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Research Article

Controlled Delivery Systems for Proteins Using Polyanhydride Microspheres

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A method to provide near-constant sustained release of high molecular weight, water-soluble proteins from polyanhydride microspheres is described. The polyanhydrides used were poly(fatty acid dimer) (PFAD), poly(sebacic acid) (PSA), and their copolymers [P(FAD-SA)]. P(FAD-SA) microspheres containing proteins of different molecular sizes—lysozyme, trypsin, heparinase, ovalbumin, albumin, and immunoglobulin—were prepared by a solvent evaporation method using a double emulsion. The microspheres containing proteins were spherical, with diameters of 50–125 μm, and encapsulated more than 80% of the protein, irrespective of the protein used. Enzymatic activity studies showed that encapsulation of enzymes inside polyanhydride microspheres can protect them from activity loss. When not placed inside polyanhydride microspheres, trypsin lost 80% of its activity in solution at 37°C at pH 7.4 in 12 hr, whereas inside the polyanhydride microspheres the activity loss was less than 10% under these conditions. About 47% of the enzymatic activity of heparinase encapsulated in the microspheres was lost at 37°C in 24 hr, while in solution it lost over 90% of its activity. The protein-loaded microspheres displayed near-zero-order erosion kinetics over 5 days as judged by the release of sebacic acid (SA) from the microspheres. The microspheres degraded to form SA and FAD monomers. All proteins were released at a near-constant rate without any large initial burst, irrespective of polymer molecular weight and protein loading. The period of protein release was longer than that of SA and continued protein release was observed even after the microsphere matrix had completely degraded. Differential scanning calorimetric studies demonstrated an interaction between protein and the FAD monomers produced with microsphere degradation. It is likely that the protein interaction with FAD monomers permits formation of water-insoluble protein aggregates which slowly dissolve and diffuse out of the matrix, leading to delayed protein release. For trypsin-loaded microspheres, trypsin lost 40% of its activity during microsphere preparation. Activity studies demonstrated that the sonication process was primarily responsible for activity loss. A reduction in the period of ultrasound exposure decreased the loss of protein activity to around 20%.

KEY WORDS: protein drug delivery system; controlled release; enzyme stability; polyanhydride; biodegradable microspheres.

INTRODUCTION

Controlled release of proteins has become an important area of research because recombinant DNA technology allows mass production of therapeutically important proteins (1,2). However, most proteins have very short *in-vivo* half-lives and consequently multiple injections are required in order to achieve desirable therapy. One way to increase the therapeutic efficacy of proteins is encapsulating them in a sustained-dosage form that is capable of releasing the bioactive macromolecules continuously, at a controlled rate, over long time periods.

Various polymer matrices have been utilized for controlled release of proteins (3–10). We have previously suggested that polyanhydrides may be a promising candidate for controlled drug delivery and recently reported on a modified solvent evaporation technique using a double emulsion to prepare polyanhydride microspheres for low molecular weight compounds (11). We now report on polyanhydride microspheres containing proteins that are prepared by this approach. This method permitted the preparation of microspheres that achieved near-constant release of proteins without any large initial burst. Polyanhydrides used for microsphere preparation were copolymers of fatty acid dimer (FAD) and sebacic acid (SA) with different molar ratios and molecular weights.

MATERIALS AND METHODS

Materials

The polyanhydrides used, poly(sebacic acid) (PSA), poly(fatty acid dimer) (PFAD), and their copolymers

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[P(FAD-SA)] with different polymer compositions and molecular weights as well as the copolymer poly[bis(*p*-carboxyphenoxy) propane sebacic acid anhydride] [P(CPP-SA)], were kindly supplied by Nova Pharmaceutical Corporation, Baltimore, MD, and synthesized by melt condensation (12). Physical properties of the polyanhydrides are shown in Table I. Proteins, chicken egg lysozyme (Grade I; M_w = 12,500), bovine pancreatic trypsin (Type I; M_w = 24,000), chicken egg albumin (ovalbumin; M_w = 45,000), bovine serum albumin (BSA; M_w = 66,000), and bovine immunoglobulin (Cohn fraction III; M_w = 140,000), were purchased from Sigma Chemical Company, St. Louis, MO. Heparin sodium (pocrine intestinal mucosa; activity, 170 U/mg USP) was obtained from Hepar Industries, Inc., Franklin, OH. Heparinase (M_w = 43,000) was prepared according to Ref. 13 and kindly supplied by Dr. Ramanth Sasisekharan, MIT. Poly(vinyl alcohol) (PVA; M_w = 77,000–79,000; 88% hydrolyzed) and acid orange 63 (AO; M_w = 832.80) were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Other chemical reagents were obtained from Sigma Chemical Company, St. Louis, MO, and used without further purification.

