Preparation of Biodegradable Polymer Microspheres Encapsulating Protein with Micron Sizes

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ABSTRACT: Biodegradable polymer poly-D,L-lactide–polyethylene glycol (PLA–PEG) was synthesized with stannous octoate ($SnOc_2$) as catalyst by a cationic ring-opening polymerization. The molecular weight of PLA–PEG is the highest at a content of 0.1% $SnOc_2$. The PLA–PEG microspheres carrying protein were prepared by a solvent evaporation composite emulsion technique with a narrow size range (1–2 μm). The sizes of PLA–PEG microspheres increased with the increase of the molecular weight of PLA–PEG. The PEG in PLA–PEG (10%) significantly improved the size control of the microspheres of the PLA family as a drug carrier matrix. Polyvinyl alcohol (PVA) aqueous solution was used as dispersion medium for microsphere preparation. The concentration of the PVA solution can affect the size of the PLA–PEG microspheres. The differential scanning calorimetry data showed that the PLA–PEG microspheres can efficiently encapsulate the protein and that the crystalline of the microspheres carrying protein was lower than that of the nonprotein-loading microspheres. The amount of protein carried in the PLA–PEG microspheres was related to the nature of the protein itself. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **66:** 583–590, 1997

Key words: PLA–PEG; microspheres; size; protein; encapsulation

INTRODUCTION

Biodegradable polymeric materials used as protein carriers have attracted much attention recently in the health care area because of the requirement of developing new efficient vaccines and therapy agents.[1–3] Conventional vaccines usually need several booster immunizations and are difficult to administer orally due to gastrointestinal destruction. These problems could be resolved by using a polymer matrix as the carrier of antigen protein[4,5] because of the protection of the polymer matrix to the protein entrapped in the carriers from the destruction of acid, base, and various proteases in the gastrointestinal system and because of a longer period of antigen release with the degradation of polymer matrix. In order to achieve an effective oral immunization, selecting some suitable biodegradable materials, preparing microspheres with suitable size, and encapsulating a certain amount of antigen in microspheres would be very important.[6,7] Zhu[8] and Deng et al.[9] previously reported a poly-D,L-lactide–polyethylene glycol (PLA–PEG) block copolymer. Because the copolymer was composed of both hydrophilic and hydrophobic segments, its degradation rate, protein loading, and microsphere forming would be easy to control. In this

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article, we report the preparation of the PLA–PEG microspheres with micron size and protein encapsulation of the PLA–PEG microspheres. This work aims to obtain a better vaccine microsphere carrier.

EXPERIMENTAL

Materials

D,L-lactic acid (85%) was produced by Chemical Factory of Hube University. Polyethylene glycol (PEG, M_w = 6,000) was purchased from Guangzhou Chemical Reagent Department and recrystallized with acetone and *n*-hexane before use. Human serum albumin (HSA) was manufactured by the Institute of Blood Transfusion, Chinese Academy of Medical Sciences. Outer membrane protein (OMP) was prepared by Chengdu Institute of Biological Products. Other reagents were commercially available.

Preparation of Block Copolymer

D,L-lactide was prepared according to the procedure of Gilding and Reed[10] with some modification of using ZnO and Sb_2O_3 as catalysts. After purification, the white products had a yield of 40%.

SPECTRUM DATA: IR (in KBr): 1764 cm^{-1} (C═O, lactone); ^{13}C-NMR (in $CDCl_3$): δ = 15. 6 ppm (CH_3), δ = 71.2 ppm (CH), δ = 178 ppm (COO).

The copolymerization of D,L-lactide and PEG was carried out according to the literature[8,9] procedure using stannous octoate as a catalyst. The purified copolymer has a yield of 90%.

SPECTRUM DATA: IR (in KBr): 1756 cm^{-1} (C═O, straight chain). ^{13}C-NMR (in $CDCl_3$): δ = 75.1 ppm (CH_2, in the middle of the polyether chain); δ = 64.5 ppm (CH_2, CH_2—CH_2—O—C(═O)-). ^{1}H-NMR (in $CDCl_2$): δ = 1.55 ppm (CH_3); δ = 3.6 ppm (CH_2); δ = 5.12 ppm (CH).

In the integral curve of ^{1}H-NMR spectrum of PLA–PEG copolymer, the ratio of the hydrogen number of each group was CH : CH_2 : CH_3 = 22 : 12 : 70.

