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Preparation and characterization of protein-loaded polyanhydride microspheres

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Abstract Poly(1,3-bis-(p-carboxyphenoxy propane)-co-(sebacic anhydride) (P(CPP-SA)) have the anhydride bonds in copolymer backbone, which are available for degradation on the base of passive hydrolysis. This chemical structure made it degraded within a short time in linear degradation rate. For this property, polyanhydrides are one of the most suitable biodegradable polymers employed as drug carriers. This paper aimed at researching the erosion and degradation of P(CPP-SA) microspheres with CPP/SA monomer ratios of 20:80, 35:65 and 50:50. In vitro protein release from the microspheres was also investigated in this paper. Human serum albumin (HSA) was used as the model protein. In this research, the microspheres degradation and drug release rate from microspheres can be adjusted by altering the CPP/SA ratios of P(CPP-SA). The features of surface erosion were observed in SEM. The structural integrity of HSA extracted from microspheres was detected by gel permeation chromatography, compared with native HSA. The results showed HSA remained its molecule weight after encapsulated.

1 Introduction

Biodegradation of polymers can be caused by enzyme, chemical, microbe or simply hydrolysis in human body. The biodegradable polymers can be eliminated from the human body by metabolism after used [1]. In recent years, biodegradable polymer microspheres used as controlled drug release system have been widely researched. Such systems can maintain appropriate drug concentration at therapeutic site of the body, protect and stabilize the active drug, and help patient compliance by reducing the frequency of administration [2–6].

The classes of polyester and polyanhydride are the common biodegradable polymers, approved by FDA to be used as medical device in human body, because of their biodegradability and non-toxicity of polymers and degradation products. Polyester and polyanhydride are widely used in controlled drug release systems. The suitable polymer as drug carrier is one which degrades linearly over time in an aqueous environment [7]. These require biodegradable polymers should degrade from surface to the core. Because surface eroding is expected to release drug at a constant rate. And, the drug release rate is directly proportional to the polymer erosion rate. Comparing with polyester, the polyanhydrides are hydrophobic, which can restrict water penetration into the bulk. Only the surface of polyanhydride matrix can contact with water in aqueous environment. And there are anhydride bonds in polymer backbone, which are high water lability. Thereby, hydrolysis of polyanhydride microspheres happens from surface to core. It is believed that polyanhydrides undergo surface erosion predominantly [8]. They degrade to their respective diacids and completely eliminate from the body within a period of weeks to years. So, it is the desirable polymer for drug controlled release system to achieve zero-order release.

Polyanhydrides are suited to control drug release as they degrade into non-toxic metabolites that are non-mutagenic, non-cytotoxic and non-inflammatory [9]. The other

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important reason is that polyanhydrides could undergo surface erosion. This unique property made drug release stably based on a zero-order kinetic rule. Moreover, hydrolysis rates can be adjusted by altering the hydrophile-lipophile of polyanhydrides [10, 11]. While, degradation time of polyanhydrides can be designed from few days to several years by varying the type of structural units and their ratios. Generally, aliphatic polyanhydrides degrade in a few days while some aromatic polyanhydrides degrade over a few years. The degradation time of copolymers made up of aliphatic and aromatic polyanhydrides can be varied in this range. These features of polyanhydrides give an opportunity to make drug release within a required times.

The most extensively researched and used polyanhydride is poly(1,3-bis-(p-carboxyphenoxy propane)-co-(sebacic anhydride) (P(CPP-SA)), whose chemical structure is shown in Fig. 1. It is an aromatic-aliphatic polyanhydride copolymer, which have been approved by FDA to be used to deliver drugs for treating brain cancer. Gliadel, a device to deliver carmustine (BCNU) to the malignant glioma tumor, is the most successful commercial application of polyanhydrides [12]. This is one of the examples where an implantable synthetic degradable polymer device has been approved for drug delivery [13–15].

