Preparation and Characterization of BSA-Loaded Microspheres Based on Polyanhydrides

Yang Yu,[1] Tingli Lu,[1] Wen Zhao,[1] Weiguang Sun,[1] Tao Chen[1,2]

[1]*Key Laboratory for Space Biosciences & Biotechnology, Faculty of Life Science, Northwestern Polytechnical University, Xi'an 710072, China*
[2]*Liposome Research Center of Shaanxi Province, Xi'an 710075, China*

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ABSTRACT: A series of biodegradable PEG-containing polyanhydrides composed of sebacic acid, 1, 6-bis(*p*-carboxyphenoxy) hexane, and poly (ethylene glycol) (PEG) were used as matrix material for BSA-loaded microspheres. The effects of polymer composition on the microsphere size, entrapment efficiency, *in vitro* degradation, and *in vitro* protein release were studied. Microspheres in the size range of 0.8–10 μm were fabricated via a modified double emulsion method and were characterized using scanning electron microscopy. As the content or the molecular weight of PEG was increased in the copolymer, a proportional increase was found in the particle size and the efficiency of BSA entrapment. The *in vitro* degradation rate of particles could be controlled by varying the polymer composition, increasing as the PEG proportion increased. *In vitro* release studies of BSA from polyanhydride microspheres revealed that the increased amounts of PEG within microspheres could accelerate the release rate of protein. These studies indicate that the PEG-containing polyanhydrides hold potential for protein delivery applications. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 352–358, 2011

Key words: polyanhydrides; protein; microspheres; degradation; release

INTRODUCTION

While small molecule weight drugs form the majority of new therapeutic agents, protein-based therapies are becoming increasingly prevalent. Unlike small molecules, proteins are complex three-dimensional molecules, whose higher-order structure as well as function is prone to chemical and physical alterations.[1] Therefore, the environment of a protein delivery vehicle is a critical parameter for successful protein release. As is well known, biodegradable polymeric microspheres have been used successfully in protein delivery, which could protect the protein from the physiological environment.[2–4] The characteristics of polymers, such as water swelling, hydrophobicity, degradation rate, and especially the affinity with protein, can be manipulated to maintain protein stability.[5,6]

Polyanhydrides are the common biodegradable polymers, and they have been investigated as useful biomaterials for protein carriers.[7–9] Compared to the most widely used poly (D,L-lactide-*co*-glycolide) (PLGA), polyanhydrides exhibit surface erosion, which may prevent moisture-induced protein aggregation. Besides, the pH of the degrading medium does not drop severely as that of PLGA,[10–12] thus providing a more suitable microclimate for protein molecules. Furthermore, polyanhydride particles could completely degrade ranging from days to several months by varying their compositions, and thus a suitable degradation time can be achieved to meet delivery needs. However, it has been observed that the increased hydrophobicity of polyanhydrides can negatively affect the stability of the incorporated protein.[13]

A promising alternative for the polymers discussed above was the use of PEG-containing polymer carriers for protein stabilization, which could usually be obtained by introducing PEG segments to the main chain of polymers.[14–17] As discussed in detail elsewhere,[18–20] the hydrophilic–hydrophobic block copolymers with PEG segments showed several advantages in protein delivery systems,

Correspondence to: T. Chen (taochen@libang.com.cn).
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including improvements in protein entrapment efficiency and protein stability through enhanced affinity between matrix polymer and protein molecules. In addition, the hydrophilic PEG segments could promote the water uptake and swelling of microspheres, so as to modulate the diffusion of proteins.[21] More recently, molecular mechanism operating behind the PEG mediated stabilization of BSA was well investigated, which further confirmed that PEG could improve the stability of protein.[22]

In this study, we reported on the entrapment of a model protein (BSA) using polyanhydrides based on hydrophilic PEG and hydrophobic P (SA-CPH). The SA-CPH-PEG copolymers varied in PEG weight ratio (2.5–30%) and PEG molecular weight (2000, 4000, and 8000), and were indicated as aPEGb, in which "a" was the PEG molecular weight, "b" the wt % PEG. The SA/CPH ratio was kept at 80 : 20. For example, 2000PEG10 was the polyanhydride composed of PEG2000 and 80 : 20 SA:CPH (1 : 10, w/w). Polyanhydrides without PEG segments were indicated as SA80CPH20. Different kinds of polyanhydride microspheres were fabricated via the double emulsion technique. *In vitro* degradation profiles were characterized by measuring the particle mass loss, the PBS medium pH decrease and the morphological change of microspheres. *In vitro* BSA release from polyanhydride microspheres were also described. The overall objective of this study was to investigate the effects of PEG segments on microsphere characteristics, including the surface morphology, encapsulation efficiency, *in vitro* degradation, and *in vitro* protein release behavior.

