Effect of Polymer Compositions on the Fabrication of Poly(*ortho*-ester) Microspheres for Controlled Release of Protein

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ABSTRACT: Eight kinds of self-catalyzed poly(*ortho*-esters) (POEs) are used to fabricate bovine serum albumin (BSA)-containing microspheres using a W/O/W double-emulsion solvent extraction/evaporation method. All eight kinds of POE polymers used in this study are shown able to form microspheres under proposed fabrication conditions. The surface morphology and inner structure of the microspheres are analyzed using scanning electron microscopy (SEM). The microspheres have a size range from 64.7 to 120.2 μm. POE with a higher viscosity leads to bigger microspheres. It was found that the POE composition has a significant effect on BSA release profiles. POEs, which are more hydrophilic and contain a greater amount of glycolide or lactate (latent acid), yield higher BSA release rates. Specifically, POE containing 1,6-hexanediol diglycolide (HD-diGL) microspheres have the highest BSA release rate after a 20-day test through a combination of surface erosion and diffusion mechanisms. POE containing a high percentage of the *trans*-cyclohexanedimethanol (CDM) segment tends to yield microspheres with a lower release rate because of its hydrophobic nature. It was also found that the BSA release rate is more rapid at 37°C than at 22°C because of faster polymer degradation and water penetration at 37°C. Experimental results suggest that various protein release rates can be achieved by using different compositions of POEs. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 80: 1630–1642, 2001

Key words: poly(*ortho*-ester)s; surface erosion; microspheres; release profile; bovine serum albumin

INTRODUCTION

Poly(*ortho*-ester)s (POEs) have received extensive attention in the controlled-release community because of their unique characteristics of surface-erosion mechanisms. POEs were first described in a series of patents assigned to the Alza Corp.[1–4] There are three distinct families of POEs. The most promising family of liner or cross-linked POEs was prepared by the addition of polyols to diketene acetal 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undercane.[5] POEs have been studied

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for the delivery of various active compounds, such as pyrimethamin,[6] 5-fluorouracil,[7–9] naltrexone,[10] contraceptive steroids,[11,12] prostaglandin,[13] and insulin.[14] Since the POE molecule contains pH-sensitive linkages, its hydrolysis rate can be increased by acidic excipients such as suberic acid or retarded by basic excipients such as magnesium hydroxide. However, diffusion of the excipients from the polymer can cause drug-release kinetics complications and leads to an excipient-depleted residual polymer in the tissue. Ng et al. developed a better approach to overcome this deficiency by incorporating a short segment of a latent acid such as a glycolic acid dimer into the POE backbone.[15] The acid produced after hydrolysis of the segment can catalyze the hydrolysis of *ortho*-ester linkages. Viscous and ointmentlike POEs have also been developed for drug delivery.[16–18] These POEs, having molecular weights of more than 30,000, are viscous at room temperature. Active agents can be easily incorporated into the polymer by physical mixing without the presence of toxic solvents and the polymer matrix containing therapeutic drugs can be injected directly. Zignani et al.[19] reviewed the synthesis and properties of various families of POEs and their usage in controlled drug release. Most of the previous work was focused on POE implant devices such as discs, rods, and tablets. However, in this study, attention was given to identify key fabrication parameters in order to maximize the potential of POEs for protein delivery. We used bovine serum albumin (BSA) as a model protein and various self-catalyzed POEs as the matrix to study the encapsulation process and release mechanism of BSA-containing POE microspheres. The POEs used here contain latent acids such as mono/diglycolide and monolactate. The effect of the POE compositions and the *in vitro* temperature on the BSA release profile was investigated. This work is essential to design POE microspheres for effective protein delivery.

EXPERIMENTAL

Materials

POEs were synthesized at Advanced Polymer Systems Inc. (Redwood City, CA). Poly(vinyl alcohol) (PVA; 87–89 mol % hydrolyzed, MW 31,000–50,000), D-trehalose dihydrate, and BSA (fraction V, 58 kDa) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dichloromethane (DCM), ethyl acetate, and tetrahydrofuran (THF) of HPLC grade were obtained from Merck (Germany) and used as received. Table I shows the composition and chemical structure of POEs used in this study.

Polymer Characterization

Molecular Weight Analysis

The average molecular weights of as-received polymer materials and microspheres were determined by a gel permeation chromatograph (GPC) (Waters 2690, USA). The raw polymers and microspheres were dissolved in tetrahydrofuran (THF) at a concentration of 0.2 % (w/v) and the solution was then filtered. A volume of a 100-μL sample was injected into the GPC and the eluting peaks were detected with a differential refractometer detector (Waters 410). The average molecular weights were calculated using polystyrene as the reference (Polymer Laboratories Inc., USA). The mobile phase used was THF at a flow rate of 1 mL/min.

Glass Transition Temperature (T_g)

The glass transition temperature (T_g) of POE was determined using a Perkin–Elmer 7-Series differential scanning calorimetry (DSC; Model DSC 4, Perkin–Elmer, USA) with 2–5 mg of the polymer. The temperature of the DSC was calibrated with an indium standard. The sample was placed in an aluminum pan and scanned from 20 to 200°C with a heating rate of 20°C/min in a nitrogen atmosphere.