This paper focused on researching the in vitro degradation and protein release of P(CPP-SA) microspheres. P(CPP-SA) with CPP/SA ratios of 20:80, 35:65, and 50:50 were investigated. At first, P(CPP-SA) microspheres loading human serum protein were fabricated by solvent evaporation method. Particle size analyzer and scanning electron microscopy (SEM) were carried out to characterize the mean size, the size distribution and the surface morphology. Next, the degradation of microspheres and the protein release in vitro were performed in phosphate buffered saline (PBS, pH 7.4) at 37°C. Mass loss, pH change and SEM were used to characterize the process of degradation; UV–vis spectrophotometer (UV) was used to detect the concentration of protein released. Surface erosion of P(CPP-SA) microspheres can be proved by SEM. The structural integrity of HSA extracted from microspheres was detected by gel permeation chromatography (GPC), compared with native HSA.

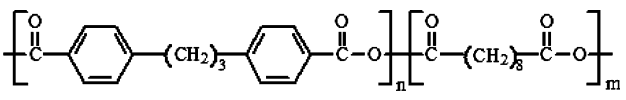

Fig. 1 The chemical structure of P(CPP:SA) copolymer

2 Experimental

2.1 Materials

The P(CPP-SA) with CPP/SA weight ratios of 20:80, 35:65, 50:50 were polymerized by a melt polycondensation process in our lab and their intrinsic viscosity [η] determined by Ubbelohde viscometer is about 0.23, 0.20, 0.19, respectively. Polyvinyl alcohol (PVA, M_n of 130,000 g mol^{-1}, degree of hydrolysis 88) was purchased from Shanghai Petrochemical Industry Company. Methylene chloride was obtained from Chengdu Kelong Chemical Reagent Company (Sichuan, China). Isopropanol was purchased from Chengdu Jinshan Chemical Reagent Company (Sichuan, China). Span 80 was purchased from Tianjin Riujinte Chemical Reagent Company. Sodium oleate was purchased from Shanghai Chemical Reagent Company. Human Serum Albumin (HSA) was purchased from Aventis Behring Gmbh, Germany. All the chemicals used in this research were analytical reagent grade from commercial market without further purification.

2.2 Microspheres fabrication

Microspheres loading HSA were prepared by double emulsion method ($W_1/O/W_2$) based on solvent evaporation as described previously [16]. The three kinds of P(CPP-SA) with CPP/SA ratios of 20:80, 35:65, 50:50 were selected as microspheres matrix. PVA was used as emulsion stabilizing agent. First, 0.15 g P(CPP-SA) was dissolved in 3 ml methylene chloride and emulsified with 500 μl of 20% aqueous solution of HSA by magnetic stirrer for 10 min to form the stable initial emulsion (W_1/O). Then, the resultant emulsion was added dropwise into 100 ml of a 3% PVA solution and emulsified for 40 min at 700 rpm using overhead stirrer to form a double emulsion system. Finally, 50 ml of a 6% isopropanol solution was poured into the double emulsion and stirred for about 2 h in fuming cupboard. After the solvent evaporated completely, microspheres were washed three times with distilled water and collected by centrifuge (Avanti^{TM} J-301, BECKMAN COULTER) at 8,000 rpm. The resultant microspheres were freeze-dried in vacuum overnight and stored at 4°C.

2.3 Scanning electron microscopy

The surface morphology of microspheres was examined by scanning electron microscope (SEM, FEI, QUANTA 200). Several solution droplets containing microspheres were placed on the SEM sample stage. The microsphere samples were sputter coated with gold after lyophilized overnight.

2.4 Particle size analyzer

The mean size and size distribution were determined by laser diffraction particle size analyzer (LA-9200, HORIBA). Microspheres were resuspended in distilled water and ultrasonic to prevent microspheres aggregation. Then, the microsphere solution was poured into the sample tank of particle size analyzer.

2.5 In vitro degradation

Preweighed microspheres were placed individually in test tube containing 10 ml of 0.1 M PBS at pH 7.4. The tubes were kept in a thermostated shaking air bath (Haerbin Dongming Medical Equipment Company) which was maintained at 37°C and 100 cycles/min. The degradation samples were washed by distilled water to remove the residual buffer salts and were collected from the tubes by centrifugation at predetermined intervals. Then the resultant samples were dried to constant weight. The degradation medium was collected to characterize the pH change of PBS. The degradation process was estimated from the morphological change of microspheres surface, the mass loss of microspheres and the pH change of PBS at predesigned intervals. The morphological change of microspheres surface can be characterized 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 PBS medium was determined by detecting the supernate using pH meter (Shanghai Leici Instrument Company) at 25°C.

