# Preparation and Characterization of Improved Microspheres Containing Bovine Serum Albumin

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**ABSTRACT:** Microspheres containing bovine serum albumin (BSA, a model drug) were prepared via double emulsion solvent evaporation using the compound of poly(3-hydroxybutyrate) (PHB) and polyethylene glycol (PEG) as the matrix. Influences of introduction of BSA and wall polymer composition on BSA-PHB/PEG microspheres characteristics were studied by means of DSC, XRD, optical microscope (OM), SEM, FTIR, etc. The crystallinity of PHB dropped when PEG was brought in the compound, and it decreased with the increasing proportion of PEG. BSA-PHB/PEG microspheres had still lower crystallinity than PHB/PEG compound and raw materials. The yield and protein loading of the microspheres reached 36.1% and 12.2%, respectively, at the optimum mass ratio, m(PHB) : m(PEG) = 4/1. FTIR results confirmed the existence of BSA in the microspheres and revealed the absence of chemical interaction between BSA and polymers. It was found that the mass ratio of PHB to PEG had direct effect on the size distribution, surface morphologies, and microstructure of microspheres. The mean particle size of microspheres ranged between 2.9 and 5.0 μm measured by optical microscopy, depending on the different proportion of PEG. The results from the OM observations combined with SEM micrographs showed that PHB/PEG microspheres were likely to have porous surface and a structure of microspheres embeded. The controlled release characteristics of the microspheres for BSA were investigated in pH 7.4 media, and the result indicated that the BSA-PHB/PEG microspheres had a quicker release rate and a higher accumulative release amount than BSA-PHB microspheres, which showed the feasibility and superiority of BSA-PHB/PEG microspheres as controlled release devices. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 818–825, 2009

**Key words:** Poly(3-hydroxybutyrate) (PHB); polyethylene glycol (PEG); bovine serum albumin (BSA); microsphere; double emulsion solvent evaporation

## INTRODUCTION

Recently, many pharmacologically active peptides and proteins are becoming gradually good candidates for therapeutic drug treatment with the development of biotechnology and genetic engineering. However, their clinical applications in oral administration have been limited due to their higher molecular weight, which makes diffusion through biological membrane difficult, and their instability in the gastrointestinal environment. Alternative administration options by frequent injection are also tedious and expensive.[1] Therefore, many researchers have paid much attentions to exploring controlled delivery systems with a longer parenteral dosing interval, which will increase patience acceptance and improve drug management.

Microspheres containing protein or peptide as controlled release devices have been widely used for the treatment of human diseases and animal health. Various techniques are available to encapsulate proteins into biodegradable microspheres. These techniques include W/O/W double emulsion, organic phase separation, supercritical fluid, and spray drying techniques.[2–5] Among them, the W/O/W double emulsion technique is a well-used process.

Both natural and synthetic biodegradable polymers have been investigated for controlled drug release.[6–8] Among these polymers, poly(3-hydroxybutyrate) (PHB) is found to be remarkable for its application in drug delivery due to its excellent biocompatibility[9] and biodegradability.[10] Hydrolytic degradation of PHB in vitro proceed to the monomer, D-(-)-3-hydroxybutyric acid. This acid is a normal constituent of blood and, in common with acetoacetate and acetone, is one of the three ketone-bodies which are produced endogenously by the process known as ketogenesis. It is therefore thought that PHB will be well tolerated in vivo.[11–13] So PHB is considered to be a good candidate for drug delivery.

However, pure PHB has several inherent deficiencies in use, including high crystallinity and stiff polymer chains, which would result in an inefficient entrapment of aimed drugs. Furthermore, PHB has a relatively slow rate of in vivo hydrolytic degradation compared with polylactides.[14] It often needs several years for body to absorb the degradation products