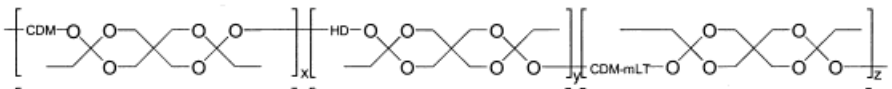
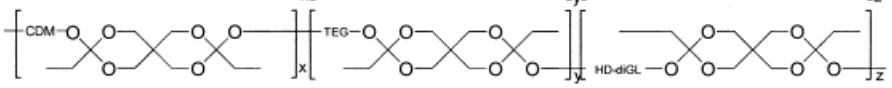
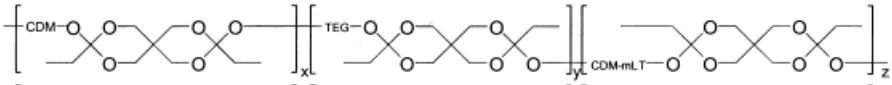
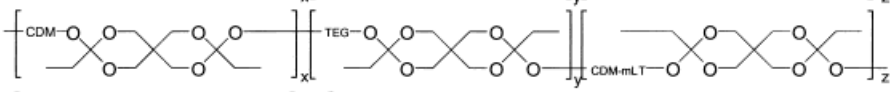
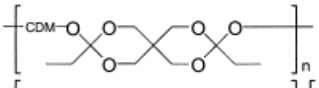
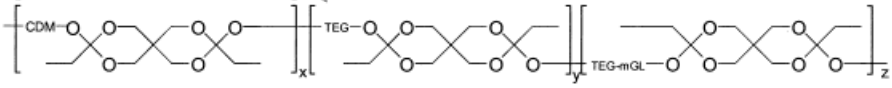
Apparent Viscosity Measurement

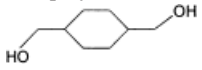
The apparent viscosity of the polymer solution (the oil phase: POE in DCM) used for microsphere fabrication was measured using an Ostwald viscometer at room temperature. The Ostwald viscometer is a U-shaped glass tube containing a capillary tube that allows liquid to flow slowly from one end to the other. The time taken for the polymer solution to flow in the Ostwald viscometer was compared to the time for DCM alone. The concentration of the polymer solution was 5% w/v. The apparent viscosity is expressed in centipoise (cP).

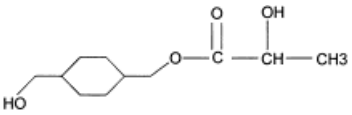
Preparation of Microspheres

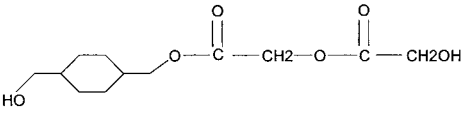
Eight kinds of POE polymers (POE1–POE8) with different compositions were used to encapsulate BSA within the microspheres. The PVA concen-

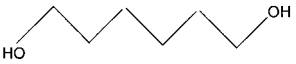
Table I Chemical Structure of POES

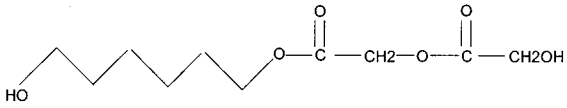
Polymer ID	Monomer Composition	T_g[a] (°C)	Chemical Structure[b,c]
POE1	CDM/HD/CDM-mLT = 65/34/1	ND	
POE2	CDM/TEG/HD-diGL = 75/20/5	ND	
POE3	CDM/TEG/CDM-mLT = 75/20/5	76	
POE4	CDM/TEG/CDM-mLT = 89/10/1	57	
POE5	CDM/CDM-diGL = 75/25	ND	
POE6	CDM/CDM-mLT = 90/10	54	
POE7	CDM (100%)	81	
POE8	CDM/TEG/TEG-mGL = 94/5/1	102	

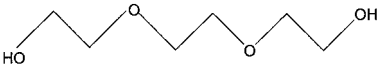
[a]ND: not detectable in the temperature range of tests.
[b]x, y, z: the ratio of each monomer in the polymer backbone.
[c]CDM: *trans*-cyclohexanedimethanol: 

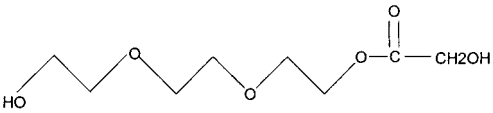
CDM-mLT: cyclohexanedimethanol monolacate: 

CDM-diGL: cyclohexanedimethanol diglycolide: 

HD: 1,6-hexanediol: 

HD-diGL: 1,6-hexanediol diglycolide: 

TEG: tri(ethylene glycol): 

TEG-mGL: tri(ethylene glycol) monoglycolide: 

tration in the outer water phase was optimized to fabricate POE microspheres. Microspheres were prepared using a double-emulsion (water-in-oil-in-water) solvent extraction/evaporation method[20] with slight modifications: Briefly, a BSA aqueous solution (the internal water phase, 20%

w/w) containing D-trehalose dihydrate (BSA:D-trehalose dihydrate = 4:1) was emulsified with a polymer solution (5 mg/mL in DCM) by sonication at 50 Hz for 10 s to form a primary emulsion. The addition of D-trehalose dihydrate is to protect BSA from degradation caused by DCM.[21] The resultant emulsion was then injected into a 250 mL aqueous solution (the outer water phase: PBS, pH 7.4, containing a specific percentage of PVA as a stabilizer) with continuous stirring at 300 rpm by a mixer (Colo-Parmer Instrument Co., Vernon Hills, IL) for 35 min. A 640 mL PBS buffer solution (pH 7.4, containing the same concentration of PVA as in the outer water phase) was then added at a constant rate with simultaneous stirring for 4 h at ambient temperature (22°C) to remove the solvent. The resultant microspheres were filtered and washed three times with PBS (pH 7.4) and vacuum-dried overnight. The final product was stored in a dessicator at 4°C.

Microsphere Characterization

Size Distribution

The sizes of the microspheres were analyzed by a laser light-scattering particle-size analyzer (Coulter LS 230, microvolume module, Coulter Corp., USA). Twenty milligrams of the micropsheres well dispersed in 1 mL of distilled water were used for the measurement. Experiments were conducted at room temperature in triplicate. Average particle size is expressed as the volume mean diameter.

Bulk Density

A known weight of microspheres was transferred to a 10-mL glass graduated cylinder and the initial volume was recorded. The cylinder was then tapped using an Autotap (Quantachrome Corp., USA) for hundreds of times until the volume of the microspheres remained constant. The bulk density was obtained by calculating the ratio of the weight to the volume in g/cc.