Preparation of Copolymer PLA–PEG Microspheres

The copolymer was dissolved into 20 mL of organic solvent of dichloride methane or a mixture of dichloride methane and acetone (1 : 1). With stirring, the organic solution of PLA–PEG was added into a bottle with 15 ml of PVA (M_w = 10,000, 88% hydrolyzed) solution (4%). In the first 20 min, the speed of stirring was set at 800 rpm and hereafter at 250 rpm for 12 h. When the solvent was completely evaporated out, the resultant microspheres were washed with distilled water and separated by centrifuge (10,000 rpm, 10 min). Particle sizes were determined with a Shimadzu SALD-2009 laser diffraction particle size analyzer. Data is as follows: $\bar{X}$ = 2.257 μm; SD = 0.2812 μm; dispersion coefficient = 0.132. The morphology assay was carried out with an Amray scanning electron microscope.

Protein Encapsulation of the PLA–PEG Microspheres

70 mg of HSA was dissolved in 1 mL of distilled water. The solution of HSA was emulsified with 20 ml of organic solution of PLA–PEG (1 g : 20 ml) by supersonic oscillation (50 W, 10 min). The W : O emulsion of HSA : PLA–PEG was added dropwise into the aqueous PVA solution (4%) and formed into a composite emulsion of water : oil : water by stirring of 800 rpm for 20 min. The latter procedure was the same as that for microsphere preparation, in which $\bar{X}$ = 2.236 μm. OMP was encapsulated by the same method.

Determination of Contact Angle of Aqueous Solution on the Polyester Film

A photomicroscope (Microscope XSZ-5, Chongqing Optical Inst. Factory) with some modification of fitting a triprism on an object flat under the object lens. The light source, prism, and object lens were placed at a right angle; and the light can give sight to the eyepiece through the prism. A polyester film was placed in the light route near the prism. A droplet of aqueous solution was dropped on the film by using a syringe with a 3# needle. The length and the height of the droplet on the film was read from the eyepiece. The contact angle between the solution and the film could be estimated by the following equation:

$$t_g\theta = h : l \qquad (1)$$

where h is the height of the droplet, and l is half the length of the droplet.

The preparation of polyester film was as follows. The organic solution of PLA–PEG (20% w : v) was poured homogeneously on a glass plate with a glassy surface. After one day, in ambient temperature and pressure, the organic solvent had been evaporated, and a film of PLA–PEG with a thickness of 0.1 mm was obtained. The film was cut into pieces of 1×0.5 cm^2 for determination of contact angle.

Differential Scanning Calorimetry Analysis of the PLA–PEG Microspheres

6 mg of lyophilized microspheres was used as a sample for differential scanning calorimeter (DSC) analysis with a Perkin-Elmer 7 Series thermal analysis system. Heating rate was 10°C/min. A heat flow–temperature curve was plotted.

DATA: ΔH: PLA–PEG–MS 25.653 J g; HSA–PLA–PEG–MS 2.234 J g; OMP–PLA–PEG–MS 2.161 J g.

Quantity of Protein in Microspheres

One hundred milligrams of lyophilized protein-carrying PLA–PEG microspheres were dissolved in 10 ml of CH_2Cl_2. When the microspheres were completely dissolved, 10 ml of phosphate buffered salt (PBS; pH = 7.4, 1 : 15M) was added in the solution. The oil : water mixture was oscillated with a supersonic oscillator for 5 min and then statically placed for allowing the aqueous phase and oil phase separate. The oil phase was separated out and again mixed with 10 ml of PBS. The abstraction was repeated once. Two aqueous abstracts were combined together, and the amount of protein in the abstract was assaied by an ultraviolet spectrophotometer (Shimadzu UV-120) at 283 nm. A standard curve of protein solution was made out with the known content solutions of protein.

Table I The Effect of Catalyst on the Yield of D,L-lactide

Sb_2O_3 + ZnO (%)	D,L-lactic acid (g)	D,L-lactic (g)	Yield (%)
0.01	98	21	21.42
0.05	98	32	32.65
0.01	98	44	44.92
0.20	98	42	42.61
0.50	98	48	49.15

Temperature (highest) is 210°C.