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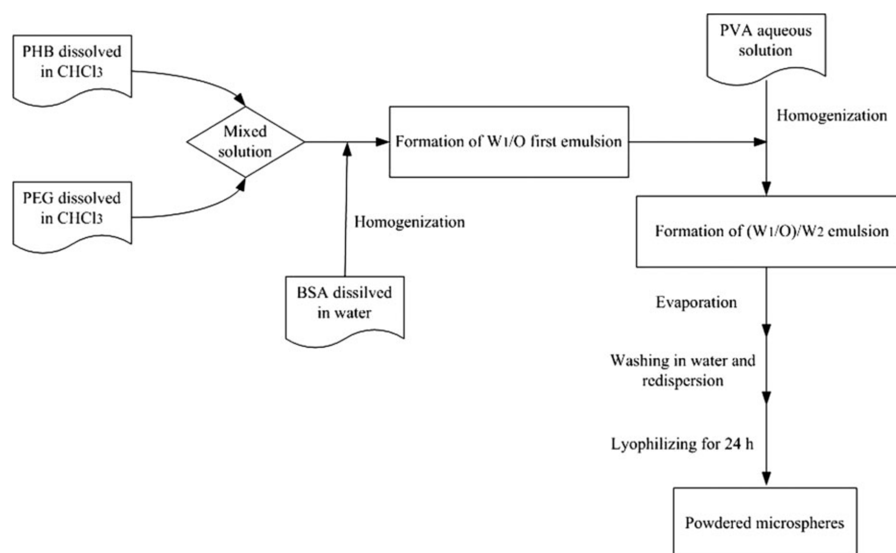


**Figure 1** Processing of microspheres via double emulsion solvent evaporation.

completely.[15] All of these are disadvantageous for the application of PHB in drug delivery system. Because PHB does not have any functional groups usable for chemical modification, PHB-based microspheres are often prepared using compound of PHB blending with other materials, such chitosan,[16] polyhydroxybutyrate-co-valerate.[17] PEG is a hydrophilic polymer which has outstanding properties, including good solubility in water and in organic solvents, a lack of toxicity, and no antigenicity, and, consequently, no immunogenicity, all of which are essential properties for pharmacy applications. However, there are few literatures focused on the introduction of PEG to improve the properties of PHB based microspheres. In this study, PHB and PEG were used to prepare the microspheres via double emulsion solvent evaporation in which a model drug bovine serum albumin (BSA) was entrapped. Influences of BSA and wall polymer composition on the properties of BSA-PHB/PEG microspheres were studied.

## MATERIALS AND METHODS

### Materials

PHB, with $M_n = 2.93 \times 10^5$, $M_w = 4.37 \times 10^5$, $M_w/M_n = 1.49$, and 96% $\sim$ 98% content by weight, was purchased from Tianlu Co. (Tianjin, China). PEG, with $M_n = 19000$, was obtained from Shanghai Chemical of China medicine Com. (Shanghai, China). Poly(vinyl alcohol), (PVA) commonly used in emulsification solvent evaporation formulations, with $M_w = 22000$, and 86%–88% degree of hydrolysis, was obtained from Shanghai Chemical of China medicine Com. (Shanghai, China) too. BSA (fraction V, 66kDa) was purchased from Sigma Chemical Company. Chloroform, one of the most extensively used formulation solvent, was purchased from Tianjin Krs Chemical Co. (Tianjin, China). All chemicals were used without further purification.

### Film preparation

Films of PHB with and without PEG were prepared by casting using the following proportions of PHB/PEG: 5/0, 4/1, 3/2, 2/3, and 1/4 (wt %). Pure PHB is represented by 5/0. The materials were dissolved in chloroform to give 3% (v/w) solutions that were stirred thoroughly at 60 $\pm$ 1°C for 10 min and then poured into Pyrex recipients after which the solvent was allowed to evaporate in a saturated atmosphere.

### Preparation of BSA-PHB/PEG microspheres

The BSA-PHB/PEG microspheres were prepared by double emulsification solvent evaporation method (Fig. 1). BSA solution ($W_1$) of 3 mL 3% w/v was injected into 15 mL 3% w/v PHB/PEG chloroform solution (O) under stirring (23,000 rpm) for 2–3 min to form the primary emulsion ($W_1$/O). Then the primary emulsion was dripped into a 150 mL 1% w/v PVA solution ($W_2$) at a rate of 5000 rpm for 4–5 min to form the secondary emulsion ($W_1$/O/$W_2$). High-speed emulsification was achieved by FJ-200 homogenizer. Both BSA solution ($W_1$) and the primary emulsion ($W_1$/O) were added by injection through 0.2 mm injector in 15 s. The finial $W_1$/O/$W_2$ emulsion was left to evaporate under magnetic stirring (500 rpm) for 4–5 h. The microspheres were separated by centrifugation at 5000 rpm for 5 min and washed three times with 100 mL of deionized water

to remove PVA residues. Finally, the microspheres were lyophilized.