Morphology Analysis

The shape, surface, and cross-section morphology of the microspheres were examined using a scanning electron microscope (SEM; Model JSM-5310, JEOL Japan). Cross sections of the microspheres were obtained by embedding certain amounts of microspheres (several milligrams) into a Lipshaw M1 embedding matrix (Shandon Lipshaw Inc, Pittsburgh, PA) under liquid nitrogen. The samples were then sectioned using a cryostat (Leica CM 3050, Leica Instrument Gmbh, Nussloch, Germany). Then, the microspheres or sectioned samples were mounted on aluminum holders and sputter-coated with gold in argon prior to the SEM examination.

Determination of BSA Content in the Microspheres: Encapsulation Efficiency Tests

The BSA content in the microspheres was determined by an extraction method. The BSA-containing microspheres (10 mg) were dissolved in 1 mL ethyl acetate and kept at room temperature for about 15 min until complete dissolution. PBS (10 mL, pH 7.4) was then added and the mixture was shaken vigorously for 2 min and kept at room temperature for 1 h. The bottom layer was taken out and filtered. The BSA content in the filtered solution was analyzed using HPLC. The encapsulation efficiency of BSA was calculated as the ratio of actual to theoretical BSA content.

In Vitro *BSA Release Study*

Thirty milligrams of vacuum-dried microspheres were dispersed in 1 mL PBS (pH 7.4). *In vitro* release tests were carried out in triplicate with continuous shaking at 37 and 22°C. The supernatant was periodically collected and replaced with fresh PBS (pH 7.4) buffer at each sampling point. The protein content in the supernatant was analyzed using HPLC. The percentage of the BSA cumulative release (w/w %) was investigated as a function of the incubation time.

RESULTS AND DISCUSSION

Polymer Characteristics

Table II shows the molecular weights and glass transition temperatures (T_g) of POEs with various compositions and their corresponding apparent viscosity. These POEs have molecular weights in the range of 18k–46k and glass transition temperatures in the range of 54–102°C. POE1, 2, and 5 do not show a visible T_g in the temperature testing range. Heller et al.[11,22] suggested that the mechanical properties of POE polymers can be controlled by an appropriate choice of the diols used in the condensation reaction. In short, an increase in the 1,6-hexanediol percentage or chain length of the diols results in a decrease in

Table II Effect of Polymer Compositions on Characteristics of Microspheres

Polymer ID	Polymer Composition	Molecular Weight (Mw)	T_g[a] (°C)	Apparent Viscosity (cP)	Microspheres Yield (%)	Mean Diamter (μm)	Bulk Density (g/cm^3)	Encapsulation Efficiency (%)
POE1	CDM/HD/CDM-mLT = 65/34/1	36k	ND	0.14	62.1	87.2	0.46	16.5
POE2	CDM/TEG/HD-diGL = 75/20/5	18k	ND	0.14	33.2	64.7	0.39	26.5
POE3	CDM/TEG/CDM-mLT = 75/20/5	32k	76	0.43	61.7	115.8	0.44	38.3
POE4	CDM/TEG/CDM-mLT = 89/10/1	37k	57	0.41	51.4	120.2	0.44	23.2
POE5	CDM/CDM-diGL = 75/25	22k	ND	—	35.8	68.0	0.48	29.0
POE6	CDM/CDM-mLT = 90/10	31k	54	0.26	42.7	108.6	0.43	21.5
POE7	CDM (100%)	40k	81	0.16	34.7	89.0	0.40	19.7
POE8	CDM/TEG/TEG-mGL = 94/5/1	46k	102	0.15	53.6	85.7	0.42	31.7

ND: not detectable in the temperature range of tests.

the glass transition temperature. The higher the polymer T_g the more rigid to the polymer chains. Highly rigid POE chains may create more difficulties both for BSA to diffuse out and for water to penetrate through the microspheres. The apparent viscosity of the polymer solutions (in the oil phase) is in the range from 0.14 to 0.43 cP (Table II). POE3 and POE4 solutions have relatively higher apparent viscosity values compared with those of POEs 1, 2, and 6–8.

Formation of Microspheres

Various operational conditions were investigated to yield spherical POE microspheres. For instance, when the PVA concentration in the outer water phase is decreased to 0.01% (w/v), spherical POE particles cannot be produced (Fig. 1). This arises from the fact that a low PVA concentration cannot yield a stable double emulsion (w/o/w). However, a high PVA concentration such as 0.2% w/v leads to the formation of POE particles with a spherical shape and smooth surface as illustrated in Figure 2.

Characterization of Microspheres

Yield, Size, Density, and Encapsulation Efficiency

Table II lists the yield, size, density, and encapsulation efficiency of various POE microspheres. The yields range from 33 to 62%. The encapsulation efficiency of BSA is between 16.5 and 38.3%. The mean diameters of the microspheres are in the range of 64.7–120.2 μm. Figure 3 illustrates the size distribution of the microspheres.

In this study, all POE microspheres were fabricated with the same conditions in order to specifically understand the effect of POE composition on the microsphere formation and the release profile. Therefore, the apparent viscosity of each polymer solution is a critical parameter to influ-

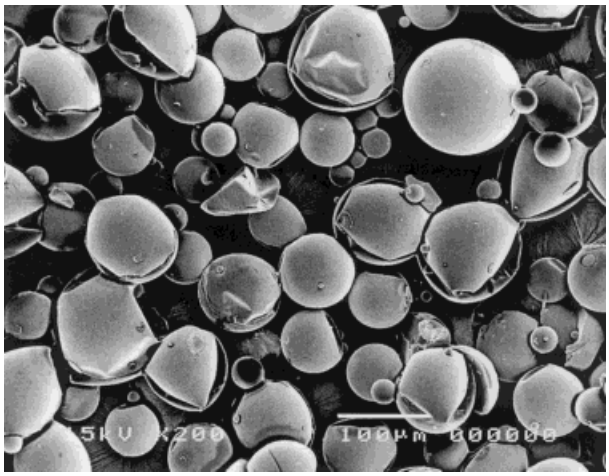


Figure 1 SEM image of POE8 microparticles prepared using POE8 (50 mg/mL in DCM) and 0.01 w/v % PVA stabilizer concentration (bar size = 100 μm).