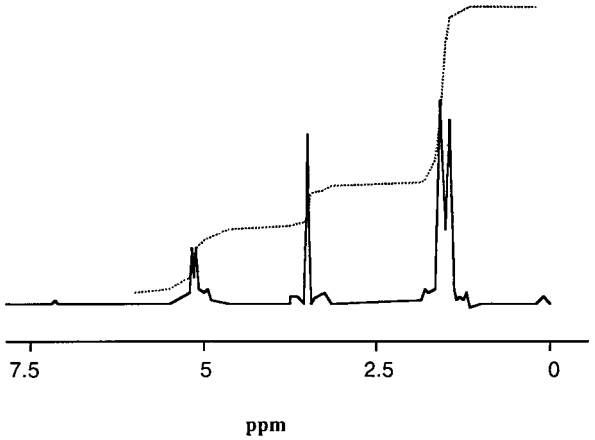


Figure 1 ^{1}H-NMR spectrum of PLA–PEG copolymer (No. 6).

RESULTS AND DISCUSSION

Synthesis of Copolymer

Because lactic acid cannot be directly polymerized into polymer with a higher molecular weight, the preparation of lactide was a necessary middle process. The copolymer PLA–PEG was synthesized with PEG as middle block, D,L-lactide as monomer, and $SnOc_2$ as catalyst by bulk copolymerization. The lactide and PLA–PEG were both perfectly characterized by infrared and nuclear magnetic resonance analysis. The yield of lactide increased with increasing the amount of catalyst in a certain range as Table I shows. The determination of molecular weight of PLA–PEG would be estimated by the integral height of hydrogen of each group in the integrating plot of ^{1}H-NMR spectrum (Fig. 1). In copolymer PLA–PEG,

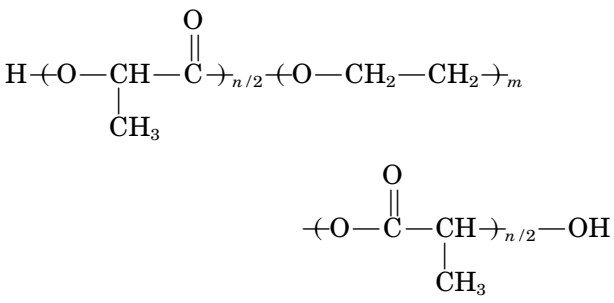


Table II The Effect of Catalyst on the Molecular Weight of PLA–PEG Copolymer

No.	Catalyst	Content (%)	Molecular Weight of PLA–PEG	Yield (%)
01	$SnCl_2$	0.01	7.1×10^3	85
02	$SnCl_2$	0.10	9.8×10^4	92
03	$SnCl_2$	1.00	6.3×10^4	96
04	$SnOc_2$	0.01	8.5×10^3	86
05	$SnOc_2$	0.10	8.8×10^4	88
06	$SnOc_2$	1.00	6.2×10^4	97

Reaction temperature is 190°C; pressure is -0.95×10^4 Pa.

Determined by ^{1}H-NMC spectra.

the ratio of integrating height of hydrogen of each group in ^{1}H-NMR spectrum should be h_1 (CH) : h_2 (CH_2) : h_3 (CH_3)$=n$: 4 m : 3 n.

Therefore, according to h_1, h_2, h_3, and the molecular weight of PEG, the amount of chain units of LA and the molecular weight of PLA–PEG could be estimated. The experimental results showed that the molecular weight of PLA–PEG was related with the amount of catalyst, 0.1% of which could obtain a polymer with a higher molecular weight, as Table II shows.