MATERIALS AND METHODS

Materials

Sebacic acid, 4-carboxybenzoic acid, succinic anhydride, and acetic anhydride were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). 1,6-dibromohexane was purchased from Aladdin Reagent Company (Shanghai, China). Hydroxyl-terminated poly (ethylene glycol) (PEG, M_n = 2000, 4000, and 8000, respectively), pyridine and polyvinyl alcohol (PVA, 99 mol % hydrolyzed) were obtained from Tianjin Kermel Chemical Reagent Company (Tianjin, China). Bovine serum albumin (BSA) was obtained from Biosharp. The BCA assay kit was purchased from Bioteke Corp. (Beijing, China). All the other chemicals were of analytical grade without further purification.

Polymer synthesis and characterization

1,6-bis(*p*-carboxyphenoxy) hexane (CPH) was synthesized according to the method described by Conix.[23] PEG diacid was synthesized by the method previously described.[24] The prepolymers of SA, CPH, and PEG diacid were synthesized by the method suggested in the literature.[25] Subsequently, the SA-CPH-PEG copolymers of different compositions were synthesized by melt polycondensation under vacuum. During the polymerization process, the weight ratio between SA and CPH in polyanhydrides was kept at 80 : 20, and the feeding PEG was varied from 2.5 to 30% with respect to the total weight amounts of SA and CPH. The reason we chose this ratio is that the 80 : 20 SA:CPH polyanhydrides have no effect on the BSA secondary structure.[13] The chemical structure of polyanhydrides was analyzed using ^{1}H-NMR spectra obtained from an AVANCF300MHZ NMR spectrometer. The peaks corresponding to SA (1.32, 1.65, and 2.44 ppm), CPH (6.95 and 7.97 ppm), and PEG (3.65 ppm) confirmed the three components in the newly synthesized polyanhydrides.

Microsphere fabrication

Microspheres were generated using a double emulsion solvent evaporation procedure.[25] A water-in-oil emulsion was obtained by sonication (50 s, 90 W) of 3 mL methylene chloride containing 100 mg of polymer with 300 μL of distilled water, or 300 μL of BSA solution (30 mg/mL). Aqueous 1% PVA (6 mL) was added, and the solution was stirred with a homogenizer (10,000 rpm for 60 s) to make a (w/o)/w emulsion. The mixture was poured into 0.1% PVA (50 mL) and stirred for 2.5 h at 300 rpm to evaporate the organic solvent. The microspheres were washed several times with distilled water and then collected by centrifugation for 10 min at 1500 × *g*. The supernatant was collected after the first centrifugation. Finally, the microspheres were suspended in 1 mL of distilled water, and freeze-dried.

Determination of microsphere size and zeta potential

The size (number and volume mean diameter) and the size distribution of microspheres suspended in water were determined by laser diffraction particle sizer (Nano-ZS, Malvern Instrument, UK). The zeta potential was measured by Malvern Zeta analyzer (Nano-ZS, Malvern Instrument, UK). The temperature was kept at 25°C during measuring.

Scanning electron microscopy

The surface morphology of microspheres was observed by scanning electron microscope (SEM, FEI, QUANTA 200). Samples were sputter-coated with a thin gold layer.