### Thermal analysis

Thermal analysis was done using a PERKIN-ELMER DSC7 differential scanning calorimeter under an atmosphere of nitrogen, at a heating rate of 10°C $min^{-1}$. Two heating cycles were used for each film prepared before. The samples were initially heated from −50°C to 200°C to eliminate the thermal history of the sample, and then cooled to −50°C at a cooling rate at 10°C $min^{-1}$ before being immediately reheated to 200°C. The second scan (−50°C to 200°C) was done at the same heating rate. The melting enthalpy of PHB was gotten from the thermograms. Then the crystallinity ($X_c$) of PHB in the blends film was calculated according to the equation below.

$$X_c = \Delta H_f \times 100/\Delta H_0 \times w(\text{PHB}) \quad (1)$$

$\Delta H_f$ = melting enthalpy of the sample ($Jg^{-1}$). $\Delta H_0$ = melting enthalpy of the 100% crystalline PHB which is assumed to be 146 $Jg^{-1}$[18] and w(PHB) is the weight fraction of PHB in the sample.

### X-ray diffraction

A D8 DISCOVER model X-ray generator with a Ni filter to provide a Cu-Ka radiation ($\lambda$ = 0.1542 nm) was used. Every scan was recorded in the range of $2\theta = 6 \sim 40°$ at a scan of 3°/min.

### OM observation microsphere formation

The formation of final microspheres was observed under an optical microscope (American Optical, Micro Star) equipped with a digital camera (Nikon 4500). Usually, the solvent is highly volatile, and the fast solvent elimination process makes it difficult to observe. To observe in detail the transformation of the second emulsion microdroplets into the final microspheres, a small amount of the final emulsion was dropped on a concave in the center of a microscope slide and sealed with cover glass. In this case, the solvent evaporation is restricted only through the thin emulsion-air interface located at the edges of the cover glass. The final microspheres are formed as the solvent is extracted out from the second emulsion microdroplet, transported through the aqueous phase and evaporated through the emulsion-air interface. Thus, the whole process is slowed down allowing a proper observation of the microsphere formation.

### Analyses of microspheres size

Freeze-dried microspheres were re-dispersed in distilled water and observed by microscopy (MicroStar, America).

### SEM observation

The morphology of the microspheres was examined by scanning electron microscopy (JSM-6700F) after coating a thin gold film.

### Interaction between BSA and polymers

A Fourier transform infrared spectrophotometer (FTIR, Perkin-Elmer Spectrum 2000) was employed to explore the interactions between BSA and polymers using potassium bromide pellets.

### Evaluation of the protein loading and yield of microspheres

A Coomassie Brilliant Blue method was adopted to determine the concentration of protein. Ten milligrams BSA-loaded microspheres were dissolved completely in chloroform with vigorous shaking at room temperature for 24 h. Then 10 mL physiological phosphate buffer solution (PBS, pH 7.4, 0.1 mol/L) was added to dissolve the BSA in the microspheres, and the resultant solution was filtrated. After that, 5 mL Coomassie Brilliant Blue reagent was added into 0.5 mL resultant solution to be detected at 595 nm by UV/Vis spectrophotometer. At last, the concentration of BSA in the solution and amount of protein in microspheres could be worked out. Yield and protein loading of microspheres were determined by eqs. (2) and (3), respectively:

$$\text{Yield of microspheres (\%)} = \left(\frac{\text{retained protein amount}}{\text{initially loaded protein amount}}\right) \times 100\% \quad (2)$$

$$\text{Protein loading (\%)} = \left(\frac{\text{amount of protein in microspheres}}{\text{amount of microspheres}}\right) \times 100\% \quad (3)$$

### In vitro BSA release

Microspheres (50 mg) were placed in triplicate into Eppendorf tubes and incubated in 10 mL release medium (PBS buffer, pH = 7.4 0.1 mol/L) under agitation (100 rpm) at 37 ± 0.5°C. At desirable time intervals, the microspheres suspension was centrifuged at 5000 rpm for 20 min. The supernatant (10 mL) was withdrawn and replaced with 10 mL fresh release medium. The amount of BSA released was determined by measuring the BSA content in the supernatant. To determine the BSA content in the