2.6 In vitro protein release

A total of 100 mg of microspheres were suspended in a test tube containing 10 ml of phosphate buffered saline (PBS, pH 7.4). These tubes were allowed to store in the same air bath as mentioned in degradation test. At predesigned intervals, 1.0 ml of supernatant was collected and 1.0 ml of fresh PBS medium was added back to the test tube. Concentration of HSA released in the supernatant was determined by measuring the absorbance at 278 nm in a UV–visible spectrumphotometer (SHIMADZU, UV-2550).

The structural integrity of HSA extracted from microspheres was detected by gel permeation chromatography (GPC, waters 2695 and 2414, Milford, MA) with an Ultrahydrogel 250 column (7.8 × 300 mm, Waters, Milford, MA), compared with native HSA. The mobile phase was distilled water with the flow rate of 0.5 ml/min. The HSA extraction from microspheres was performed by dissolving a preweighed amount of microspheres in methylene chloride followed by extracting three times with distilled water.

3 Results and discussion

The P(CPP-SA) microspheres loading HSA were prepared by double emulsion method ($W_1/O/W_2$) based on solvent evaporation. As shown in Fig. 2, the microspheres exhibited spherical structure and rough surface morphology. Some folds can be observed on microspheres surface. It was attributed to the fast degradation rate of anhydride bonds on the surface during the fabrication [17]. The fabrication of P(CPP-SA) microspheres were performed in solution while the anhydride bonds are labile in aqueous environment. As we can see from the figure, the microspheres surface morphology became smooth as the content of CPP increased. It indicated that the amount of CPP in P(CPP-SA) can also affect the surface morphology. It was attributed to the hydrolysis rate of P(CPP-SA). Since CPP anhydride was more hydrophobic than SA, 50:50 P(CPP-SA) was more hydrophobic than 20:80 P(CPP-SA) and 35:65 P(CPP-SA). The more hydrophobic the polyanhydride was, the more difficult the cleavage of anhydride bonds in aqueous environment. The P(CPP-SA) microspheres containing more CPP structural units degraded more slowly. Thereby, the 50:50 P(CPP-SA) microspheres had the smoothest surface at initial time.

The size distribution determined by laser diffraction particle size analyzer was shown in Fig. 3. The mean size of microspheres was about 10 μm and the size distribution was from 3 to 30 μm. As shown in Fig. 3, P(CPP-SA) microspheres with different monomers ratios had almost the same mean size and size distribution. So, the composition of copolymer can not affect the mean size and size distribution. The method of fabrication is the key to control the microspheres size and size distribution.

Scanning electron microscopy was carried out to observe the morphological change of microspheres incubated in PBS at pH 7.4 during the degradation. The microspheres were intact spherical structure and slight rough surface morphology before incubated into degradation media, shown in Fig. 2. The morphological change after 10 and 15 days degradation was shown in Fig. 4. The surface morphology of P(CPP-SA) microspheres with CPP/SA ratios of 20:80, 35:65, 50:50 changed after 10 days degradation shown in Fig. 4a–c, respectively. As we can see, the surface layer of microspheres was destroyed first. 20:80 P(CPP-SA) microspheres surface peeled off layer by layer shown in Fig. 4a. While, the 35:65 P(CPP-SA) and 50:50 P(CPP-SA) microspheres surface became granular in appearance shown in Fig. 4b, c, respectively. It indicated that the degradation occurred on microspheres surface first. And the features of surface erosion were obvious. After 15 days degradation, there were few microspheres can be observed in scope shown in Fig. 4d–f, respectively. The surface morphological change of 20:80 P(CPP-SA)