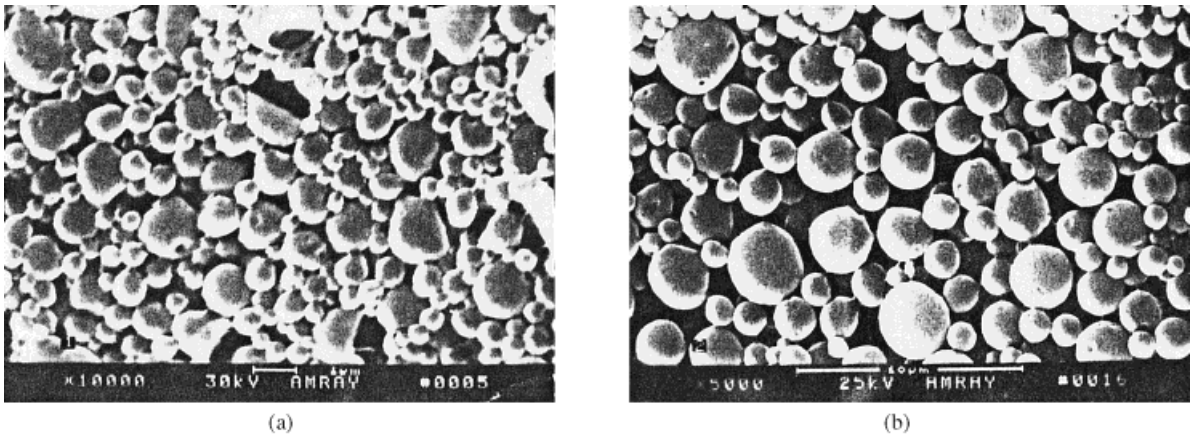
Preparation of PLA–PEG Microspheres

By the solvent evaporation method, the PLA–PEG microspheres with micron size could be prepared (Fig. 2). In our experiments, we found that the dispersion medium was a key factor for particle size control. Polyvinyl alcohol (PVA) with molecular weight of 5000–10,000 and 88% hydrolyzed was fit to microsphere preparation. If the molecular weight of PVA is higher or lower than this range and the hydrolyzation of PVA is higher or lower than 88%, the effect of dispersion of PVA would be poor, and the resultant microspheres would be difficult of purification. By determining the contact angle θ of PVA solution on hydrophobic film of polyester (Table III), it was found that the θ gradually decreased with an increase in the concentration of PVA in solution. This suggested that the compatibility of PVA solution and oil polyester is improved with the increase of the concentration of PVA in solution. Consequently, the interface tension between PVA solution and polyester organic solution was decreased, and the size of PLA–PEG microspheres decreased with the increase of the concentration of PVA in solution as dispersion medium. Figure 3 plots the relationship between the concentration of PVA in dispersion medium solution and the sizes of the resultant PLA–PEG microspheres.

Table III The Contact Angle of PVA Solution on the Film of Polyester

Content of PVA (mg mL)	Contact Angle (θ)
20	81.8
40	71.4
70	65.9
90	62.4

20°C; 0.1 MPa.



(a) (b)

Figure 2 Scanning electron micrograph of PLA–PEG microspheres with an average size of (a) 0.9 and (b) 2.3 μm.

In addition, because of no charge or ionicity, the PVA would not lead to a denaturation of protein and protect the activity of antigen in the system of microsphere preparation.

The polymer used as microsphere matrix has a significant effect on the size of microspheres. By comparing the PLA homopolymer microspheres and the PLA–PEG copolymer microspheres prepared at the same procedure conditions (stirring speed, solvent, concentration of dispersion medium, etc.), it was found that PLA–PEG microspheres could reach a quite small size of 0.1 μm with a narrow size distribution; but the PLA microspheres could only reach a size of 1.4 μm with a wide size distribution (Fig. 4). The molecular weight of the polymer is also a factor influencing the size of microspheres. As Table IV shows, the size of the PLA–PEG microspheres would be increased by increasing the molecular weight of PLA–PEG. Besides, using mix solvent of CH_2Cl_2 and acetone was more advantageous to prepare microspheres with sizes < 2 μm than using single CH_2Cl_2 because the acetone was able to improve the dissolubility of the organic solution of PLA in aqueous dispersion medium and reduce the interface tension between the oil and water phases. In our work, preparation of microspheres with sizes < 10 μm only need a general electromagnetic stirrer at stirring speed < 1000 rpm. In consideration of the toxic problem, the residual organic solvent should be removed out as completely as possible. The dispersion of microsphere preparation should be stirred for at least 12 h,[2] even though CH_2Cl_2 and acetone are both extremely volatile solvents.