Instrumentation

The molecular weight and polydispersity of polyanhydrides were determined on a Perkin–Elmer GPC system consisting of the series 10 pump and the 3600 Data Station with refractive index detection (the LC-25 RI detector). Samples were eluted in chloroform through a Phenogel 5-μm column (Phenomenex, Torrance, CA) at a flow rate of 0.90 mL/min. The molecular weights were determined relative to polystyrene standards (Polyscience, Pennsylvania; molecular weight range of 2500 to 500,000) using CHROM 2 and GPC 5 computer programs (Perkin–Elmer).

Thermal analysis was performed on a Perkin–Elmer DSC-2 differential scanning calorimeter using a heating rate of 5°C/min.

Microsphere Preparation

Polyanhydride microspheres were prepared as reported previously (11) by a solvent evaporation method using a double emulsion. Briefly, 100 μL of aqueous solution containing the desired amount of proteins (W_1) was added to 1 mL of polymer/methylene chloride solution (O) and the mixture was emulsified by probe sonication (Model VC-250, Sonic & Material Inc.) at output 50 W for various periods of time up to 30 sec to form the first inner emulsion. Immediately, the first emulsion (W_1/O) was poured into 2 mL of 1% PVA aqueous solution saturated with methylene chloride (W_2) and vigorously mixed using a vortex mixer at maximum speed for 20 sec to form the double emulsion. Then, the resulting double emulsion was poured into another 100 mL of 0.1% PVA aqueous solution and stirred for 3 hr enabling complete evaporation of methylene chloride. The microspheres were washed several times with double-distilled water by centrifugation and freeze-dried into powdered microspheres. Polyanhydride microspheres with different proteins at different loadings as well as with some protein stabilizers (e.g., BSA, glycine, sucrose) were prepared similarly, but always the total amount of stabilizer, protein, and the polymer was adjusted to 250 mg. Five proteins (see Materials) were used for microsphere preparation and their loading in the final preparation was 1, 2, and 4%.

Table I. Physical Properties of Polyanhydrides

Polymer	Molecular weight[a] M_w	Molecular weight[a] M_n	Polydispersity	Physical properties and appearance
PSA	29,400	16,500	1.78	Fragile
P(FAD-SA)				
8:92	23,700	10,100	2.35	Flexible
25:75	42,900	17,200	2.49	Flexible
25:75	29,000	13,400	2.16	Flexible
25:75	19,700	9,400	2.10	Flexible
25:75	12,300	7,000	1.76	Flexible
44:56	18,000	9,800	1.83	Flexible
PFAD	16,100	7,500	2.15	Clear liquid
P(CPP-SA)				
20:80	36,500	16,300	2.24	Fragile

[a] Determined by GPC analysis.

Microsphere shape and size distribution were estimated using light-microscopic pictures of sample polyanhydride microspheres according to a reference scale. At least 500 microspheres per sample were examined to estimate the size distribution based on a number average.

Protein Recovery from Polyanhydride Microspheres

The amount of protein encapsulated in polyanhydride microspheres was determined by the following two methods: by recovering the protein from the microspheres and by calculating the total amount of protein released. In the former method, 2 mL distilled water was added into 2 mL methylene chloride in which the microspheres had been previously dissolved and then the protein was extracted into 2 mL distilled water. The amount of protein in the aqueous solution was determined by its absorbance at 280 nm (Perkin–Elmer UV Spectrophotometer 553). Because SA, a water-soluble degradation product of microspheres, absorbs in the UV region, it can interfere with UV analysis of proteins. To estimate the protein amount accurately, an SA standard curve at 280 nm was prepared, and based on the extinction coefficient obtained from this curve, a calculated optical density was subtracted to compensate for SA.

To examine the decrease in biological activity of trypsin during microsphere preparation, enzymatic specific activity (enzymatic activity/mg protein) was assayed after each process of microsphere preparation. The amount of trypsin was determined from its absorbance at 280 nm and the enzymatic activity was measured using *p*-toluenesulfonyl-L-alginyl-methylester (TAME) as a substrate (14).

Enzymatic Activity of Proteins in P(FAD-SA) Microspheres

Enzymatic activity of proteins in aqueous solution and in P(FAD-SA) microspheres was examined at 37°C. Trypsin, 50 μg/mL, in 0.1 *M* phosphate buffer (pH 7.4) as well as 200 mg of microspheres at a 2% trypsin loading in 4 mL of the same buffer were incubated separately at 37°C. Activity studies for heparinase was performed under the following

conditions: 50 μg/mL of heparinase in 0.1 *M* MOPS ((3-([*N*-morpholino]propane-sulfate) sodium salt) buffer (pH 7.0) with 5 m*M* calcium acetate and 50 mg of microspheres with 10 μg of heparinase in 1 mL of the same buffer at 37°C. Aliquots were taken at different time intervals to measure specific activity.

The microspheres sampled were collected by centrifugation (2000 rpm, 2 min, 4°C), washed two times with distilled water to remove salts, and freeze-dried. The dry microspheres were dissolved in 1 mL methylene chloride and the trypsin was extracted into 1 mL distilled water. The specific activity of the extracted trypsin was determined as above. The activity of heparinase was determined by measuring the UV absorbance of heparin-degraded products at 232 nm (15) and the protein amount was determined from its absorbance at 280 nm to estimate heparinase specific activity.