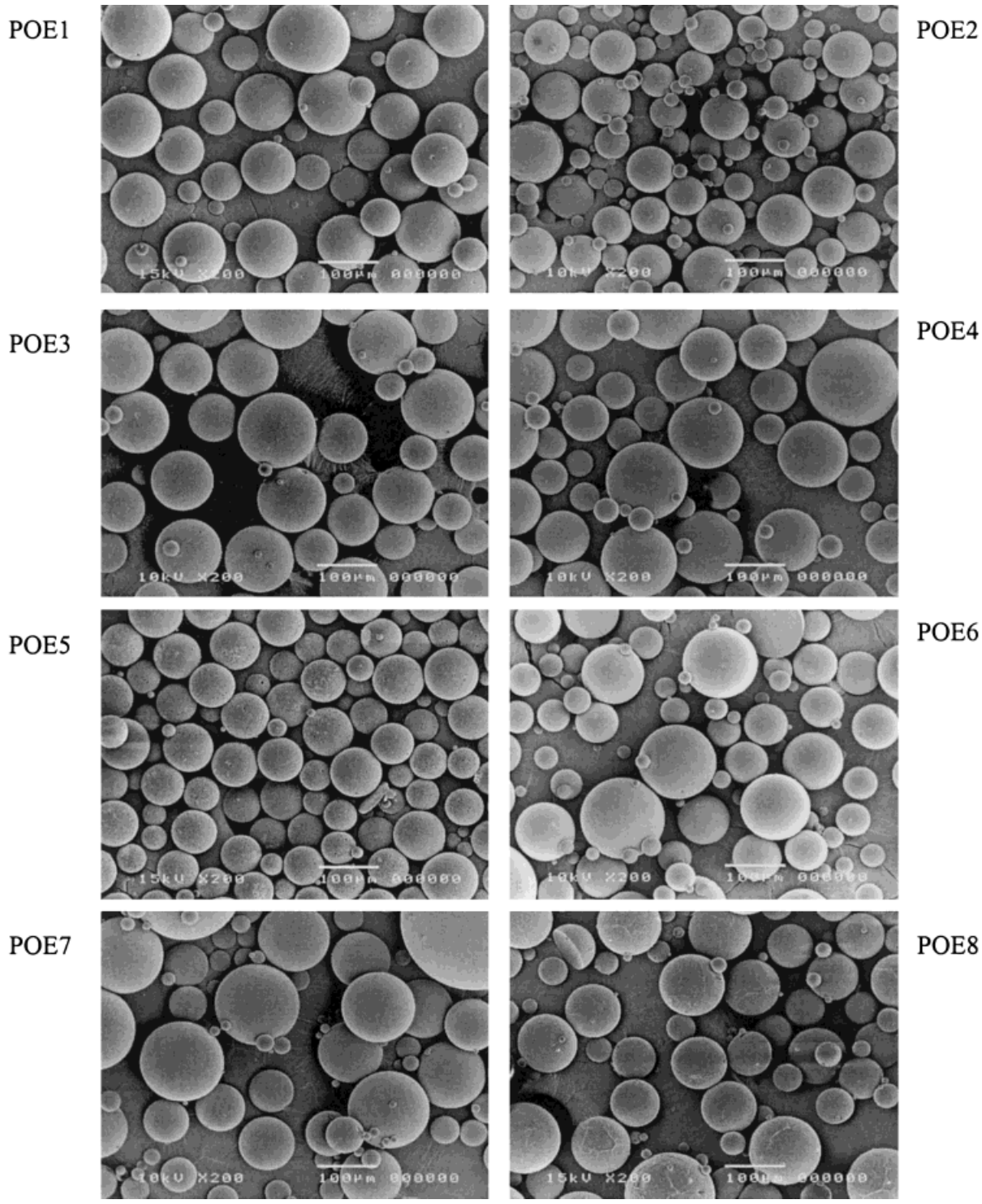


Figure 2 SEM images of BSA-loaded POE microspheres prepared using POE (50 mg/mL in DCM) and 0.2 w/v % PVA stabilizer concentration (bar size = 100 μm).

ence the size and size distribution of the microspheres. From Table II, it can be seen that the size of the microspheres is obviously related to the apparent viscosity of the polymer solution. POE3, POE4, and POE6, with relatively higher viscosities, yield larger sizes of microspheres compared

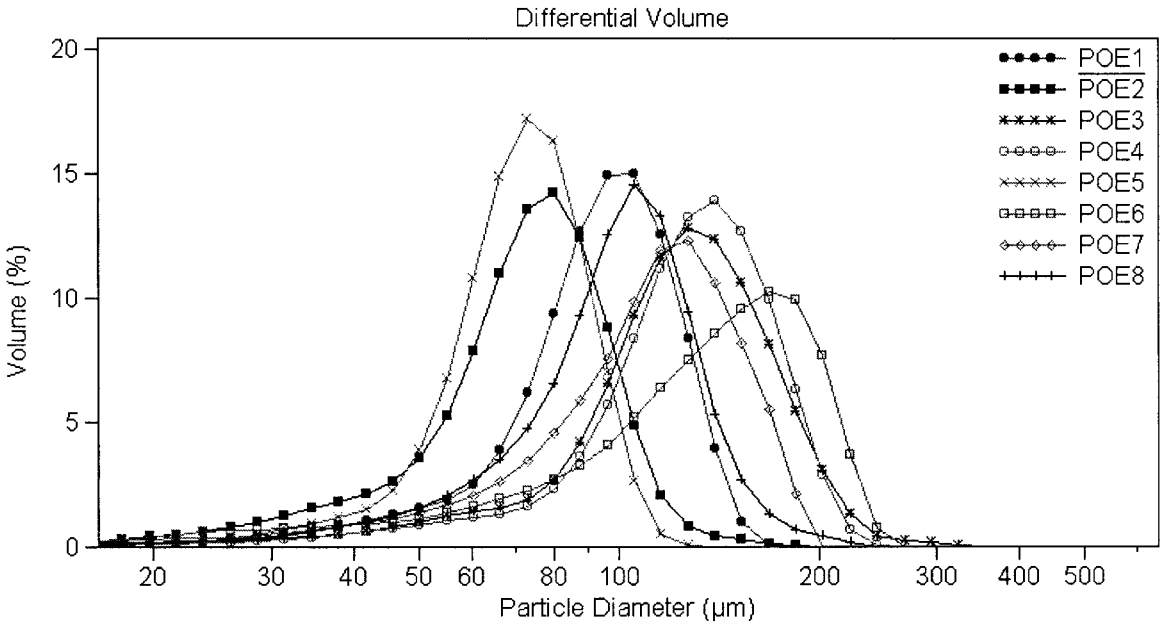


Figure 3 Size distribution of BSA-loaded POE microspheres. The particle size is expressed as the volume mean diameter (μm).

