with various chitosan concentrations were studied (Fig. 6). Compared with the release rates from the uncoated alginate microcapsules,

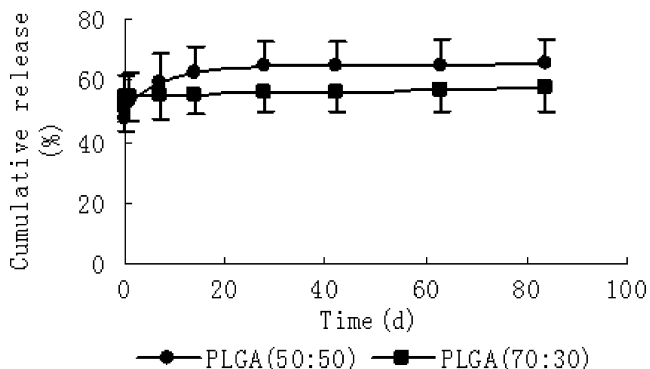


Fig. 5. The cumulative releases of BSA from conventional microspheres of PLGA (50:50) and PLGA (70:30).

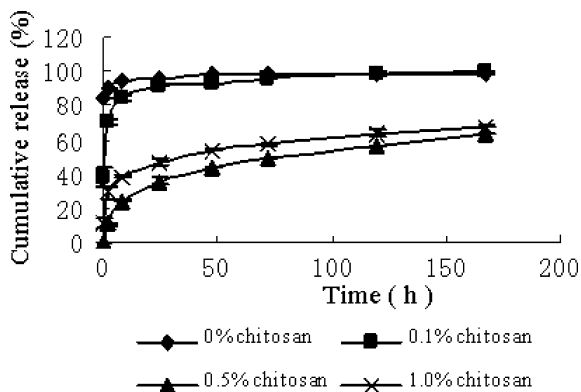


Fig. 6. Release profiles of BSA-loaded microcapsules prepared with 1% alginate and different concentrations of chitosan in saline.

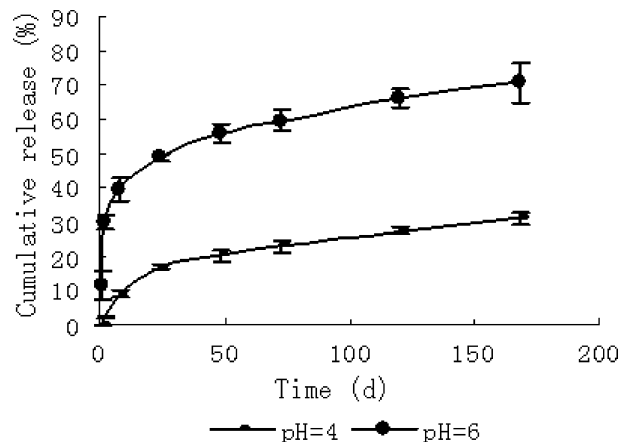


Fig. 7. BSA release percent from alginate–chitosan microcapsules prepared under pH 4 and 6 of chitosan solution. Alginate 1% (w/v) and chitosan 1% (w/v) were selected.

90.61% at 2 h and 99.50% at 48 h, respectively, the release rates of BSA from 0.1% chitosan-coated alginate microcapsules were slightly delayed, 70.50% at 2 h and 93.20% at 48 h. When the chitosan concentration was elevated to 0.5%, the release rate of protein was greatly decreased, whereas no further retardation effect was seen after the chitosan concentration increased to 1.0%. In addition, the influence of the chitosan solution’s acidity (Fig. 7) was evaluated. The protein release rate of the microcapsules coated with pH 6 chitosan solution was higher than that with pH 4 chitosan solution ( $48.90\% \pm 0.89\%$  vs.  $16.97\% \pm 0.42\%$  at 24 h,  $p < 0.001$ ,  $70.74\% \pm 5.67\%$  vs.  $31.15\% \pm 1.89\%$  at 168 h,  $p < 0.05$ ), while the encapsulation efficiency presented no difference from each other.

Based on the retardation of alginate–chitosan microcapsules above, the burst releases of BSA in alginate–chitosan–PLGA (50:50) composite microspheres were 23.96% at 1 h, 29.82% at 3 h, 34.35% at 8 h, and 38.89% at 24 h, obviously lower than those in conventional PLGA (50:50) microspheres, which were 47.73%, 49.80%, 51.88%, and 52.83%, respectively. Both of the cumulative releases were similar at 9 week. From their release profiles, the following release rate in composite seemed to be slightly larger than that in conventional PLGA microspheres (Fig. 8).