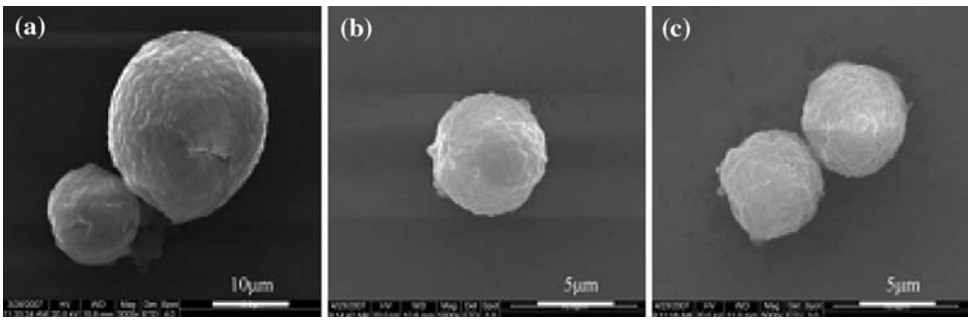

Fig. 2 The surface morphology determined by scanning electron microscopy of 20:80 P(CPP:SA) (**a**), 35:65 P(CPP:SA) (**b**), 50:50 P(CPP:SA) (**c**) microspheres entrapped HSA

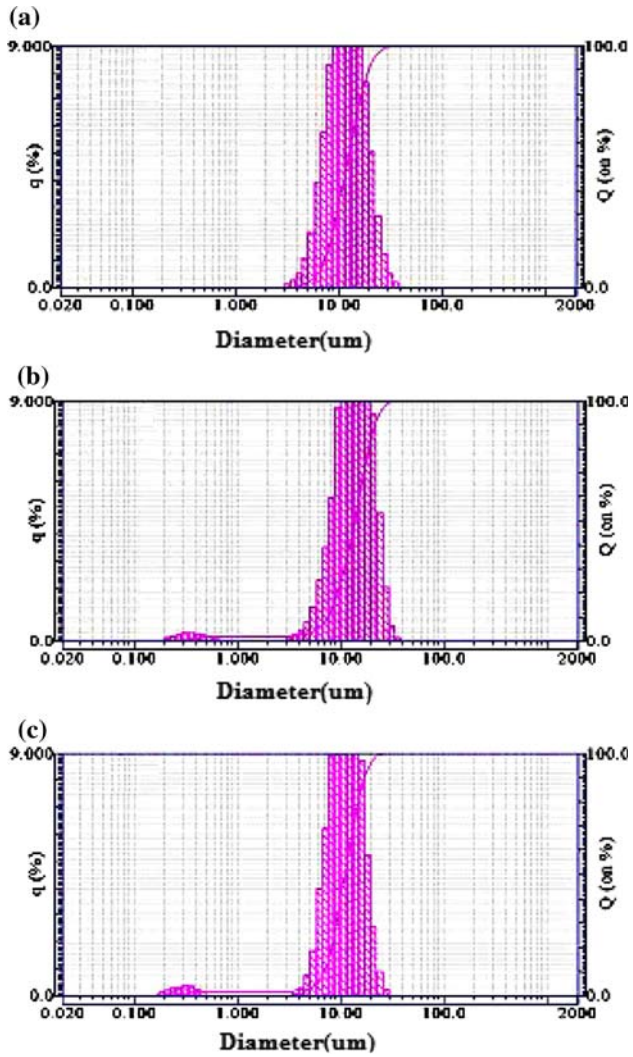

Fig. 3 The size distribution determined by laser diffraction particle size analyzer of 20:80 P(CPP:SA) (**a**), 35:65 P(CPP:SA) (**b**), 50:50 P(CPP:SA) (**c**) microspheres entrapped HSA

microspheres was clearer and faster than that ones prepared by 35:65 P(CPP-SA) and 50:50 P(CPP-SA) during the degradation. Therefore, P(CPP-SA) became more instabilization as the content of SA increased. It was because that there were more SA–SA bonds in P(CPP-SA) as increasing the content of SA. The more SA content, the more SA–SA bonds in P(CPP-SA) copolymer. SA is a kind of aliphatic anhydrides, which can degrade in a few days. Comparing with CPP–CPP bonds, SA–SA bonds are more labile in aqueous environment [18]. Thereby, degradation rate of 20:80 P(CPP-SA) microspheres was the fastest among them.