Polymer Degradation and Protein Release Studies

Three types of degradation studies were performed (11). In the first, polyanhydride degradation products were measured spectrophotometrically. Polyanhydride microspheres (5 mg) were suspended in 2.5 ml 0.1 *M* phosphate-buffered solution (pH 7.4) and the buffer was changed periodically to approximate perfect sink conditions. During the degradation study, the frequency of changing the buffer solutions was adjusted to ensure that the concentration of the protein and degradation products was below 10% of the saturation values at all times. The degradation kinetics were followed by measuring the UV absorbance of SA and CPP monomers in HLPC (11,16). The second type of degradation study was to estimate molecular weights of polyanhydrides before and after microsphere preparation and during degradation studies. The microspheres sampled were collected according to the method described in the above section. The dry microspheres were dissolved in chloroform to measure the molecular weight of their matrix polymer by GPC. In the third study, morphology and degradation of the microspheres were observed by SEM.

Protein release studies from polyanhydride microspheres were performed under the same conditions as the degradation studies. The amount of protein released was determined by UV absorbance at 280 nm. The degradation and release experiments were done independently in triplicate.

RESULTS

Characteristics of P(FAD-SA) Microspheres

Polyanhydride microspheres containing proteins were spherical, irrespective of the type of proteins encapsulated (Fig. 1). Size distribution measurements for several microspheres showed that more than 90% of the microspheres had diameters ranging from 50 to 125 nm.

In the present preparation method, the size of the resulting microspheres depended mainly on the mixing conditions used in the preparation of the inner emulsion. Since polyanhydride solutions were transparent, it was easy to observe the microspheres during different stages of preparation. When the inner emulsion was prepared by vortex mixing, the resulting microspheres were larger with a large inner emulsion. It was found that when prepared by probe soni-

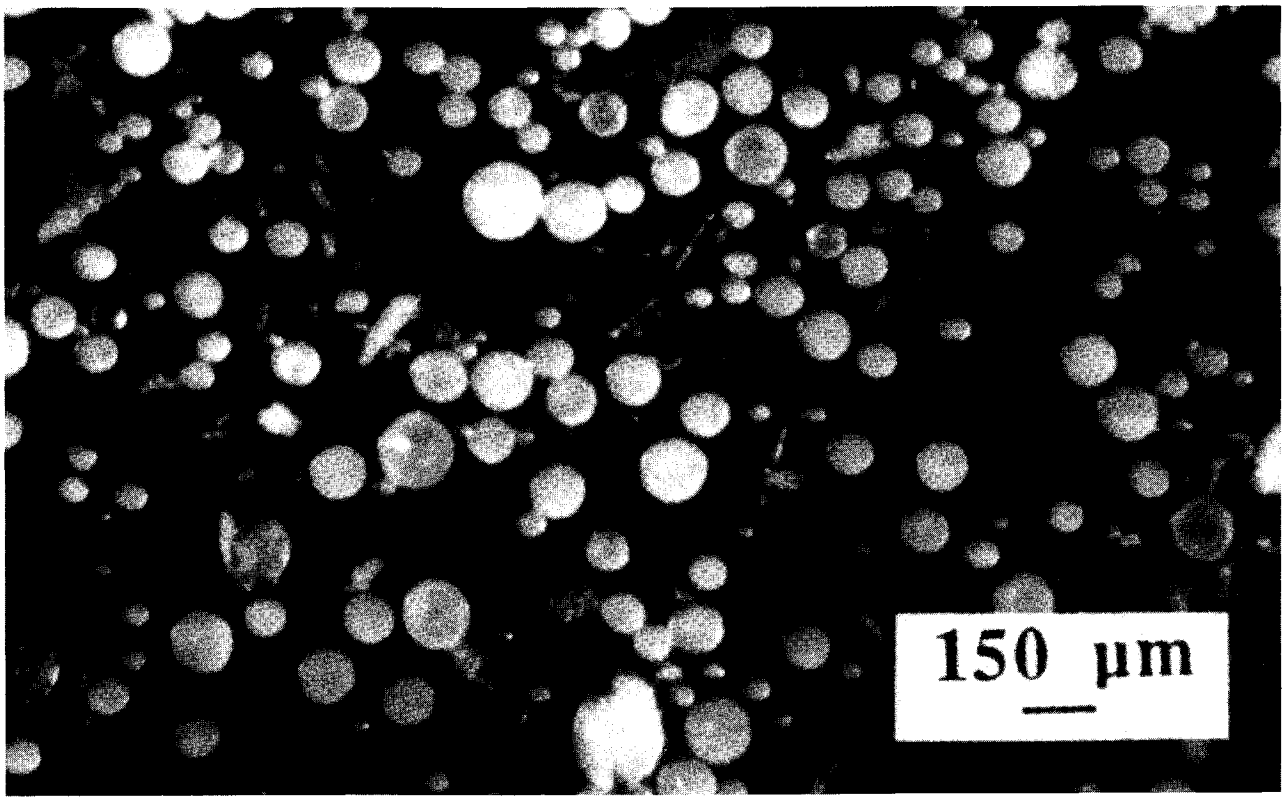


Fig. 1. Micrograph of P(FAD-SA) microspheres by phase-contrast light microscopy.