to the others. The POE2 solution has the lowest viscosity (0.14 cP), yielding the smallest size of microspheres (64.7 μm), which is due to that a less viscous polymer solution is easier to break up into smaller droplets at the same input power for mixing. Figure 3 also shows that POE3, POE4, and POE6 have a broader range of size distribution. Microspheres harden more rapidly with more viscous POE solutions. This may result in the microspheres having a broader size distribution since shearing forces have a limited effect on microsphere size once the microspheres harden.

Many factors can affect the encapsulation efficiency. It is noticed from Table II that POE3 yields the highest encapsulation efficiency (38.3%). As presented above, POE3 provides a more viscous polymer solution. This may result in a rapid polymer precipitation and fast microsphere hardening. Thus, it may be more difficult for BSA to diffuse out into the outer water phase. The other possible reason is that POE3 has a high content of hydrophilic segments such as triethylene glycol (TEG) and cyclohexanedimethanol monolactate (CDM-mLT) in the backbone. These segments may interact with BSA and prevent BSA from diffusing out, resulting in a higher encapsulation efficiency. For this reason, POEs 2 and 4–7 yield an encapsulation efficiency in the following order: POE7 < POE6 < POE4 < POE2 < POE5, which is the same order as the content of the hydrophilic segment in the polymers. However, when the hydrophilic segment content is too high, rapid water penetration may lead to a greater amount of BSA lost in the outer water phase. Therefore, POE1 yields a very low encapsulation efficiency. In addition, the low viscosity of POE1 may cause slower microsphere hardening, resulting in lower encapsulation efficiency. The high encapsulation efficiency yielded by POE8 may be due to fast hardening of nascent microspheres during the fabrication process.

Surface Morphology and Inner Structure

SEM micrographs in Figure 2 demonstrate that all the POE polymers used in this study yield microspheres with smooth surfaces. It is noticed from Figure 4 that all the eight kinds of microspheres have a porous inner structure. However, pores are not evenly distributed. More and bigger pores are distributed within the inner region of the microspheres. There is a relatively thicker dense layer underneath the skin of POE1, 4, 7, and 8 microspheres. POE2, 5, and 6 microspheres seem to have a more uniform and porous inner structure. The formation of the inner structure is a complicated process. Stability of the primary emulsion significantly affects the inner structure of the resultant microspheres. A stable primary emulsion could yield a uniform inner structure. However, a less stable primary emulsion and less viscous polymer solution may allow inner water