Mass loss of microspheres during the degradation was shown in Fig. 5. For microspheres prepared by P(CPP-SA) composed of different content of SA and CPP, the curves of mass loss were divided into two phases as shown in Fig. 5. The mass of microspheres decreased sharply in the initial 5 days. And then, only a slight mass loss was observed in the following period. Especially, there was no evident mass loss shown in the curves after 20 days. The mass loss of 20:80 P(CPP:SA) microspheres decreased fastest at both phases. The mass loss percent of 20:80 P(CPP-SA) microspheres, 35:65 P(CPP-SA) microspheres and 50:50 P(CPP-SA) microspheres at initial 5 days are 58.9%, 42.6%, 34.1%, respectively. The results corresponded to Fig. 4.

Degradation of the polymer designates the process of a polymer chain cleavage [19]. While erosion is the sum of all processes that lead to mass loss from polyanhydride matrix [20]. The mass loss of P(CPP-SA) microspheres in the initial phase might result from low molecular weight part of copolymer dissolving into the degradation media and the cleavage of the more labile SA–SA bonds. In the following phase, the mass loss may be due to the hydrolysis of high molecule weight copolymer into oligomers or monomers. Therefore, it also explained that the 20:80 P(CPP-SA) microspheres erode fastest.

The decrease of degradation media pH versus incubation time was displayed in Fig. 6. The pH value of degradation media decreased rapidly during the initial 5 days and then the decrease of degradation media pH slowed down. The results also agreed with Fig. 5. Finally, there was no significant pH change can be detected after 20 days. In the initial 5 days, there was little difference in pH change among P(CPP-SA) microspheres with CPP/SA ratios of 20:80, 35:65, 50:50. And then, pH value of 20:80 P(CPP-SA) microspheres decreased faster than others. After 20 days, the pH value of 20:80 P(CPP-SA), 35:65 P(CPP-