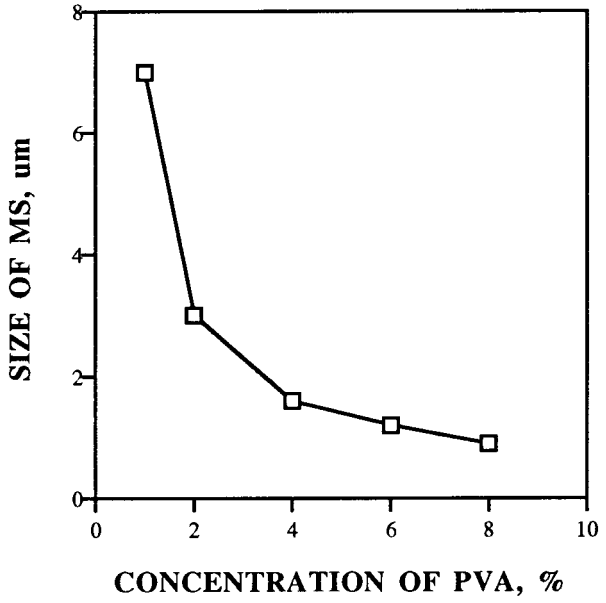


Figure 3 The plot of the size of PLA–PEG microspheres produced at different concentrations of dispersion medium PVA solution.

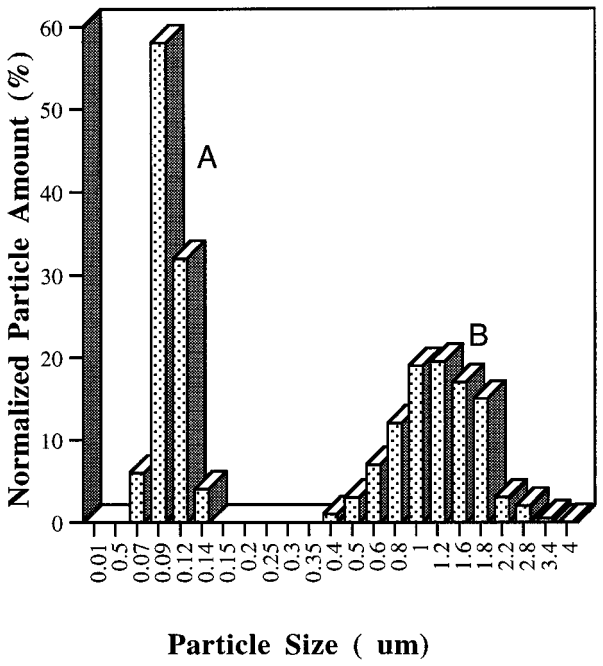


Figure 4 Particle size distribution of (A) PLA–PEG microspheres with an weight-average size of 0.1 μm and (B) PLA microspheres with an weight-average size of 1.4 μm determined with a Shimadzu SALD-2009 laser diffraction particle size analyzer. Procedure conditions are as follows. Emulsion stirring speed = 1000 rpm; concentration of PVA solution = 8%; solvent = a mixture of CH_2Cl_2 and acetone (1 : 1).

Encapsulation of Protein in PLA–PEG Microspheres

By the water : oil : water composite emulsion technique, protein can be carried in the PLA–PEG microspheres. DSC data showed that the melting point T_m and the fusion heat ΔH of protein-carrying PLA–PEG microspheres were both depressed compared to nonprotein-carrying PLA–PEG microspheres. Especially, the heat of fusion of protein-carrying microspheres was depressed by 90%. In the protein-carrying PLA–PEG microspheres, the protein could be considered as a diluent in a heterogeneous polymer diluent system. According to Flory–Huggins expression for the free energy of mixing,

$$\frac{1}{T} - \frac{1}{T_m^0} = \frac{R}{\Delta H}\frac{V_2}{V_1}(v_1 - \chi v_1^2) \qquad (2)$$

Table IV The Effect of Molecular Weight of the Copolymer PLA–PEG on the Size of the PLA–PEG Microspheres

Polymer Batch	Molecular Weight	Volume-average Size ($\bar{X}$, μm)	δ	$\delta/\bar{X}$
01	7.1×10^3	3.20	1.148	0.359
04	8.5×10^3	4.63	1.249	0.270
06	6.21×10^4	3.21	0.807	0.251
03	6.30×10^4	5.99	1.839	0.308
05	8.8×10^4	5.47	1.312	0.240
02	9.7×10^4	7.85	2.396	0.305

Dispersion medium is PVA solution (4%); stirring speed is 800 rpm; ratio of W to O is 2 : 1; solvent is CH_2Cl_2.

where T and T_m^0 are the melting points of the polymer-protein mixture and the pure polymer, respectively; V is molar volume; 1 and 2 are the polymeric matrix and protein encapsulated, respectively; v is the volume fraction; and χ is the polymer–protein interaction parameter. So the difference of the melting points and the fusion heat between PLA–PEG microsphere and protein-carrying PLA–PEG microspheres suggested a difference of composite and structure between the two microspheres. Moreover, the more endothermic peaks of the protein-carrying PLA–PEG microspheres in the DSC plots (Fig. 5) also represented a heterogeneous system of protein-carrying microspheres; consequently, PLA–PEG microspheres indeed efficiently encapsulated protein.