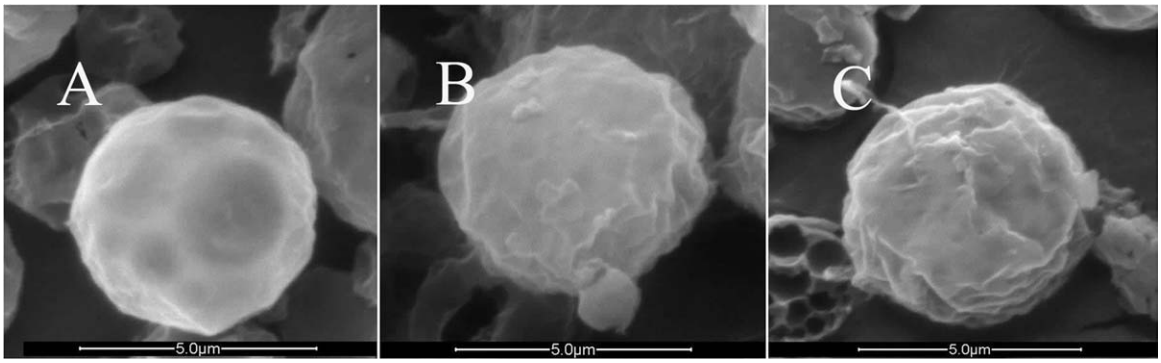

Figure 1 Scanning electron micrographs of the surface of BSA-containing microspheres prepared from 2000PEG2.5 (A), 2000PEG5 (B), and 4000PEG5 (C).

Determination of the BSA content in the microspheres

The amount of BSA entrapped in microspheres was determined by measuring the difference between the total amount of protein incorporated in the particle preparation medium and the amount of nonentrapped protein remaining in aqueous medium after the encapsulation process.[13,26] During the fabrication process, the microspheres were obtained by centrifugation, and the supernatant was collected to determine the amount of protein not encapsulated. The mass of protein encapsulated in microspheres could be easily calculated by mass balance, and then the protein encapsulation efficiency was determined by dividing the mass of the loaded protein by the initial mass of protein. The entrapment efficiency was slightly overestimated because the protein that was lost while washing the microspheres was not accounted for when calculating the encapsulation.

In vitro degradation

The degradation behavior of microspheres was evaluated by the particle mass loss, the pH change of degradation medium, and the morphological change at predesigned intervals. Polyanhydride microspheres (10 mg) were placed individually in test tube containing 5 mL of 0.1*M* PBS at pH 7.4. The sample tubes were incubated in 37°C under continuous shaking (100 rpm). At predetermined intervals, microspheres were centrifuged (1500 × *g* for 5 min), washed three times and dried to constant weight. The morphological change of microspheres can be observed by SEM. Mass loss was determined gravimetrically by comparing the dry weight remaining at a specific time with the initial weight. The pH change of degradation medium was obtained by detecting the pH value of the supernatant at 25°C.

In vitro protein release

BSA-loaded microspheres (20 mg) were immersed in 1 mL PBS (pH 7.4) containing 0.1% (w/w) sodium dodecyl sulfate (SDS). Tubes were continuously shaken at 37°C, and samples were taken at various time points after the suspension was centrifuged. The concentration of protein in each sample was measured by the BCA protein assay upon removal. The amount of protein released was normalized by the amount of protein initially loaded into the microspheres. The release experiments were done independently in triplicate.

RESULTS AND DISCUSSION

Microsphere characterization

Typical methods for microsphere fabrication include double emulsion (a.k.a., water-in-oil-in-water (w/o/w)),[27] spray drying, [28] and cryogenic atomization (CA).[8] The last two methods require special equipment while double emulsion methods do not; however, care must be taken when encapsulating proteins via the double emulsion technique because the presence of a water/oil interface is potentially detrimental for protein stabilization. In this study, P (SA-CPH-PEG) microspheres containing BSA were prepared by a modified w/o/w method in the same condition. As seen in Figure 1, different surface structures of microspheres were observed corresponding to different polymer compositions. Microspheres prepared from 2000PEG2.5 copolymers presented a smooth surface [Fig. 1(A)], whereas the 2000PEG5 microspheres were much rougher [Fig. 1(B)]. As molecular weight of PEG increased to 4000, the particle surface got even rougher and more folds could be observed [Fig. 1(C)].