cation, a microfine inner emulsion was formed and the overall microsphere size was much smaller. Figure 1 indicates that no difference in size distribution was observed for the microspheres prepared using ultrasound exposure for more than 10 sec. However, the size of microspheres became somewhat bigger when the exposure period of sonication was less than 10 sec (data not shown). In the present experiment, the sonication exposure for the microsphere preparation was 30 sec unless otherwise mentioned. The scatter of the microsphere size among preparation procedures was small and the size distribution was not affected by the type of proteins encapsulated. The percentage protein recovered in the microspheres was about 85%, irrespective of the type of polyanhydrides and proteins. When the microspheres were dissolved in methylene chloride, it was possible to extract all protein into the aqueous phase. However, the protein amount per microspheres determined by this extraction method was less than that calculated from the total amount of protein released, although it was enough to perform the enzymatic activity studies. The latter method gave the trapping efficiency of protein to be 80% for the protein-loaded microspheres, irrespective of the type and loading of proteins used.

Enzymatic Activity of Proteins in P(FAD-SA) Microspheres

To study the enzymatic activity of proteins encapsulated in polyanhydride microspheres, trypsin was encapsulated in microspheres and its activity was followed for 2 days. The results (Fig. 2A) show that the enzyme lost less than 20% of its activity following microencapsulation. Figure 2A showed that trypsin lost 80% of its activity in solution at 37°C in 12 hr but activity loss of trypsin encapsulated in microspheres was less than 10% under these conditions. The encapsulation of trypsin inside microspheres was effective in reducing its activity loss; more than 67% of the activity was detected even after 2 days. Moreover, a similar effect of microsphere encapsulation on heparinase was observed. The heparinase entrapped inside the microspheres lost 47% of its activity in 1 day at 37°C, while heparinase in solution lost nearly its entire activity (over 90%) over this time period. This result suggests that encapsulation of proteins inside polyanhydrides may stabilize their activity.

Degradation Characteristics of P(FAD-SA) Microspheres

The release curve of sebacic acid (SA) from different polyanhydride microspheres with 2% BSA loading is shown in Fig. 2B. SA was released at a near-constant rate, although an initial lag phase before degradation was observed for the polymer with the lower sebacic acid content. The degradation rates were enhanced by copolymerization with sebacic acid. This result may be explained in terms of the higher