TABLE I
DSC Results of the Various PHB/PEG Blends

| m(PHB) : m(PEG) | $T_m$ (°C) | $\Delta H_f$ ($Jg^{-1}$) | Crystallinity (%) |
|---|---|---|---|
| 5/0 | 176.5 | 78.63 | 53.9 |
| 4/1 | 171.6 | 67.16 | 36.8 |
| 3/2 | 163.7 | 48.11 | 19.8 |
| 2/3 | 165.4 | 32.10 | 8.8 |
| 1/4 | 166.3 | 14.57 | 2.0 |

supernatant, 5 mL Coomassie brilliant blue was added into 0.5 ml supernatant solution to be detected at 595 nm by UV/Vis spectrophotometer.

## RESULTS AND DISCUSSIONS

### Crystallization states of PHB

To evaluate the effect of PEG additive on the thermal and crystal properties of PHB, films made from PHB and PEG blends were investigated by DSC. The DSC results (Table I) showed that the melting temperature of the blends decreased with increasing concentration of PEG in the blends. The plasticizer PEG probably weakened the intermolecular forces between the adjacent polymer chains. Consequently, there was a change in free volume that reduced the melting temperature of the system. However, PEG that crystallized before the PHB ($T_c$ of PEG = 50–60°C) could influence the lamellae formation of the polymer. The performed nucleation of the additive probably reduced the mobility of the PHB and then retarded the lamellae formation. According to Yoshie et al., thinner lamellae has lower melting temperature.[19] The crystallinity of PHB in the sample was determined according to eq. (1). During non-isothermal crystallization, the crystallization rate decreased in the presence of PEG. Consequently, nucleation was hindered and promoted the formation of small spherulites, thereby increased the flexibility of the blends compared with the pure PHB, which may be favorable for entrapment of model drugs.

The crystallization states of PHB in raw materials, PHB/PEG compounds, and BSA-PHB/PEG microspheres were shown in Figure 2. The XRD pattern of pure PHB had characteristic peaks when the 2θ were 13° and 17°. Compared with the XRD pattern of the pure PHB, it was evident that the location of peaks in the XRD patterns of the PHB/PEG compound and BSA-PHB/PEG microspheres was the same, and no new peaks appeared. However, the intensity of the peaks decreased. PHB was still less crystalline as microspheres than compound, which further indicated that BSA had the same effect as PEG on decreasing the crystallinity of PHB.

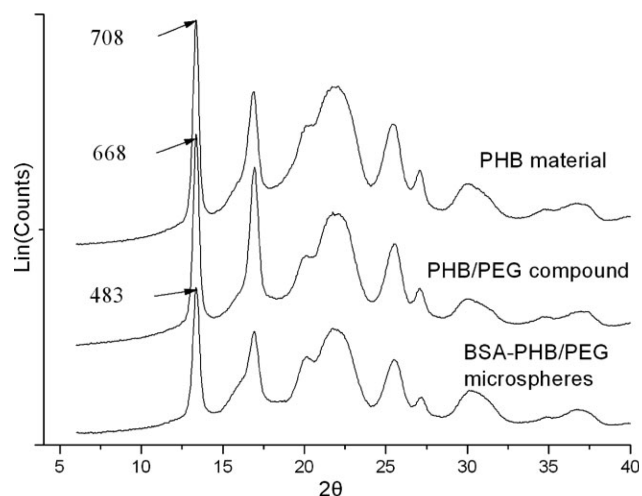


**Figure 2** XRD patterns of the raw material and microspheres.

### Entrapment of BSA

FTIR was used to explore the interactions between BSA and polymers matrix of the microspheres. Figure 3 showed infrared spectra of pure PHB, PHB/PEG compound, BSA, and BSA-PHB/PEG microspheres. Pure PHB illustrated a carbonyl group at 1718 $cm^{-1}$ and an aether group at 1276 $cm^{-1}$. The aether group is also the characteristic peak of PEG. As a result, PHB/PEG compound showed almost the same peaks as PHB. The FTIR spectrum of BSA had an amide carbonyl group at 1658 $cm^{-1}$ and a carboxyl group at 1540 $cm^{-1}$. From the FTIR spectrum of the BSA-loaded microsphere, it was observed that there were no significant changes in these bands. So it was thought that PHB/PEG microspheres could envelop BSA for a certainty, and strong chemical interactions between BSA and