Results in Table I indicated that the zeta potential of microspheres prepared from various copolymers was –(8.9−14.1) mV, the yield 30−65%, and the

TABLE I
Characteristics of BSA-Loaded Polyanhydride Microspheres Prepared from Polymers of Different Copolymer Composition

Polymer	d_n[a] (μm)	d_v[b] (μm)	Zeta potential (mV)	Yield (%)	PDI
SA80CPH20	1.01	1.07	−8.9	63 ± 9	0.326
2000PEG2.5	1.80	1.82	−13.2	65 ± 6	0.305
2000PEG5	1.25	1.27	−9.4	61 ± 8	0.314
2000PEG10	2.16	2.32	−10.8	56 ± 7	0.289
2000PEG20	2.50	2.65	−14.1	30 ± 9	0.351
4000PEG10	1.46	1.50	−10.6	52 ± 6	0.293
8000PEG10	1.51	1.57	−11.9	46 ± 10	0.347

[a] Number mean particle size.
[b] Volume mean particle size.

polydispersion index (PDI) about 0.300. The typical size distribution of these microspheres was in the range of 0.8−10 μm, with the majority of the population in the 1−3 μm range. As an example, the size distribution of 2000PEG10 microspheres is shown in Figure 2. There was no significant difference between number and volume mean particle size, indicating that the particle size distribution was unimodal. Particle size was related to the mixing rate used in the preparation of the inner emulsion. When the inner emulsion was prepared by probe sonication, the overall microsphere size was much smaller.[4] Seen from these different microspheres, a clear trend of increasing the size of microspheres as their PEG contents increased was observed. Also, the particle size increased with increase in PEG molecular weight (Table I). As already noticed by others,[21] microspheres prepared from copolymers with PEG 4000 were larger than that with PEG 600 and PEG 1000. A possible explanation for this result is that the particles were in the swollen state during the process of size determination, when the hydrophilic PEG segments promoted the water uptake and swelling of particles. Therefore, larger particles could be observed with large PEG amounts due to their high degree of swelling.

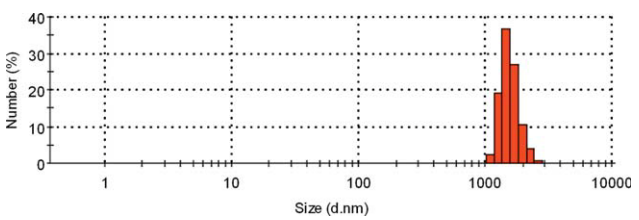

Figure 2 Particle-size distribution of BSA-loaded microspheres prepared with 2000PEG10. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

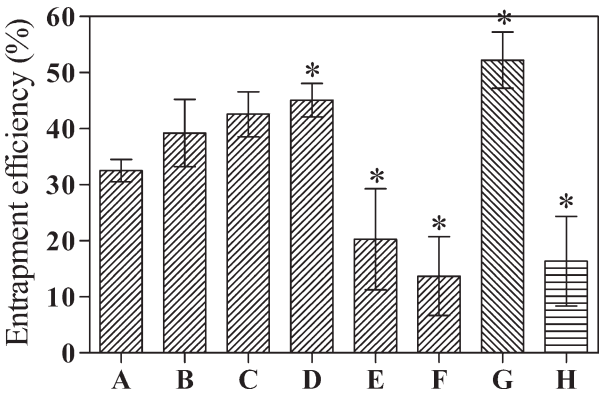

Figure 3 Effect of copolymer composition on BSA entrapment. The BSA entrapment efficiency of microspheres prepared from SA80CPH20 (A), 2000PEG2.5 (B), 2000PEG5 (C), 2000PEG10 (D), 2000PEG20 (E), 2000PEG30 (F), 4000PEG10 (G), and 8000PEG10 (H). (*) indicated statistically significant differences when compared to SA80CPH20 ($P < 0.05$).

Effect of copolymer composition on BSA entrapment

To investigate the protein entrapment efficiency of polyanhydride microspheres, a series of copolymers, with different composition, were used as matrix materials. As shown in Figure 3, the encapsulation efficiency of P (SA-CPH-PEG) microspheres with different PEG2000 contents from 2.5 to 10% is higher than that of P (SA-CPH), indicating that the incorporated PEG could enhance a slightly increase in protein entrapment efficiency. However, as the PEG2000 content in the microspheres was increased to 20% and beyond, the protein encapsulation efficiency decreased steadily. Compared with 2000PEG10 microspheres (45.1%), 4000PEG10 showed higher encapsulation efficiency (52.2%). When PEG8000 was incorporated into copolymers, the entrapment efficiency decreased significantly.