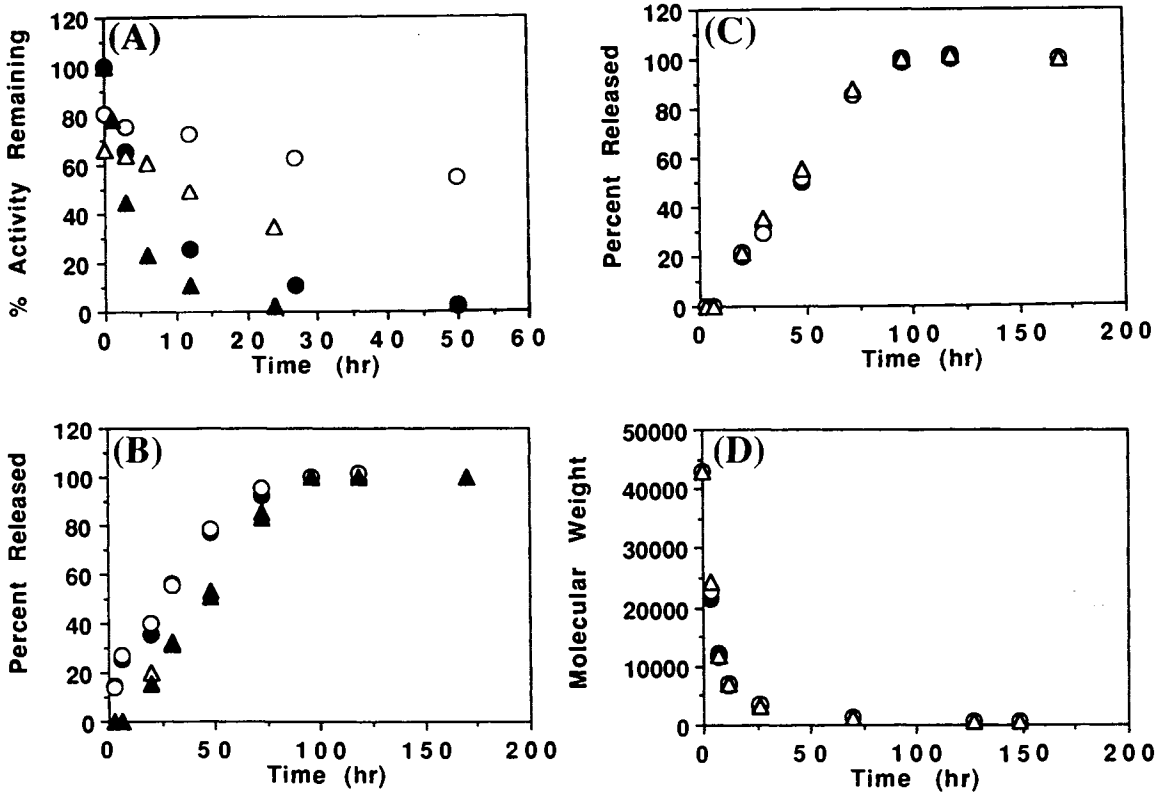


Fig. 2. (A) Stability of trypsin (circles) and heparinase (triangles) at 37°C in buffer (●, ▲) and in P(FAD-SA) 25/75 microspheres (○, △). Sonication period, 10 sec; M_w = 42,900. (B) Degradation profiles of P(FAD-SA) microspheres with different monomer compositions in 0.1 *M* phosphate buffer, pH 7.4, at 37°C: (○) PSA, (●) P(FAD-SA) 8/92, (△) P(FAD-SA) 25/75, and (▲) P(FAD-SA) 44/56 microspheres (2% BSA loading). (C) Degradation profiles of P(FAD-SA) 25/75 microspheres with different proteins in 0.1 *M* different buffers at 37°C: (○) trypsin, (●) BSA, and (△) immunoglobulin. M_w = 42,900; 2% protein loading. (D) Molecular weight change of P(FAD-SA) 25/75 microspheres in 0.1 *M* phosphate buffer at 37°C: (○) trypsin, (●) BSA, and (△) immunoglobulin (2% protein loading).

hydrophobicity of FAD compared to SA. No effect of the protein type on the erosion profile of microspheres was observed (Fig. 2C). The erosion rate of P(FAD-SA) microspheres was not altered by increasing the protein loading from 2 to 8% (data not shown).

Figure 2D shows a typical curve of molecular weight loss of P(FAD-SA) constituting microspheres with 2% loadings of trypsin, BSA, or immunoglobulin. The molecular weight of the matrix polymers decreased rapidly within the initial 72 hr and, after 127 hr in buffer, became about 600, which corresponds to the molecular weight of FAD monomer. Moreover, the molecular weight of the original P(FAD-SA) was 42,900 and no loss of polymer molecular weight was observed during microsphere preparation. In addition, no difference in degradation profiles of the microsphere matrix itself was observed between the microspheres, irrespective of the exposure period of ultrasound during microsphere preparation, compared from the standpoint of SA release and polymer molecular weight (data not shown).

SEM Observation of P(FAD-SA) Microspheres

Figure 3 shows scanning electron micrographs of P(FAD-SA) microspheres with loadings of 2% BSA at different degradation stages. The microspheres were prepared from P(FAD-SA) of molecular weight 42,900 and a 25/75 FAD/sebacic acid molar ratio. Immediately after preparation, the microspheres are spherical in shape and the external surfaces appear smooth (Fig. 3A). No traces of protein are found on the outer surfaces. A cross section of the microspheres before degradation revealed a homogeneously dense structure (Fig. 3B). A SEM photograph of the microsphere cross section after 44 hr of degradation shows that only the microsphere surface was attacked. The surface possessed small pores and an irregular structure (Fig. 3C). A cross section of the microspheres after 44 hr (Fig. 3D) showed that a dense internal core remained in the central part of the microsphere matrix. After 130 hr of degradation (Fig. 3E), the spherical shape of the microspheres was no longer observed, and instead some oily substances remained. A similar profile of microsphere degradation was observed for microspheres with different proteins. Again, the presence of protein in the microsphere matrix had no effect on the degradation profile of the microspheres.

Release Characteristics of P(FAD-SA) Microspheres

Release curves for the BSA-loaded microspheres prepared from P(FAD-SA) with different molecular weights are shown in Fig. 4A. BSA was released from microspheres at a near-constant rate without any large initial burst. No effect of polymer molecular weight on BSA release was observed. Figure 4B shows the influence of loading on BSA release from the microspheres. Differences in BSA loading had no effect on the release profile, although there was slightly faster release from the microspheres with the highest loading.

Figure 4C shows the release profile of various proteins from P(FAD-SA) microspheres. Every protein was released at a near-constant rate without any large initial burst and similar release rates were observed for the microspheres containing different proteins.

In Figure 4D, BSA release from different polyanhydride microspheres is shown. The release rate from the P(FAD-SA) microspheres decreased with increasing amounts of FAD in the copolymer. This may be due to the more hydrophobic nature of the FAD monomer. Release periods of several days to a couple of weeks were possible for microspheres of an injectable size (<150 μm).