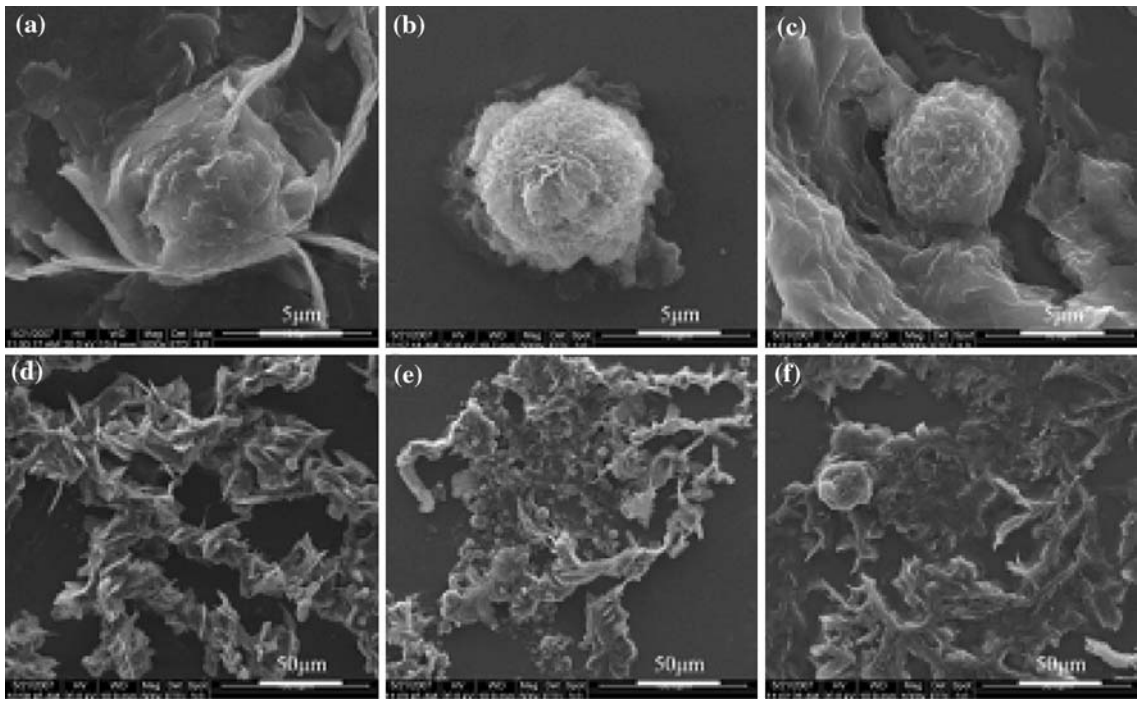

Fig. 4 The surface morphology change of microspheres detected by SEM for **a**, **d** 20:80 P(CPP:SA), **b**, **e** 35:65 P(CPP:SA), and **c**, **f** 50:50 P(CPP:SA) after 10 and 15 days degradation

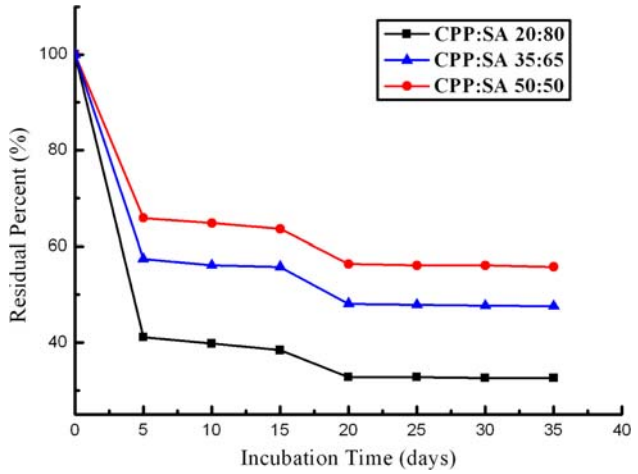

Fig. 5 The percent residual weight of polyanhydride microspheres containing HSA in PBS at pH 7.4

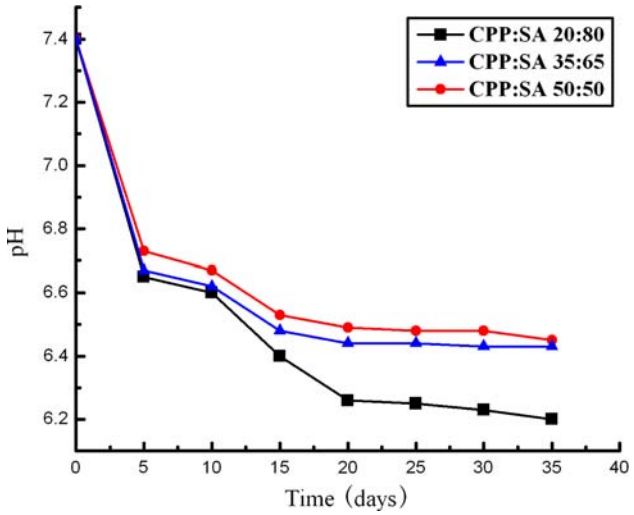

Fig. 6 The pH change of degradation media versus incubation time

SA) and 50:50 P(CPP-SA) microspheres decreased to 6.23, 6.44, 6.49, respectively. The pH change of degradation media was due to the degradation of P(CPP-SA), which generated many acidic monomers or oligomers dissolving into degradation media.

In summarization, mass loss and pH change decreased rapidly at the initial 5 days during the degradation. At this stage, labile SA–SA bonds on the microsphere surface cleaved first. While the microspheres surface eroded first and became granular in appearance. Then, water began to penetrate into the sub-surface and the hydrolysis occurred there. Thus, the microspheres eroded layer by layer time and again, which was called surface erosion. The SA–SA bonds are the most labile among the SA–SA bonds, CPP–CPP bonds and SA–CPP bonds existed in P(CPP-SA) copolymer. In the first phase, SA segments would be prior to hydrolysis compared with CPP segments owing to more hydrophilic for SA than CPP. And in the following period, the degradation presented slow-motion moment. The degradation rate is dependent on the content of CPP monomer,