All of the decrease of entrapment efficiency described earlier may be attributed to the premature release of BSA during the 2.5-h solvent evaporation in PBS. The more hydrophilic the copolymer matrix is, the more easily the protein molecules diffuse out during microsphere hardening. As for P (SA-CPH-PEG) with PEG2000 content of below 10%, the increase in encapsulation efficiency with increase in PEG content may be a result of the existence of certain amount of hydrophilic PEG segments in the copolymer chains, which improved the affinity of copolymer with protein. These results are in agreement with previous publications.[29]

Effect of copolymer composition on degradation

Degradation experiments of PEG-Containing polyanhydrides were conducted by measuring the mass

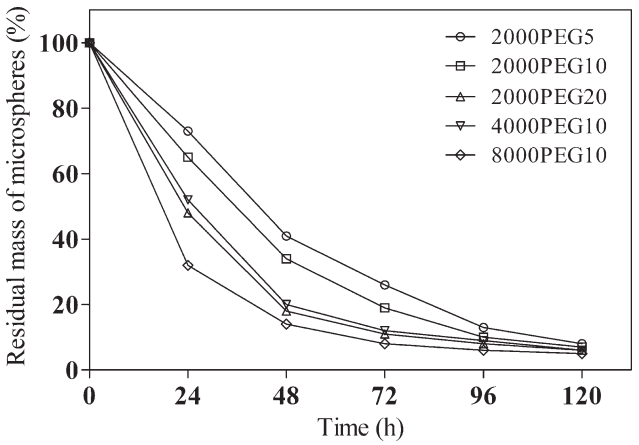

Figure 4 Effect of copolymer composition on the degradation of microspheres. The percent residual weight of P (SA-CPH-PEG) microspheres incubated in PBS at 37°C.

loss of microspheres and the pH value change of PBS solutions. Figure 4 shows the degradation profiles based on mass loss data. After 96 h of degradation, the cumulative percentage mass loss was over 90% for all cases. The mass of 8000PEG10 microspheres decreased sharply in the initial 24 h, while a slight mass loss was observed with 2000PEG5 microspheres. The rate of mass loss depended on microsphere composition, increasing when the PEG proportion in the microspheres increased. For example, the percent mass loss during the period of 24 h was 35% for 2000PEG10 microspheres and 52% for 2000PEG20 microspheres. In addition, the degradation rate of 8000PEG10 microspheres was faster than that of 4000PEG10 microspheres.

The final *in vitro* degradation products of polyanhydride microspheres are SA, CPH and PEG. It is well known that the rates of hydrolysis of SA-SA and SA-CPP bonds were higher than that of CPP-CPP bonds,[30] for higher solubility led to higher dissolution rate (The solubility of SA and CPP monomers were about 1 mg/mL and 0.02 mg/mL).[11,31] As PEG was more water soluble than SA and CPH, the PEG-PEG bonds should be more labile than the other bonds in P (SA-CPH-PEG) copolymer, and thus the increase in PEG segments of polyanhydrides could result in a faster degradation rate of microspheres. This result appears to be consistent with the data reported before.[14]

Figure 5 displays the decrease of the medium pH versus incubation time. The pH value of PBS solutions during the degradation was always higher than 7.10. Unlike PLGA, which caused a dramatic pH drop in the release medium after incubation,[10] a relatively neutral pH was retained in the degradation medium for all polyanhydride formulations, thus providing a suitable environment for proteins. As seen in Figure 5, irrespective of copolymer composition, the pH values dropped sharply to the minimum in 24 h and then gone back up gradually from 48 h. For different amounts of PEG in the copolymers, there was some slight difference in pH changes. Higher the PEG content or the PEG molecular weight, the faster the pH decreased (24 h). Judging from Figures 4 and 5, it was found that the pH change was related to the mass loss of microspheres. Specifically, as PEG segments increased, the mass of microspheres gradually reduced, and the pH values got lower accordingly. The low pH value was attributed to the high degradation rate of microspheres, which resulted in more acid monomers. The PBS solutions were always changed at predetermined intervals during degradation. After 48 h, the erosion rate of microspheres was getting slower, and the pH did not drop severely as before. Thus, the pH values showed a gradually increased tendency.

Figure 6 shows the morphology changes of the degraded microspheres. The microspheres were intact spherical structure at 0 h [Fig. 6(A1,B1,C1)]. After 24 h in releasing medium, a number of pores were seen scattered all over the particles. Most microspheres prepared from 2000PEG2.5 and 2000PEG5 still retained spherical structure [Fig. 6(A2,B2)]. However, few spherical particles could be found in 4000PEG5 [Fig. 6(C2)]. During the following 48 h, only porous remnants of particles remained [Fig. 6(A3,B3,C3)]. These results indicated that the increased PEG segments could accelerate the hydrolysis rate of polyanhydrides.