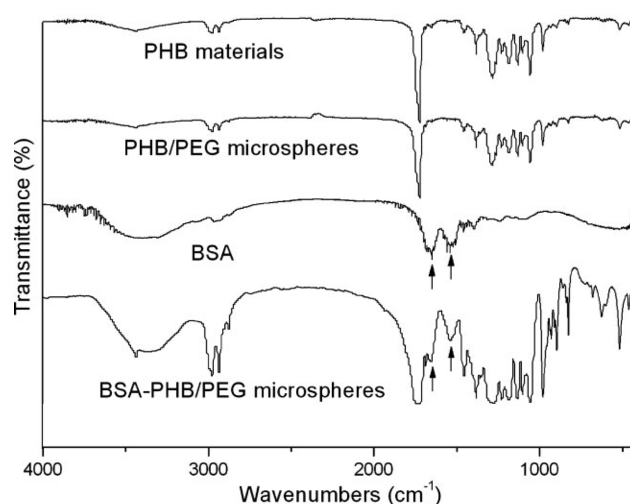


**Figure 3** FT-IR spectra of the raw material and microspheres.

TABLE II
Effect of m(PHB) : m(PEG) on the Properties of Microspheres

| Sample | m(PHB) : m(PEG) | Yield of microspheres (%) | Protein loading (%) | Particle diameter (μm) |
|---|---|---|---|---|
| A | 1/4 | 4.7 | 10.0 | 2.9 |
| B | 2/3 | 15.7 | 11.3 | 3.0 |
| C | 3/2 | 21.0 | 12.3 | 3.7 |
| D | 4/1 | 36.1 | 12.2 | 4.5 |
| E | 5/0 | 64.2 | 11.9 | 5.0 |

polymers were absent which attested the chemical stability of BSA in the PHB/PEG microspheres.

Five kinds of microspheres with different mass ratio of PHB to PEG were prepared, which had the average particle diameter from 2.9 to 5.0 μm. The yield and protein loading of the BSA-PHB/PEG microspheres were showed in Table II, and every sample thereinto was tested for three times. From the Table, yield of microspheres enhanced clearly with the increase of PHB. However, the protein loading had no distinct changes. Taking both the yield and protein loading of the BSA-PHB/PEG microspheres into account, the optimum mass ratio was fixed on m(PEG) : m(PHB) = 1/4.

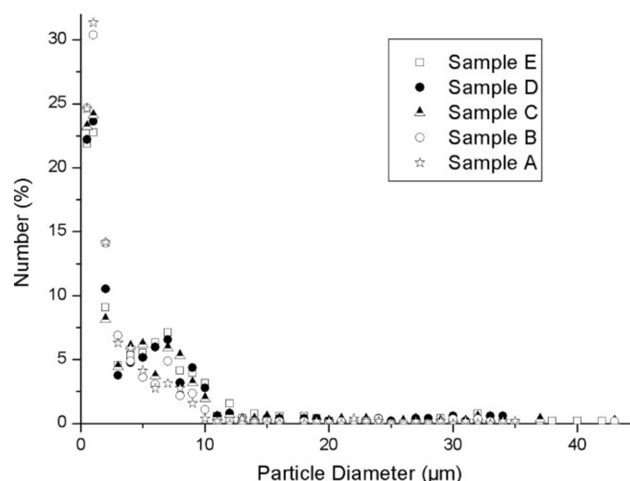


**Figure 4** Effect of PEG proportion on particle diameter distribution of microspheres.

### Structure of BSA-PHB/PEG microspheres

The particle diameter distribution of the five kinds of microspheres prepared were showed in Figure 4. Although the curve trends were nearly the same, it was found that the mass ratio of PHB to PEG had direct effect on the size distribution. There were more small mocropsheres and less big microspheres