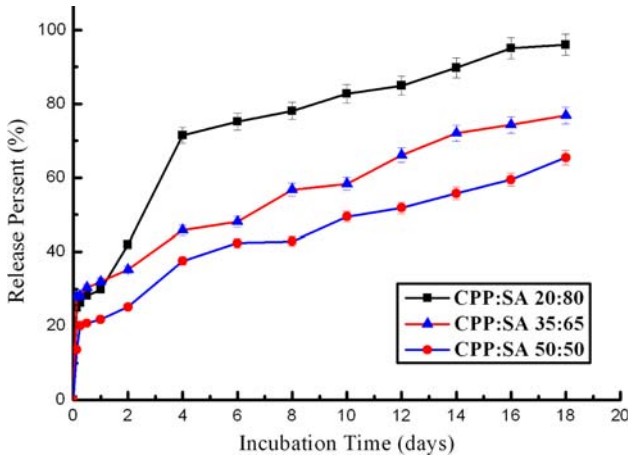

Fig. 7 Percent release of HSA from microspheres incubation in PBS at 37°C

slower degradation rate were observed for more CPP content.

In this study, HSA was encapsulated in 20:80 P(CPP-SA) microspheres, 35:65 P(CPP-SA) microspheres and 50:50 P(CPP-SA) microspheres, which exhibit different hydrophilic–hydrophobic property. As the content of SA segments increased in P(CPP-SA), the hydrophilicity of P(CPP-SA) also increased. The encapsulation efficiency of P(CPP-SA) copolymers with CPP/SA ratios of 20:80, 35:65, 50:50 microspheres is 35.32%, 35.93%, 48.58%, respectively. It indicated that the hydrophobic interaction between the P(CPP-SA) and HSA affected the encapsulation efficiency.

The percent release of protein from all kinds of microspheres against incubation time was shown in Fig. 7. The HSA release profiles of all samples consist of a burst release followed by a gradual release phase. As seen in Fig. 7, the burst release degree of P(CPP-SA) microspheres with CPP/SA ratios of 20:80, 35:65, 50:50 is 29.8%, 31.9%, 21.8%, respectively. The burst release was associated with the protein dispersing on microspheres surface or sub-surface, which was proportional to surface area of microspheres. As seen in Fig. 3, the mean size and size distribution of the three kinds of microspheres were almost same. So the amount of HSA adsorbed on microspheres surface were also