Correlation of Degradation and Release Characteristics of P(FAD-SA) Microspheres

Figure 5A shows AO, BSA, and sebacic acid release from P(FAD-SA) microspheres. For microspheres containing low molecular weight, water-soluble AO, the AO release pattern followed closely that of polymer degradation and both are almost complete in 100 min. In addition, SEM observations suggested surface erosion of the microspheres (Fig. 3). These results indicate that diffusional escape of AO from the microsphere matrix is minimal and that the release is controlled by matrix degradation. Interestingly, a much longer release of BSA was observed from the P(FAD-SA) microspheres, although they exhibited a similar sebacic acid release profile to that of the AO-loaded microspheres.

AO and BSA release as well as sebacic acid release from PSA microspheres is shown in Fig. 5B. Both AO and BSA were released at a near-constant rate without any initial burst, and no difference in release rates was observed. The release profile of AO and BSA closely followed that of sebacic acid from the AO- and BSA-loaded microspheres, respectively, although the SA release from both microspheres was similar. AO and BSA release from P(CPP-SA) microspheres was also examined (Fig. 5C). Although AO tended to be released somewhat faster than BSA, no significant lag of BSA release compared to AO release was observed (Fig. 5A). CPP was released similarly from the two types of microspheres, irrespective of substances released. Although CPP release lags behind the AO and BSA release, the correlation between the two profiles is clear.

Degradation studies of P(FAD-SA) microspheres by GPC and SEM demonstrated that the FAD monomers produced with microsphere degradation were left behind because of their oily nature and poor water solubility in contrast to highly water-soluble SA monomers. Thus, it is possible that an interaction between the FAD monomers and the protein molecules may cause continued protein release even after the polymer matrix has completely degraded. DSC analysis was performed to investigate the interaction of proteins to FAD monomers. An endotherm was observed for unencapsulated BSA around 60°C, corresponding to the melting of the crystalline region of the protein. Degradation studies demonstrated after 130 hr in buffer the microspheres were completely degraded to remnants composed of the protein and FAD monomers. In order to simulate the physicochemical situation in the microsphere matrix after complete disappearance of SA monomers from the polymer matrix, a similar procedure of microsphere preparation was performed using BSA aqueous solution and methylene chloride solution with or without FAD monomers in place of P(FAD-SA). The resulting powdered BSA also displayed an endotherm around 60°C, although the endotherm became somewhat broader. Interestingly, the BSA-released endotherm disap-