When the ratio of PLA to PGA in PLGA increased to 70:30, the burst release of BSA reduced more obviously. The initial release was 7.97% at 1 h and 21.31% at 24 h. In addition, the cumulative release of BSA was 33.37% at 13 week. Compared with 70.60% for PLGA (50:50) at 13 week (Fig. 9), there was statistical difference ( $p < 0.05$ ).

We think that the sensitivity of alginate–chitosan microcapsules to  $\text{Na}^+$  and  $\text{PO}_4^{3-}$  in PBS may accelerate the hydrate process of the gel. Consequently, the release of BSA will be stepped up in PBS. Saline was selected to



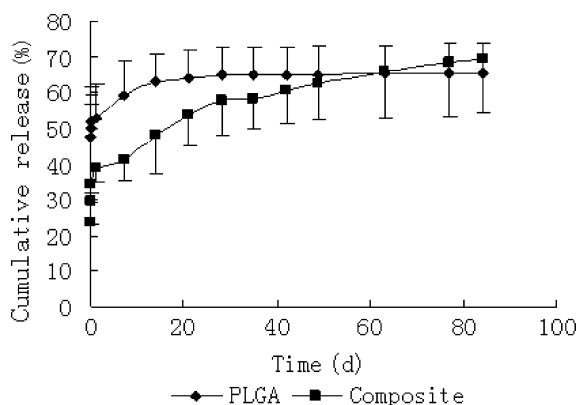


Fig. 8. Release profiles of BSA from the composite microspheres of alginate-chitosan-PLGA (50:50) and conventional PLGA (50:50) microspheres in PBS.

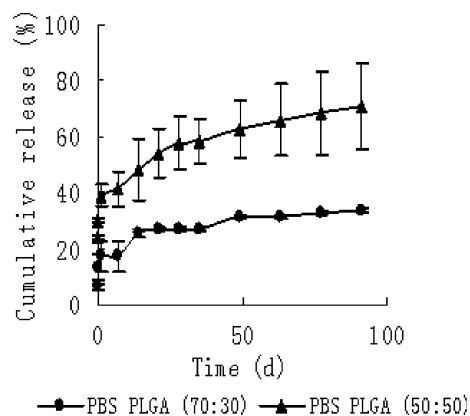


Fig. 9. The release profiles of BSA from the composite microspheres of alginate-chitosan-PLGA (50:50) and alginate-chitosan-PLGA (70:30) in PBS.

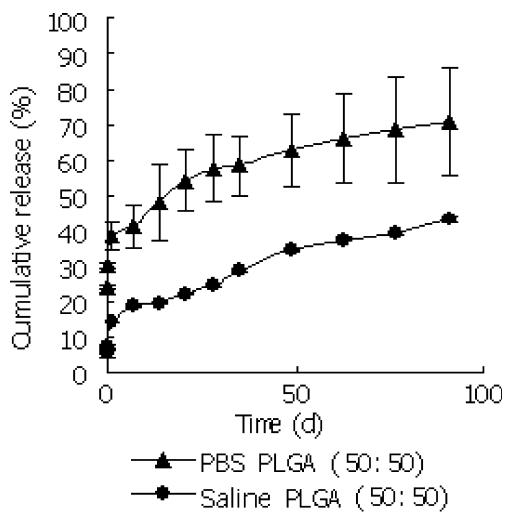


Fig. 10. Release profiles of BSA from alginate-chitosan-PLGA (50:50) microspheres in two different release media of PBS and saline.

replace PBS as release medium. The release of PLGA (50:50) composite microspheres in saline was 5.38% at 1 h, 14.03% at 24 h, and 43.14% at 13 week, meanwhile its release in PBS was 23.96%, 38.89%, and 70.60%, respectively (Fig. 10). It was the same with PLGA (70:30) composite microspheres. Nevertheless, the release profiles of conventional PLGA microspheres were almost the same both in saline and in PBS (data not shown).