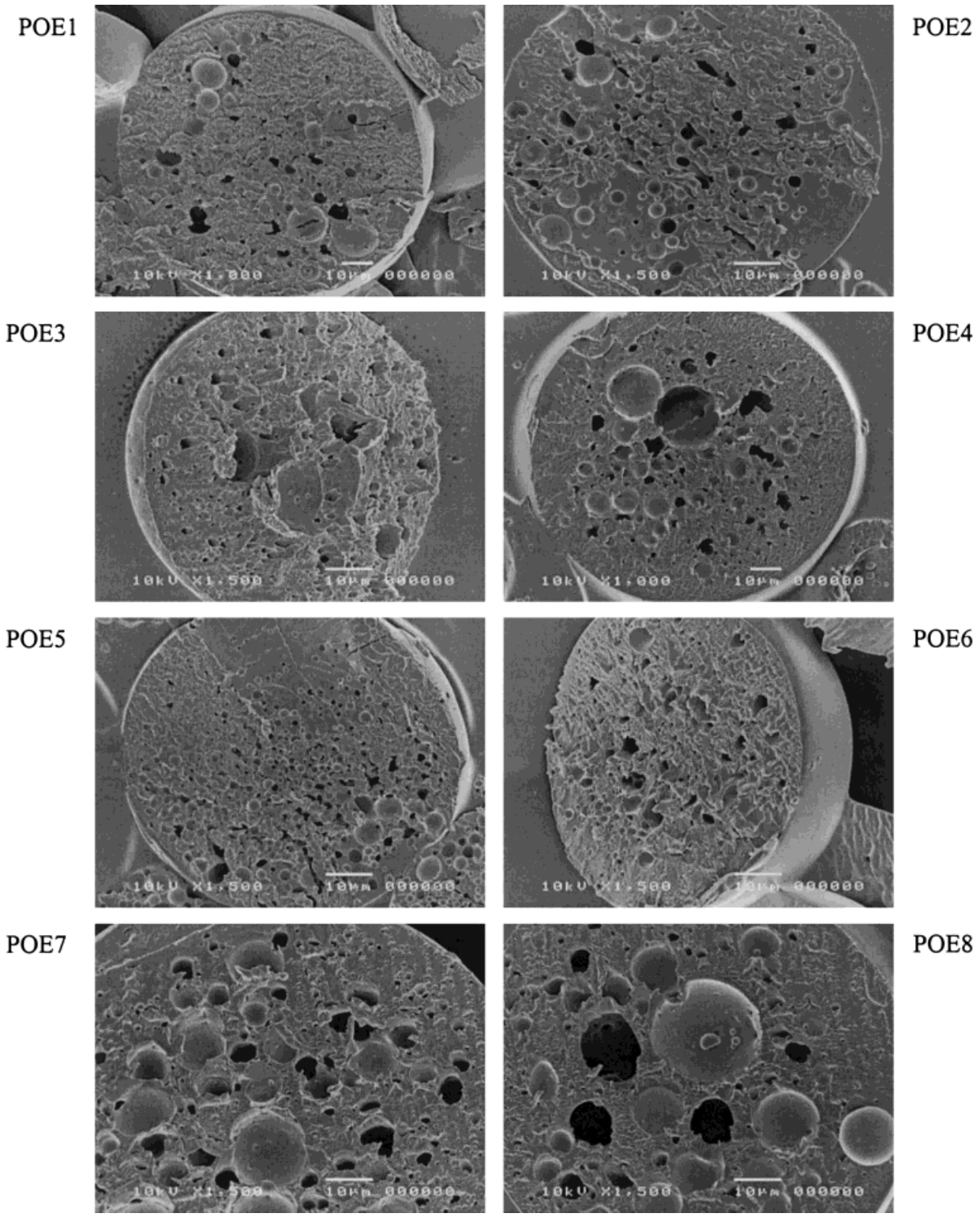


Figure 4 SEM cross-section images showing the internal structure of sectioned, porous microspheres prepared using POE (50 mg/mL in DCM) and 0.2 w/v % PVA stabilizer concentration (bar size = 10 μm).

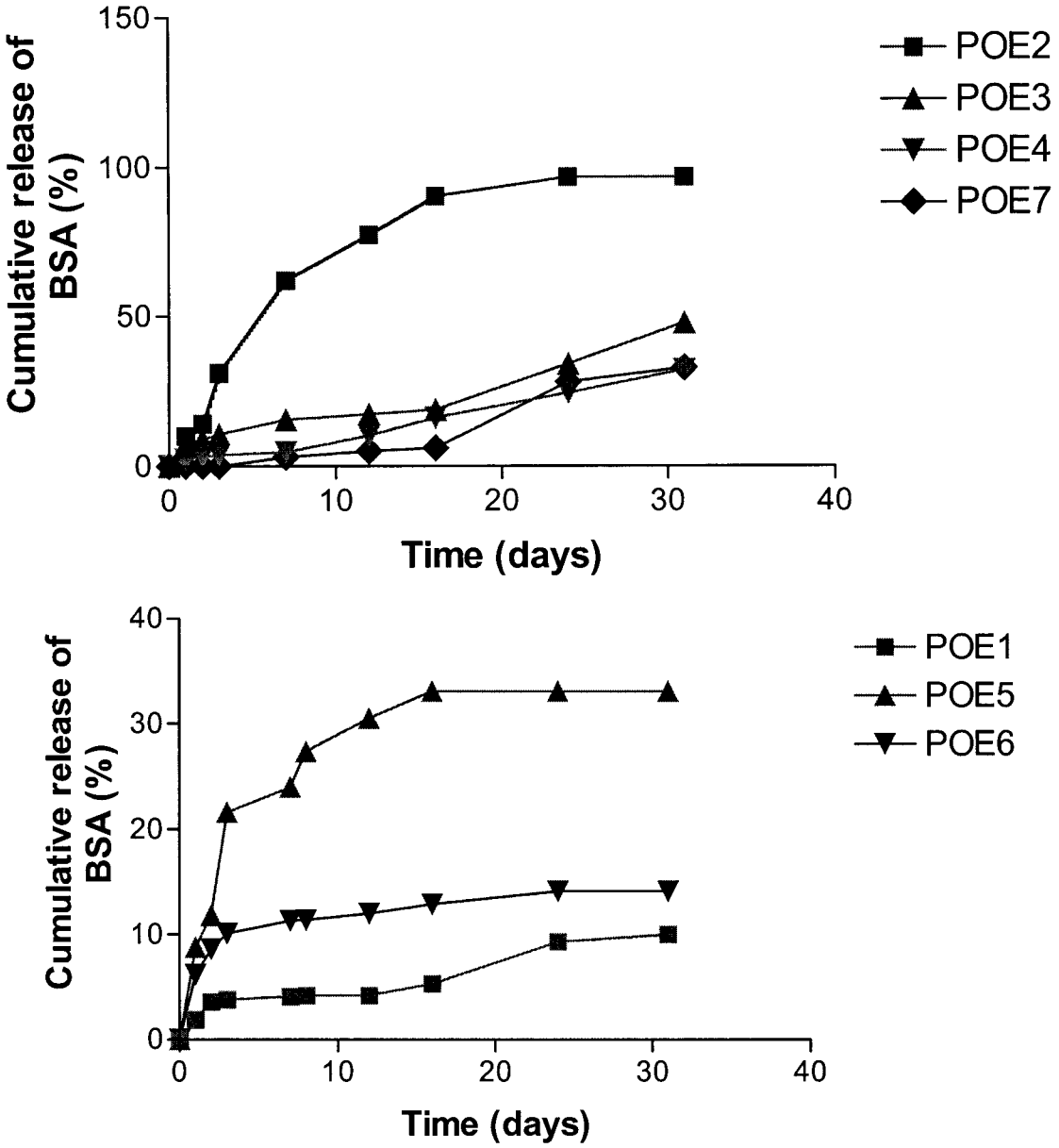


Figure 5 *In vitro* BSA release profiles from POE microspheres at 37°C.

droplets to coalesce one another during the formation process of the microspheres. This results in an inner structure characterized with big pores at the interior and a dense sublayer underneath the skin formed at the later stage. The inner structure and surface morphology have a great impact on the BSA release properties.

In Vitro *Release Profiles*

In this work, *in vitro* release analyses of the eight kinds of POE microspheres were carried out to explore the effect of polymer compositions on the release profiles of BSA. The influence of *in vitro* temperature was also investigated.