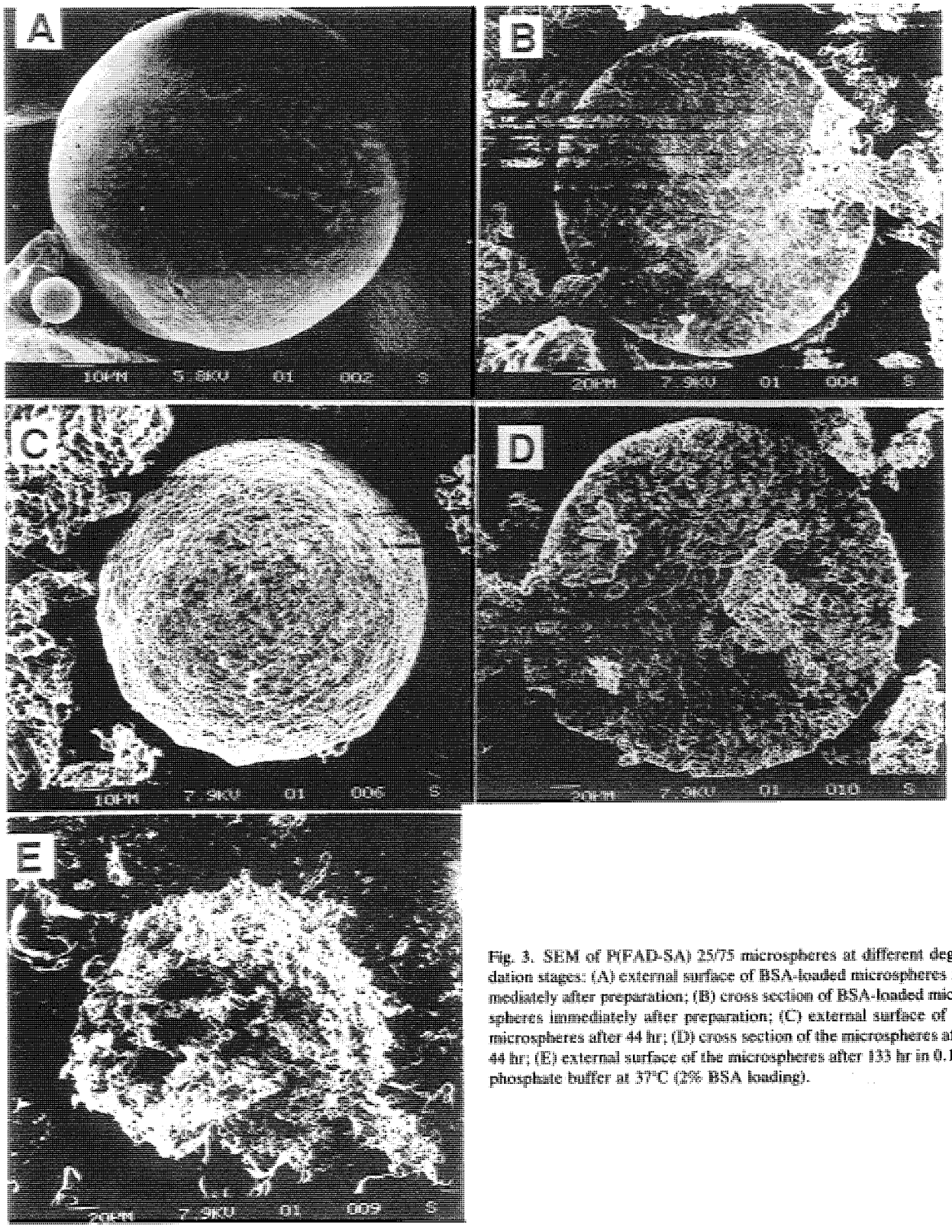


Fig. 3. SEM of P(FAD-SA) 25/75 microspheres at different degradation stages: (A) external surface of BSA-loaded microspheres immediately after preparation; (B) cross section of BSA-loaded microspheres immediately after preparation; (C) external surface of the microspheres after 44 hr; (D) cross section of the microspheres after 44 hr; (E) external surface of the microspheres after 133 hr in 0.1 *M* phosphate buffer at 37°C (2% BSA loading).

peared when FAD monomers coexisted with BSA during microsphere preparation, suggesting the possibility of an interaction between BSA and FAD monomers. In addition, FAD monomers did not exhibit any peak over a temperature range from −25 to 150°C.

Enzymatic Activity of Trypsin During Microsphere Preparation

In order to examine the activity loss of trypsin during the microsphere preparation process, the remaining activity

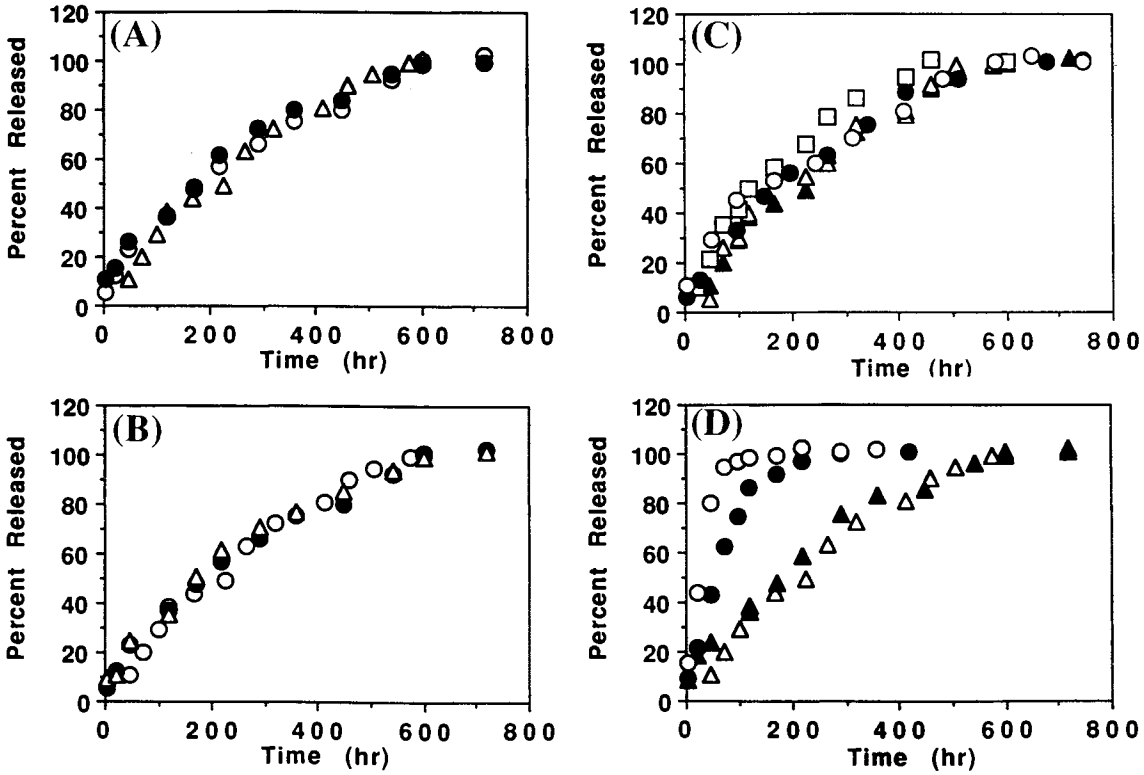


Fig. 4. (A) Release of BSA (2% loading) from microspheres of P(FAD-SA) 25/75 of different molecular weights in 0.1 *M* phosphate buffer at 37°C: (○) M_w = 12,300, (●) M_w = 29,000, and (△) M_w = 42,900. (B) Release of BSA from P(FAD-SA) 25/75 microspheres at different AO loadings in 0.1 *M* phosphate buffer at 37°C; (○) 1%, (●) 2%, and (△) 4% BSA loadings (M_w = 19,700). (C) Release of proteins from P(FAD-SA) 25/75 microspheres in 0.1 *M* phosphate buffer at 37°C: (○) lysozyme, (●) trypsin, (△) ovalbumin, (▲) BSA, and (□) immunoglobulin. M_w = 42,900; 2% protein loading. (D) Release of BSA (2% loading) from microspheres of various polyanhydrides in 0.1 *M* phosphate buffer at 37°C: (○) PSA, (●) P(FAD-SA) 8/92, (△) P(FAD-SA) 25/75, and (▲) P(FAD-SA) 44/56 microspheres.