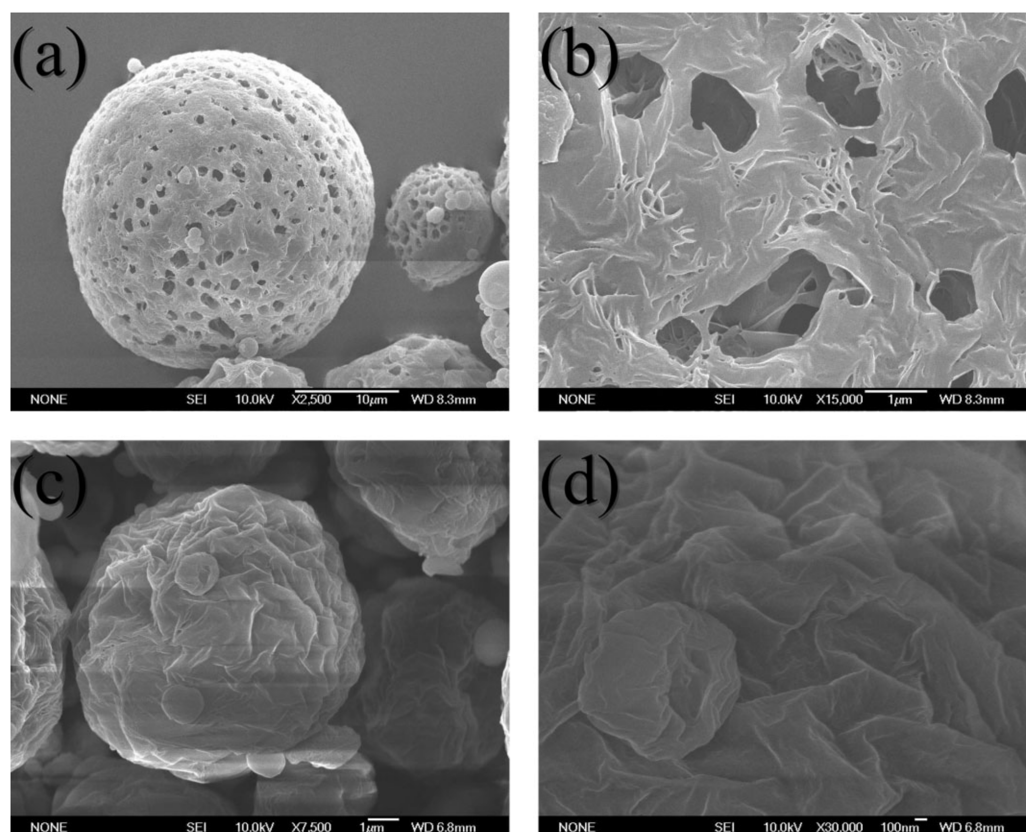


**Figure 5** SEM micrographs of the whole and partial external morphologies of microspheres, (a) and (b) belong to sample D, (c) and (d) belong to sample E.

when the PEG proportion was enhanced, which accorded with the result of average particle diameter (Table II).

SEM micrographs of sample D and E were showed in Figure 5. It was found that participation of PEG had important influence on the surface morphologies and microstructure of microspheres. Microspheres prepared using pure PHB as the matrix (sample E) had a rough and wrinkled surface, which could be explained from the crystallization rate in the transformation process of microdroplets into microspheres that introduced microstructural phase differences in the different microdroplets wall. The rough surface morphologies of the polymeric microspheres had been considered to be basically as a result of the high crystallinity of PHB and its different crystallization degree in different orientations. Although the PHB/PEG microspheres (sample D) had a porous surface. A possible reason was that PEG in the wall polymer composition of microspheres was eroded and dissolved in the process of microspheres formation, which would have a pores-making effect when the ratio of PEG was proper. However, the erosion and dissolving effect were so strong that it was impossible to form integrated microspheres when the ratio of PEG was excessively high (Fig. 6, left).

At the same time, it was found that the PHB/PEG microspheres were likely to have a structure of microspheres-embeded. When the ratio of PEG was too high (Fig. 6, left) to form integrated microspheres, some smaller microspheres leaked out from the cracked microspheres. It was also observed from the porous surface that there were smaller microspheres in the relatively big microspheres (Fig. 6, right). Hence, the smaller microspheres in the SEM micrographs were not only shaped directly out of raw materials but also produced by the cracked microspheres, which explained the result of particle diameter distribution too.

The transformation of the second emulsion microdroplets into the final microspheres was observed in this study. We had recorded the whole combination process of the second emulsion microdroplets several times, and Figure 7 was one of the OM observation records. This track record indicated that the second emulsion microdroplets might not only entrap several the first emulsion microdroplets, but also have the probability to incorporate more than one of the second emulsion microdroplets. So the microstructure of BSA-PHB/PEG microsphere was not the core-wall type, but mostly likely to be complicated microspheres-embeded structure with multi-layers.