Effect of Polymer Compositions. Figure 5 shows the BSA release profiles of POE1–7 microspheres at 37°C. Compared to PLGA microspheres,[23,24] the initial BSA bursts from most of the POE microspheres are much lower. It also can be seen from the figure that the microspheres can release proteins at a sustained manner. BSA release profiles from POE3 and 4 microspheres are pseudolinear. It is very inter-

Table III GPC Molecular Weight Analysis of Microspheres

Polymer ID	Polymer Composition	Molecular Weight (Polymer as Received) (MW)	Molecular Weight of Microspheres (Before Release) (MW)	Molecular Weight of Microspheres (After 1-Mo Release) (MW)
POE1	CDM/HD/CDM-mLT = 65/34/1	36k	23k	22k
POE2	CDM/TEG/HD-diGL = 75/20/5	18k	16k	16k
POE3	CDM/TEG/CDM-mLT = 75/20/5	32k	37k	32k
POE4	CDM/TEG/CDM-mLT = 89/10/1	36k	30k	26k
POE5	CDM/CDM-diGL = 75/25	22k	18k	13k
POE6	CDM/CDM-mLT = 90/10	30k	26k	22k
POE7	CDM (100%)	40k	36k	30k
POE8	CDM/TEG/TEG-mGL = 94/5/1	46k	42k	39k

esting that the BSA release from POE7 and POE8 microspheres is delayed for about 10 days, then followed by a rapid and constant release (Figs. 5 & 8). One possible reason is that POE7 and 8 are more hydrophobic than are other POEs so that water takes some time to penetrate into the skin polymer matrix and then let BSA diffuse out. In addition, the denser layer underneath the skin of POE7 and 8 microspheres may also result in slower initial water penetration.

Figure 5 shows that the BSA release rate is in this order: POE3 > POE4 > POE7. It is noticed from Table I that the ratio of the hydrophobic CDM segment content in the polymer backbone increases from POE3 to POE7. Since the water penetration rate and polymer degradation rate decrease with an increasing CDM segment content, this may be one of the reasons that POE7 has a lower BSA release rate. POE2 has a faster BSA release than that of POE3 even though they have the same content of CDM, which may be due to that the 1,6-hexanediol diglycolide (HD-diGL) in the POE2 backbone is more susceptible to water penetration and polymeric skin degradation than CDM-mLT in the POE3 backbone. It is also noticed from Figure 5 that more than 90% BSA is released from POE2 microspheres during a 20-day release. However, after 1-month release, there is no obvious change in the surface morphology and molecular weight (Table III) except that the microspheres become smaller. This strongly suggests that the BSA release from the POE2 microspheres undergoes a combined erosion and diffusion mechanism.

Figure 5 demonstrates that POE5 microspheres yield a much faster BSA release compared to POE6 and POE1 microspheres. After hydrolysis, cyclohexanedimethanol diglycolide (CDM-diGL) in the POE5 backbone can produce a greater amount of glycolic acid than CDM-mLT in POE6. Consequently, POE5 has a higher degradation rate, resulting in a more rapid BSA release. It is also observed from Figure 5 that only 10% of BSA is released from POE1 microspheres, which may be due to its denser inner structure (Fig. 4)

From our experimental results, it was found that the morphology of the microspheres does not show a significant change after 1-month *in vitro* release at 37°C, as illustrated in Figure 6. In addition, Table III shows that there is only a slight decrease in the molecular weight of most POE microspheres. However, the sizes of the microspheres become slightly smaller as analyzed by the particle-size analyzer (Fig. 7). These results suggest that POEs may undergo a surface-erosion mechanism. Since there is a certain content of glycolide or lactate (latent acid) in the POE, polymer chains hydrolyze at the skin and produce acid when put in PBS, which further catalyzes the hydrolysis of *ortho*-ester linkages in the outer skin layer. When the morphology of the POE microspheres and the content of latent acid are readily controlled, a surface erosion mechanism may be able to be achieved. Our results from the POE microspheres are consistent with the previous work on other shapes of solid POE devices[19] with the evidence of no molecular weight or morphology change after *in vitro* release.

Effect of In Vitro *Release Temperature on Release Profile. In vitro* release is conducted at 22 or 37°C since this work is intended to be used for marine fish and humans. The temperature of a marine

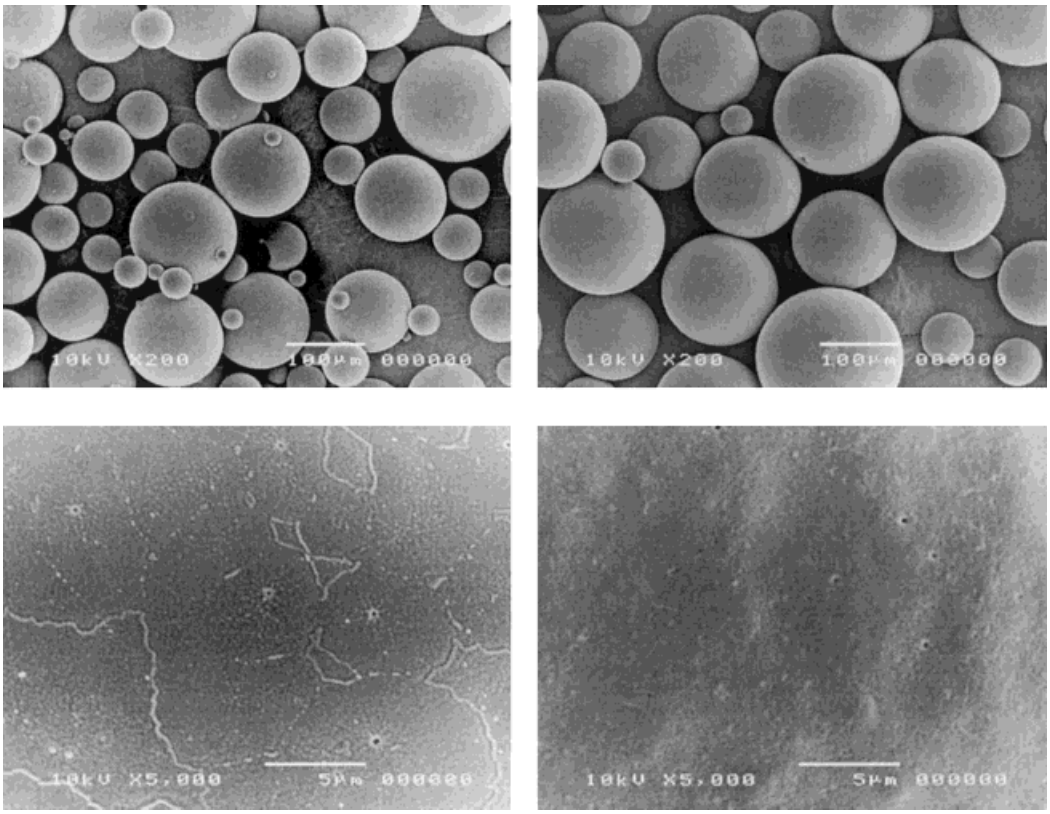


Figure 6 SEM surface analysis of POE4 microspheres before and after 1-month release at 37°C. Top: bar size = 100 μm; bottom: bar size = 5 μm)