By ultraviolet spectrum analysis, the quantity of protein in an aqueous abstracted sample of protein-carrying PLA–PEG microspheres solved in organic solvent (Figs. 6 and 7) showed that the encapsulation effect of HSA and OMP in PLA–PEG microspheres is different. The amount of OMP in PLA–PEG microspheres is lower than 1%, and the encapsulation efficiency of it is lower than 10%. However, the amount of HSA in PLA–PEG microspheres is near 5%, and the encapsulation efficiency of it is over 50%. The reason resulting in this difference was due mainly to the natures of the proteins themselves. The OMP used in this work was actually a mixture of OMP and some lipidic scraps of cell membrane, which were used together as a vaccine. The HSA was a purier protein, and its hydrophility and compatibility with organic phase were better than OMP, which could be determined from the comparison between

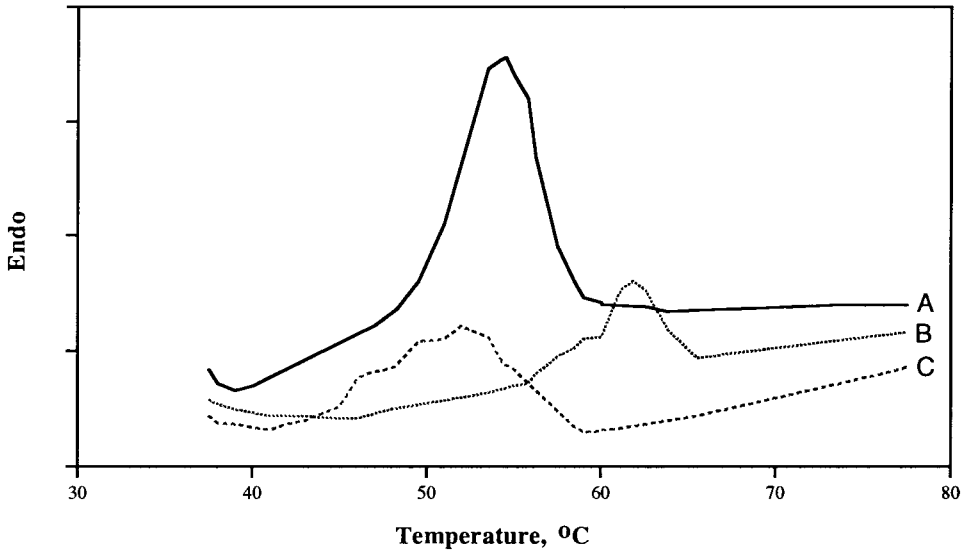


Figure 5 DSC plots of (A) PLA–PEG microspheres, (B) HSA-loading PLA–PEG microspheres, and (C) OMP-loading PLA–PEG microspheres.