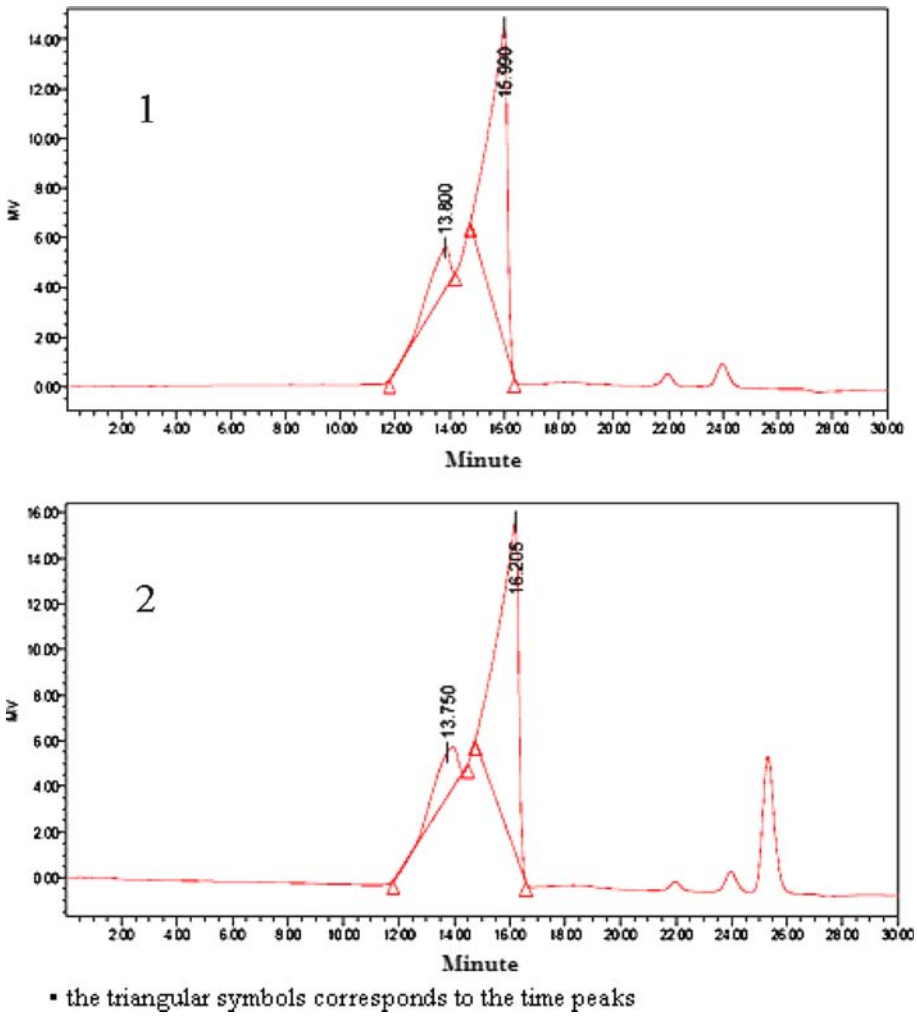

Fig. 8 The GPC results of (1) native HSA, (2) HSA extracted from 20:80 P(CPP:SA) microspheres

▪ the triangular symbols corresponds to the time peaks

same. In the following period, P(CPP-SA) microspheres with CPP/SA ratios of 20:80, 35:65, 50:50 exhibited 96%, 76.8%, 65.4% HSA gradual release, respectively. The gradual release phase was caused by HSA contained in microspheres releasing resulted from the degradation of P(CPP-SA). Therefore, the faster degradation of P(CPP-SA), the faster HSA released from the microspheres. It was also concluded that HSA release rate could be adjusted by varying the CPP/SA monomer ratios in P(CPP-SA).

The structural integrity of HSA extracted from polyanhydride microspheres was detected by gel permeation chromatography, compared with native HSA. The GPC results of all samples, which corresponded to the protein molecular weight, were almost identical. Here, the GPC result of HSA extracted from the 20:80 P(CPP-SA) microspheres was shown in Fig. 8. As we can see in Fig. 8—1, native HSA showed two major peaks at 13.800 and 16.990 min, respectively. And, HSA extracted from the 20:80 P(CPP-SA) microspheres also showed two major peaks at 13.750 and 16.205 min, respectively (Fig. 8—2). It was almost identical with the native HSA. It suggested that no remarkable chemical polymerization, non-covalent aggregation and molecular hydrolysis occurred during the encapsulating process.

4 Conclusions

The P(CPP-SA) microspheres fabricated by double emulsion method based on solvent evaporation exhibited spherical structure and rough surface morphology with the mean size 10 μm and the size distribution from 3 to 30 μm. Next, the degradation and the protein release in vitro were performed in PBS (pH 7.4). As the results shown, the P(CPP-SA) degradation and HSA release were related to CPP/SA monomer ratio. 20:80 P(CPP-SA) was more hydrophilic than 35:65 P(CPP-SA) and 50:50 P(CPP-SA). Thereby, degradation rate of 20:80 P(CPP-SA) was the fastest. And, the features of surface erosion were observed in SEM.

There are three kinds of bonds in P(CPP-SA), SA–SA bonds, CPP–CPP bonds and SA–CPP bonds. Among these bonds, the order of anhydride bonds lability was determined: SA–SA $\approx$ SA–CPP $\geq$ CPP–CPP. Therefore, The SA–SA bonds cleaved faster than other bonds in the degradation. More SA–SA bonds in P(CPP-SA) as increasing the content of SA. Hence, the P(CPP-SA) microsphere became more labile as the content of SA increased in the copolymer structure. This study also showed the degradation time and protein release time could last about 2 weeks. The degradation rate could be altered by varying the SA content in P(CPP-SA). Moreover, the release profile could also be optimized by the same way. To sum up, polyanhydride copolymers are desirable biodegradable polymer for biomedical applications as protein carriers.

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