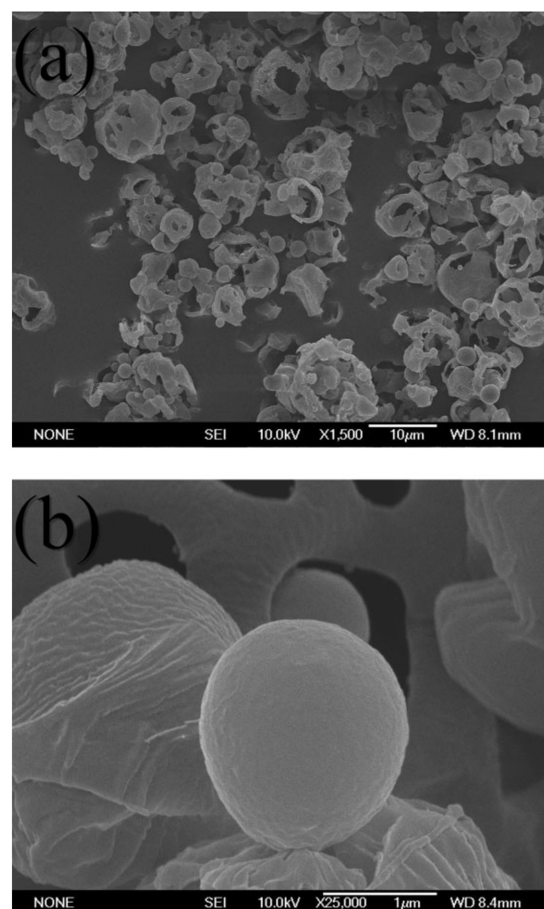


**Figure 6** SEM micrographs of microspheres with different microstructures, left: cracked microspheres; right: microspheres-embeded structure.

### In vitro release studies

Contrast of the release conducts for BSA between PHB microspheres (sample E) and PHB/PEG microspheres (sample D) was studied in this work (Fig. 8). It is obvious that the slope of the release curves is increased when PEG was introduced into the matrix of the microspheres. PHB microspheres had a relatively slow release rate, the release curve almost leveled off after 10 days and the accumulative BSA released finally was only close to 50%. Although the PHB/PEG microspheres had a much quick release rate. About 60% of BSA was released from the microspheres at the first 7 days, and the accumulative release amount of BSA reached 70% around after 29 days. It indicates that the BSA-PHB/PEG microspheres had a quicker release rate and a higher accumulative release amount than BSA-PHB microspheres.

There are two factors that work on the release of drugs entrapped in the microspheres, one is diffusion of the drug, the other is degradation of the microsphere's matrix. Both of them are highly correlative with the structure of the microspheres. PHB, as a biodegradable polymer, is generally degraded very slowly in vitro.[14,15] Furthermore, PHB microspheres are likely to have a dense core and massive wall because of its good crystallization,