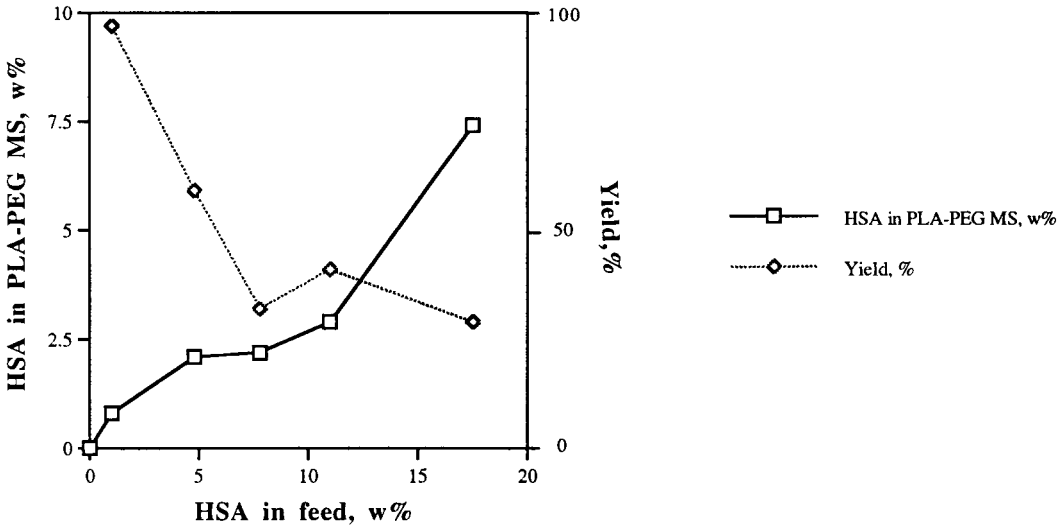


Figure 6 Content and encapsulation yield of HSA in PLA–PEG microspheres.

the contact angles of the two protein solutions with polyester film (Table V). In conclusion, biodegradable copolymer PLA–PEG can be prepared by bulk polymerization with $SnOc_2$ as catalyst. The molecular weight of PLA–PEG was controlled by the amount of catalyst. By the O : W emulsion solvent evaporation technique, and with PVA solution as dispersion medium, PLA–PEG microspheres with micron size can be prepared out. The size of PLA–PEG microspheres can be controlled by modifying the concentration of PVA solution, the class of polymers, the molecular weight of PLA–PEG, and the solvent. The PLA–PEG copolymer is more advantageous to microsphere preparation than PLA homopolymer. The PLA–PEG microspheres can efficiently encapsulate protein, and the amount of protein encapsulated in the PLA–PEG microspheres increased by increasing the amount of protein in the feed; but the yield of encapsulation would be decreased. The natures of the protein affects the encapsulation. Because of encapsulation of protein, the bulk of the microspheres became a heterogeneous mixture system in which the protein was mixed as a diluent.

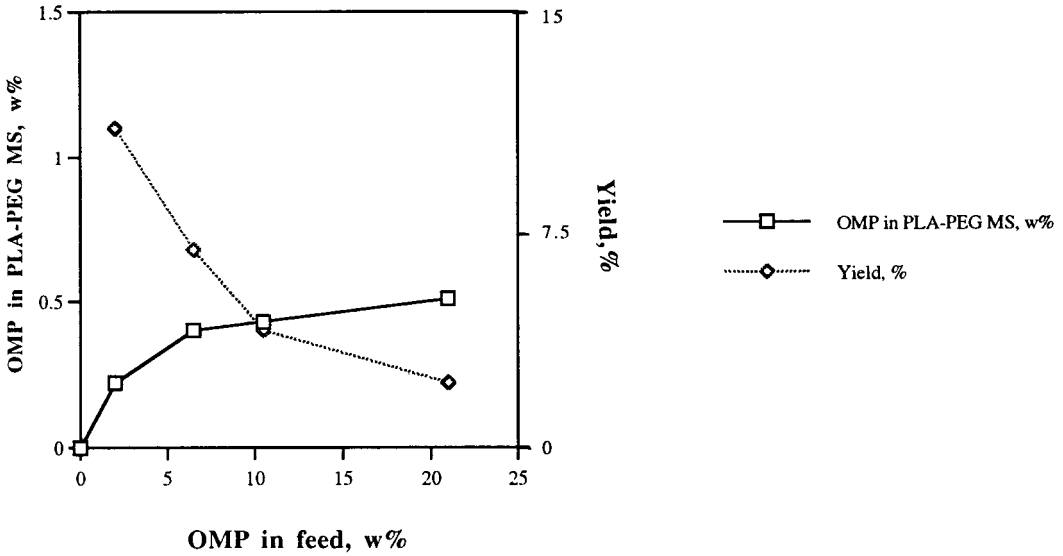


Figure 7 Content and encapsulation yield of OMP in PLA–PEG microspheres.

Table V The Contact Angle θ of Protein Solution on Polyester Film

	Concentration of Protein (mg mL)	Contact Angle (θ; in degrees)
HSA	2.0	50.4
HSA	4.8	47.2
HSA	7.7	47.4
HSA	9.2	43.2
OMP	2.0	57.8
OMP	4.0	60.0
OMP	6.6	61.2
OMP	10.0	64.2

20°C; 0.1 MPa.

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