## Discussions

Either natural or synthetic biodegradable polymer is ideal for protein or peptide drug delivery systems. Compared with non-degradable polymers, they have many advantages. For instance, convenience of delivery, improvement of the patient's compliance, maintenance of the drug concentration in the latter release phase as well as possible pulsatile release, etc. [17,18]. However, neither hydrophilic polymeric systems nor hydrophobic ones may be considered suitable candidates for protein delivery when they are used alone [19]. PLGA as a hydrophobic polymer is an ideal carrier to achieve sustained drug release, but its incompatible property and low entrapment ability make it unacceptable. The combination of gelatin nanoparticles or agarose hydrogel and PLGA microspheres had advantages of both the hydrophilic and the hydrophobic systems [19,20]. This kind of new composite microsphere had high protein loading efficiency and the capability of preventing the denaturation of protein, but the size of the composite microsphere was too large (150–175  $\mu\text{m}$ ). Moreover, the release rate of protein in the composite PLGA microspheres was larger than that in the conventional PLGA microspheres [20]. Authors think that the rapid absorption and swell of hydrophilic gelatin result in the instant protein diffusion. The same phenomenon was found in this study. BSA was released 90.61% from calcium alginate microcapsules at 2 h and 99.50% at 48 h, almost released completely. The highly porous structure of alginate microcapsules made the release faster.

Thus, the objective of this study was to delay the rapid protein release by coating the alginate microcapsules with chitosan. The enhancement of tensile strength of the membrane coat was achieved by adjusting the concentration and the pH of chitosan solution [21]. Electrostatic interaction of the alginate carboxyl groups with a polycationic amine coat decided the density of the membrane. The  $pK_a$  of chitosan and alginate are 6.3 and 3.5, respectively. At higher pH, chitosan has less free amine groups due to the increased deprotonation. As the result, the extent of the interaction between alginate and chitosan is reduced, and the alginate-chitosan complex layer becomes less dense [22]. It was for the same reason that the release characteristics of alginate-chitosan

microcapsules varied with the concentrations of chitosan (shown in Fig. 6). The alginate microcapsules coated with 0.1% chitosan still showed greater burst release of BSA than those prepared with 0.5% or 1% chitosan solution. Of course, the interaction to form the strong membrane needed corresponding anionic carboxyl groups and cationic amine coat. If the alginate carboxyl groups were counteracted thoroughly, we think that the higher chitosan concentration was not necessary.

When PBS was as a release medium, calcium ions in the microcapsules are replaced by sodium ions in PBS and then combined with phosphate ions, another ion in PBS, to form the insoluble  $\text{Ca}_3(\text{PO}_4)_2$ . The combination reversely accelerated the ionic exchange to release BSA more rapidly. Thus, the initial burst release and the cumulated release amounts were at higher levels compared with those in saline solution, which suggested that the release characteristics of protein delivery system between *in vitro* and *in vivo* might be quite different. It will be our next aim to study the *in vivo* property of the composite microspheres.

The burst release of BSA from conventional PLGA microspheres prepared by w/o/w emulsification was severe [23,24]. Proteins that are either adsorbed on the surface or loosely associated with the surface are responsible for the burst release. Moreover, proteins embedded in the surface layer can escape from the polymeric matrices through the pores and cracks that formed during the fabrication process, thereby increasing the amount of the initial release [25]. If BSA was first encapsulated in calcium alginate and then coated with chitosan will overcome the problem and the double-walled alginate–chitosan microcapsules were further entrapped into PLGA to form the composite microspheres will furthermore improve the microspheres, it is not difficult to understand the lower burst release from the composite microspheres. On the other hand, Castellanos et al. [26] proved that the incomplete release of entrapped protein was correlated with the amount of insoluble protein aggregation and/or the protein instability issue. From the release trends of conventional PLGA microspheres and the composite ones, the release from composite microspheres thereafter was more complete (Fig. 8). It can be anticipated that this kind of novel composite microspheres may give protein a proper protection. With the ratios of PLA to PGA changed, the controlled release formulations can afford desired release rates.

In this study, to obtain a high entrapment efficiency of protein, we constructed a composite microsphere. First, the much smaller calcium alginate microcapsules were obtained by a modified emulsification method in an isopropyl alcohol-washed way, and then the alginate–chitosan microcapsules were formed by chitosan coating. These double-walled microcapsules were further incorporated in the PLGA to form the composite

microspheres. The drug encapsulation efficiency was increased to more than 80%, as well as the burst release was decreased to smaller than 15% in saline within 24 h. The desired release profiles could be obtained by regulating the ratios of PLA and PGA in the composite microspheres. This kind of novel composite microspheres may be a promising delivery system for water-soluble proteins and peptides.

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