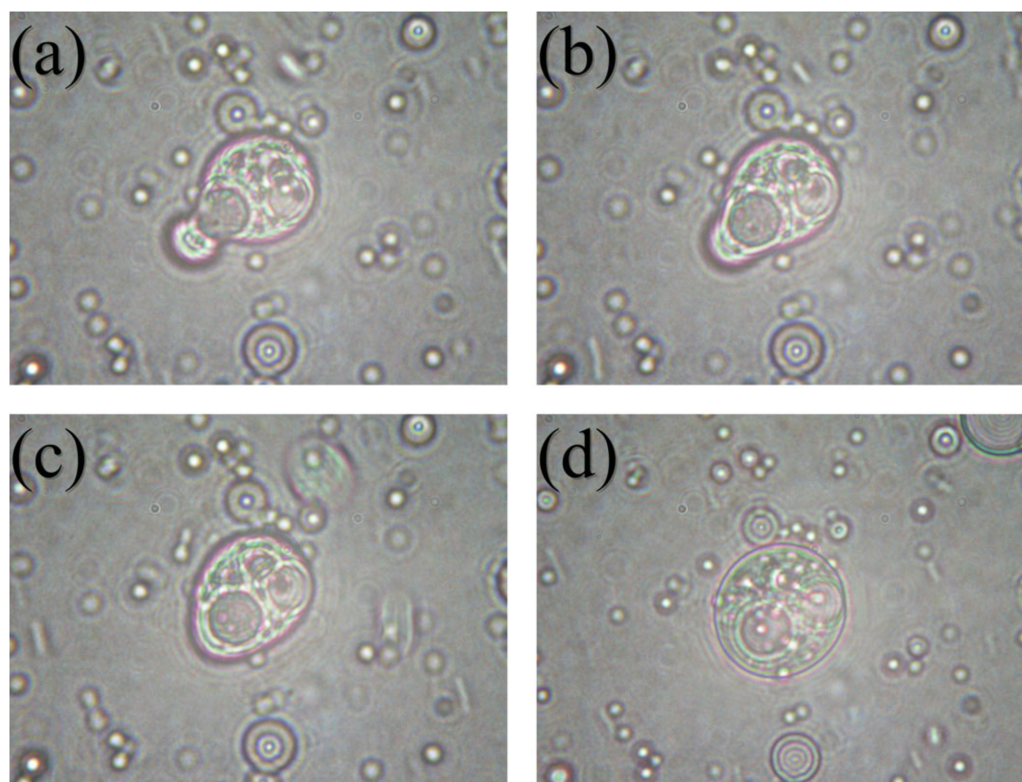


**Figure 7** OM pictures of an integrated combination process of the second emulsion microdroplets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

whose architecture is disadvantageous to encapsulate drugs and difficult for drugs entrapped to release out. Consequently, it is presumed that the good release characteristic of PHB/PEG microspheres in vitro owed to its porous surface and special microspheres-embeded structure, which highly enhanced the surface-to-volume ratio and degradability of microspheres. Further, in vitro and in vivo studies are needed to fully elucidate the dissolution and biodegradability of microspheres.

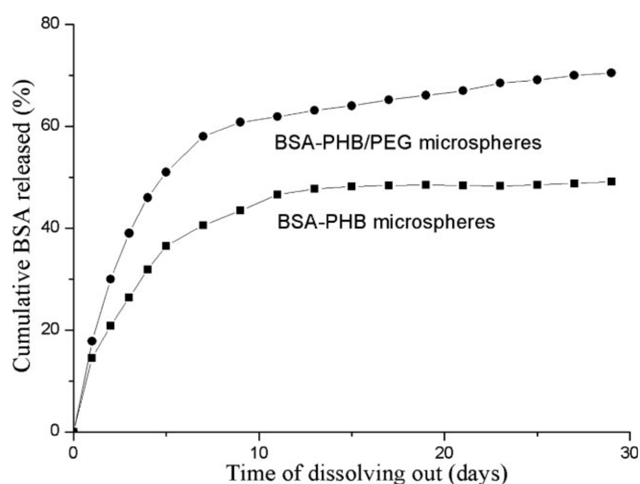


**Figure 8** Release profiles for BSA from PHB and PHB/PEG microspheres.

## CONCLUSIONS

BSA-PHB/PEG microspheres were prepared successfully via double emulsion solvent evaporation. The crystallinity of PHB decreased with the increasing proportion of PEG in the PHB/PEG compounds, and BSA-PHB/PEG microspheres had still lower crystallinity than PHB/PEG compound and raw materials. The yield and protein loading of the microspheres reached 36.1% and 12.2%, respectively, at the optimum mass ratio, m(PHB) : m(PEG) = 4/1. FTIR results confirmed the existence of BSA in the microspheres and revealed the absence of chemical interaction between BSA and polymers. It was found that the mass ratio of PHB to PEG had direct effect on the size distribution, surface morphologies, and microstructure of microspheres. The mean particle size of microspheres ranged between 2.9 and 5.0 μm measured by optical microscopy, depending on the different proportion of PEG. SEM micrographs combined with OM pictures indicated that PHB/PEG microspheres were likely to have porous surface and a structure of microspheres embeded. The results of BSA release in vitro indicated that the BSA-PHB/PEG microspheres had a quicker release rate and a higher accumulative release amount than BSA-PHB microspheres, which showed the feasibility and superiority of BSA-PHB/PEG microspheres as controlled release devices.

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