fish body in tropical areas is close to 22°C. Figure 8 compares the release profiles of POE8 at these two temperatures. It can be seen that after a 10-day delay, most of the BSA is released at 37°C during the following 20 days. However, there was a very little amount of BSA released at 22°C during the 30-day test because of slow water penetration, hydrolysis, and BSA diffusion at 22°C.

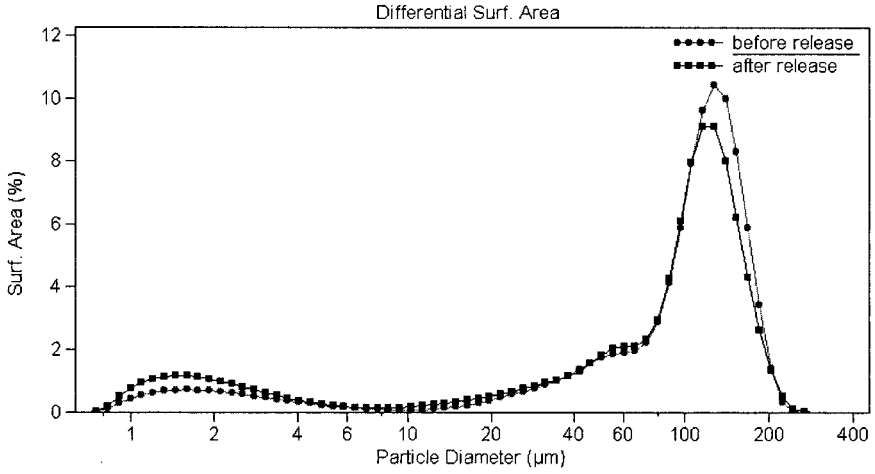


Figure 7 Size distribution of POE4 microspheres before and after 1-month release at 37°C.

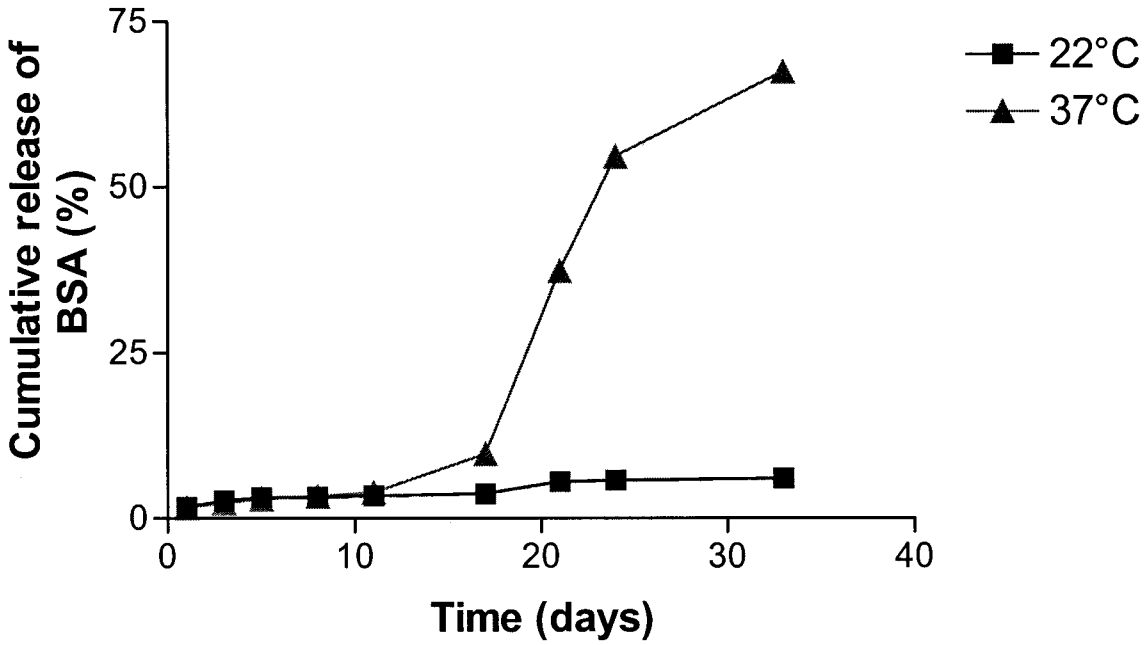


Figure 8 Effect of *in vitro* release temperature on BSA release profile from POE8 microspheres.

CONCLUSIONS

POE microspheres have been successfully fabricated to encapsulate protein. The release of BSA from POE microspheres was characterized. The experimental results indicated that POEs with higher viscosities lead to bigger microspheres. POE3 and POE4 microspheres yield pseudolinear release profiles. POE containing HD-diGL microspheres have the highest BSA release rate after a 20-day test. Since there is no obvious change in the surface morphology and molecular weight except that the microspheres become smaller after a 1-month release, the results suggest that the BSA release from these POE microspheres may undergo a combined erosion and diffusion mechanism.

Microspheres made from POEs containing a high percentage of CDM segments tend to have slower release profiles of BSA, probably because of the hydrophobic nature of the resultant microspheres. When the CDM composition in POEs reaches 94%, there is a 10-day delay in the release because it takes time for water penetration, hydrolysis, and BSA diffusion. In addition, release profiles at 22 and 37°C are quite different.

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