Effect of copolymer composition on BSA release

Release profiles of BSA from polyanhydride microspheres were investigated in 0.1% (w/w) SDS (Fig. 7). All of the release patterns consisted of a burst release followed by a gradual release phase. It

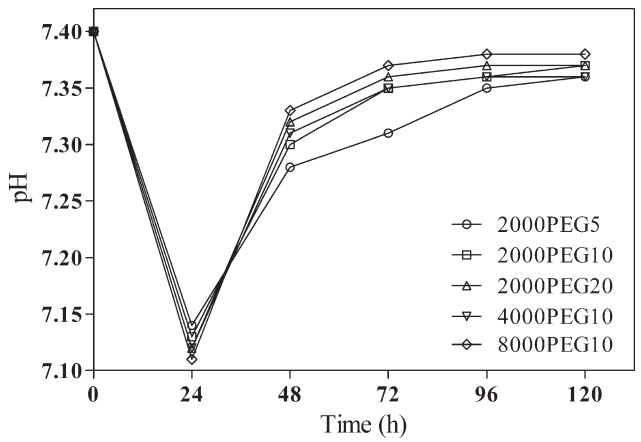

Figure 5 Effect of copolymer composition on the degradation of microspheres. The pH change of degradation medium incubated with P (SA-CPH-PEG) microspheres.

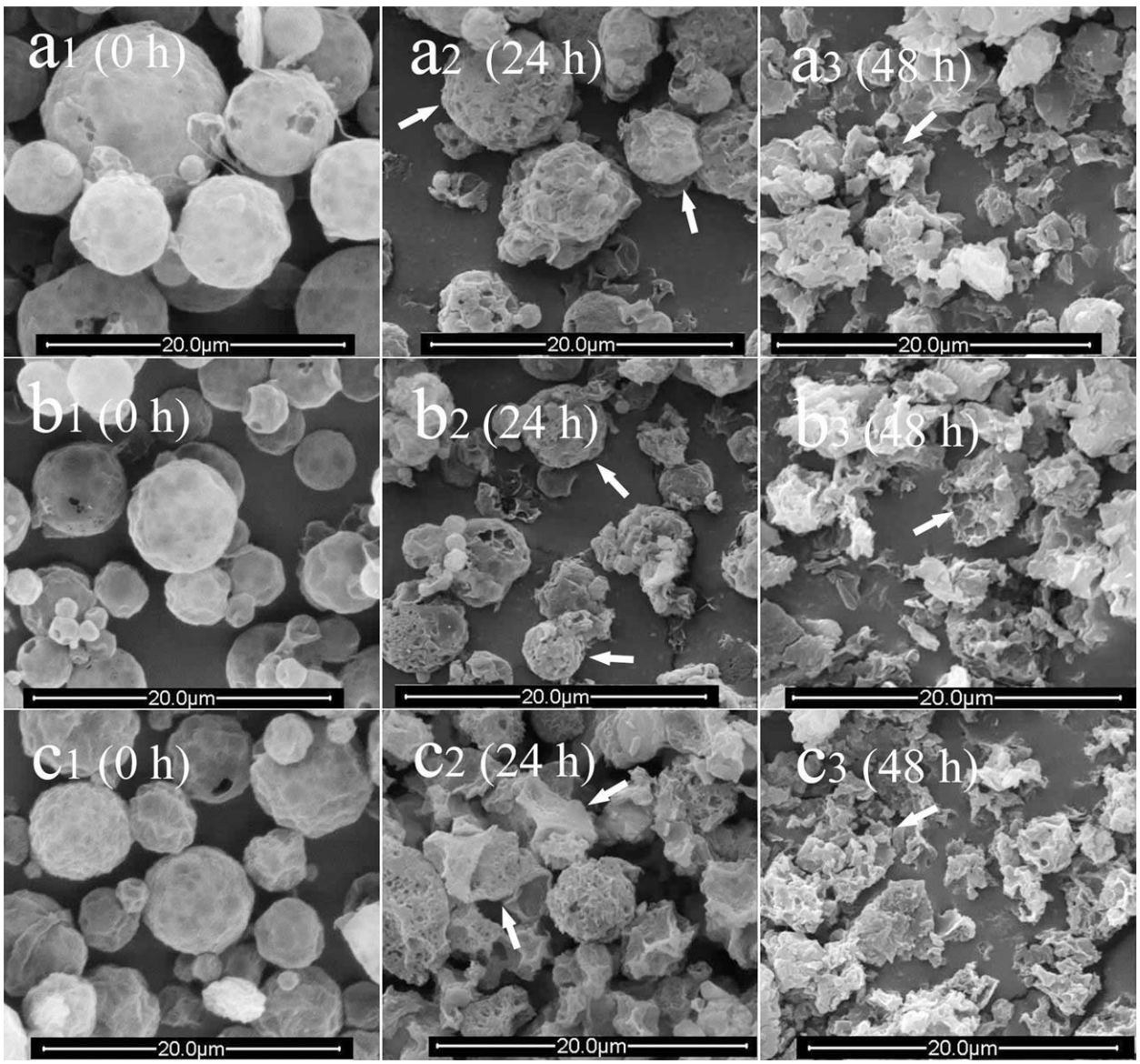

Figure 6 The surface morphology change of microspheres detected by SEM for 2000PEG2.5 (A1–A3), 2000PEG5 (B1–B3), and 4000PEG5 (C1–C3) at 0, 24, and 48 h.

is believed that the protein burst is due to protein release from the microsphere surface. The percent protein release from 2000PEG5, 2000PEG10, and 2000PEG20 microspheres in 24 h was 38.5, 44.9, and 58.8%, respectively. Release from copolymer 8000PEG10 was the fastest among all formulations in this study, with 72.2% of the protein release within 24 h. The majority of the protein encapsulated in 8000PEG10 was released after 48 h, whereas 2000PEG5 could continue for about 120 h. Such release behaviors were consistent with the *in vitro* degradation profiles of microspheres.

The extent of initial burst increased when the PEG proportion in polyanhydrides increased, which was supported by the protein release from 2000PEG5, 2000PEG10, and 2000PEG20 microspheres. The

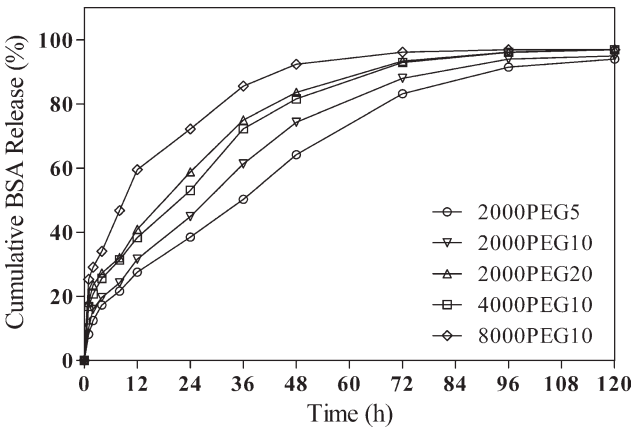

Figure 7 Effect of copolymer composition on BSA release. Cumulative release of BSA from P (SA-CPH-PEG) microspheres at 37°C in PBS with constant agitation (100 rpm).

comparison of profiles of BSA release from 4000PEG10 microspheres with that from 8000PEG10 microspheres showed that the PEG molecular weight could accelerate its initial release significantly. Similar results have also been obtained with other particle types.[32] It can be concluded from this part of the study that the polymer composition is an effective tool to modulate release of proteins from P (SA-CPH-PEG) microspheres.

CONCLUSIONS

In this study, PEG-containing polyanhydrides were successfully applied as matrix materials for protein-loaded microspheres. The *in vitro* degradation and *in vitro* protein release profiles of polyanhydride microspheres could be precisely tailored by variation of the PEG amounts within copolymers. Although this class of polyanhydrides could enhance the affinity between matrix polymer and protein molecules, not all polyanhydrides were suitable vehicles for protein delivery. For example, microspheres with high PEG content (above 10%) and large PEG molecular weight (such as PEG 8000) degraded too fast to delivery protein drugs. In our future research, it is of great interest to investigate the effect of polyanhydrides on the structure and stability of BSA. In addition, we will use nonaqueous techniques (a.k.a., solid-oil-oil (s/o/o), CA) for fabricating microspheres, which could avoid the water/organic interface and the hydrolysis of copolymers during preparation process.

References

1. Cleland, J.; Powell, M.; Shire, S. Crit Rev Therap Drug Carrier Syst 1993, 10, 307.
2. Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L.; Langer, R. Pharm Res 1991, 8, 713.
3. Cho, S.; Song, S.; Choi, Y. Arch Pharm Res 2000, 23, 385.
4. Tabata, Y.; Gutta, S.; Langer, R. Pharm Res 1993, 10, 487.
5. Li, X.; Deng, X.; Huang, Z. Pharm Res 2001, 18, 117.
6. van de Weert, M.; Hennink, W.; Jiskoot, W. Pharm Res 2000, 17, 1159.
7. Hanes, J.; Chiba, M.; Langer, R. Biomaterials 1998, 19, 163.
8. Lopac, S.; Torres, M.; Wilson-Welder, J.; Wannemuehler, M.; Narasimhan, B. J Biomed Mater Res Part B: Appl Biomater 2009, 91, 938.
9. Weiner, A.; Bock, E.; Gipson, M.; Shastri, V. Biomaterials 2008, 29, 2400.
10. Fu, K.; Pack, D.; Klibanov, A.; Langer, R. Pharm Res 2000, 17, 100.
11. Gpferich, A.; Langer, R. J Polym Sci Part A: Polym Chem 1993, 31, 2445.
12. Zhu, G.; Schwendeman, S. Pharm Res 2000, 17, 351.
13. Determan, A.; Trewyn, B.; Lin, V.; Nilsen-Hamilton, M.; Narasimhan, B. J Controlled Release 2004, 100, 97.
14. Avgoustakis, K.; Beletsi, A.; Panagi, Z.; Klepetsanis, P.; Karydas, A.; Ithakissios, D. J Controlled Release 2002, 79, 123.
15. Mder, K.; Bittner, B.; Li, Y.; Wohlauf, W.; Kissel, T. Pharm Res 1998, 15, 787.
16. Tobio, M.; Gref, R.; Sanchez, A.; Langer, R.; Alonso, M. Pharm Res 1998, 15, 270.
17. Torres, M.; Determan, A.; Anderson, G. Biomaterials 2007, 28, 108.
18. Deng, X.; Zhou, S.; Li, X.; Zhao, J.; Yuan, M. J Controlled Release 2001, 71, 165.
19. Jiang, W.; Schwendeman, S. Pharm Res 2001, 18, 878.
20. Li, X.; Zhang, Y.; Yan, R.; Jia, W.; Yuan, M.; Deng, X.; Huang, Z. J Controlled Release 2000, 68, 41.
21. Bezemer, J.; Radersma, R.; Grijpma, D.; Dijkstra, P.; Van Blitterswijk, C.; Feijen, J. J Controlled Release 2000, 67, 249.
22. Rawat, S.; Raman Suri, C.; Sahoo D. K. Biochem Biophys Res Commun 2010, 392, 561.
23. Conix, A. Macromol Synth 1966, 2, 95.
24. Geckeler, K.; Bayer, E. Polym Bull 1980, 3, 347.
25. Fu, J.; Fiegel, J.; Hanes, J. Macromolecules 2004, 37, 7174.
26. Bouillot, P.; Ubrich, N.; Sommer, F.; Minh Duc, T.; Loeffler, J.; Dellacherie, E. Int J Pharm 1999, 181, 159.
27. Manoharan, C.; Singh, J. J Pharm Sci 2009, 98, 4237.
28. Gen, L.; Demirel, M.; Yazan, Y. Arch Pharm Res 2006, 29, 921.
29. Deng, X.; Li, X.; Yuan, M.; Xiong, C.; Huang, Z.; Jia, W.; Zhang, Y. J Controlled Release 1999, 58, 123.
30. McCann, D.; Heatley, F.; D'emanuele, A. Polymer (Guildford) 1999, 40, 2151.
31. Muggli, D.; Burkoth, A.; Anseth, K. Mater Res 1999, 46, 271.
32. Matsumoto, J.; Nakada, Y.; Sakurai, K.; Nakamura, T.; Takahashi, Y. Int J Pharm 1999, 185, 93.