was assayed after each process of microsphere preparation. The result is shown in Table II. The trypsin loading for the microspheres was 2%. It is apparent that most of the trypsin activity was lost by the process of probe sonication for 30 sec (step C), in contrast to other processes occurring during microsphere preparation. Neither contact of trypsin aqueous solution with methylene chloride solution nor the freeze-drying process brought about a significant activity loss (steps B, D, and E). In addition, the percentage of trypsin activity remaining in the microspheres was around 58% when assayed after washing prior to lyophilization, indicating no activity loss through the washing processes of the microspheres.

Table III shows the effect of ultrasound exposure period on trypsin activity loss during microsphere preparation. The activity remaining of trypsin increased from 58.9 to 80.5% as the exposure period of sonication was reduced from 30 to 10 sec. Moreover, 10 and 30 sec of sonication of the trypsin solution without methylene chloride reduced its activity to 56.2 and 78.0%, respectively. This indicates that the presence of methylene chloride hardly affects the loss of trypsin activity in the sonication process. It is likely that ultrasound exposure is the cause of protein activity loss in the present experiment. We also examined the effect of stabilizers on protein activity loss during sonication. In the present study, BSA, glycine, and sucrose, known protein stabilizers, were used (17). However, no reduction in activity loss was achieved.

DISCUSSION

Polyanhydrides have been extensively studied as vehicles for the release of bioactive molecules (3,7,11,16,18,19). In particular, the polyanhydride P(CPP-SA) has been used, experimentally as well as clinically, for the treatment of neurological disorders and brain tumors (18,19). However, the polymer becomes brittle and eventually fragments after exposure to water. This property may cause water-soluble drugs to be released more rapidly than the polymer erodes at high drug loadings. Copolymers of fatty acid dimer (FAD), derived from naturally occurring oleic acid and sebacic acid (SA), P(FAD-SA), have been developed as new polyanhydrides as an optimal carrier for water-soluble compounds. Films of P(FAD-SA) were flexible, with a low melting point, but became soft and left oily, poorly water-soluble FAD monomers after exposure to 0.1 *M* phosphate buffer solution pH 7.4 at 37°C. Near-constant release of water-soluble dyes *in vitro* has been reported from microspheres (11). In addition, the polymer has shown to be capable of controlled release of some water-soluble drugs in the rat brain (20).

The present study demonstrates that the solvent evaporation method using a double emulsion is promising for

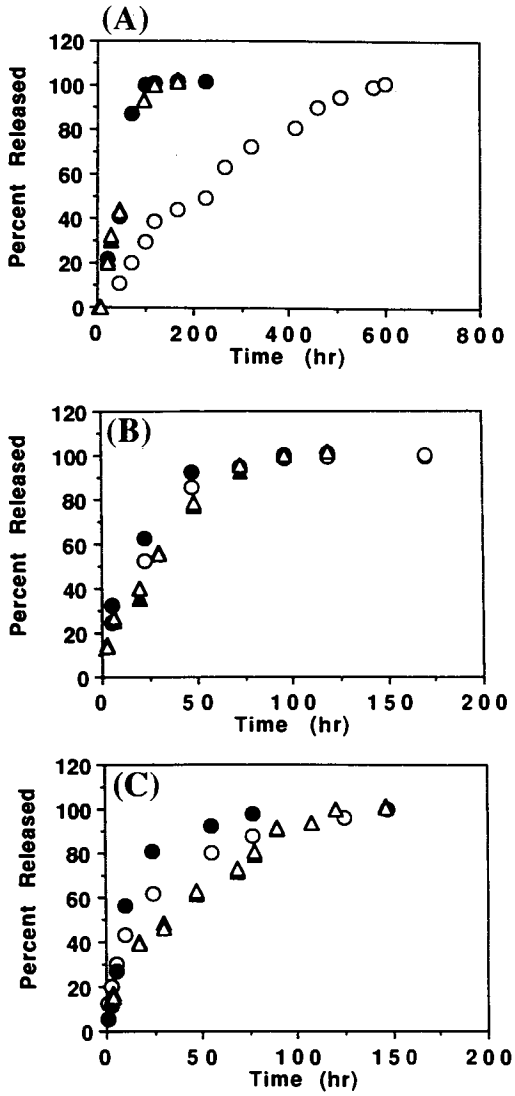
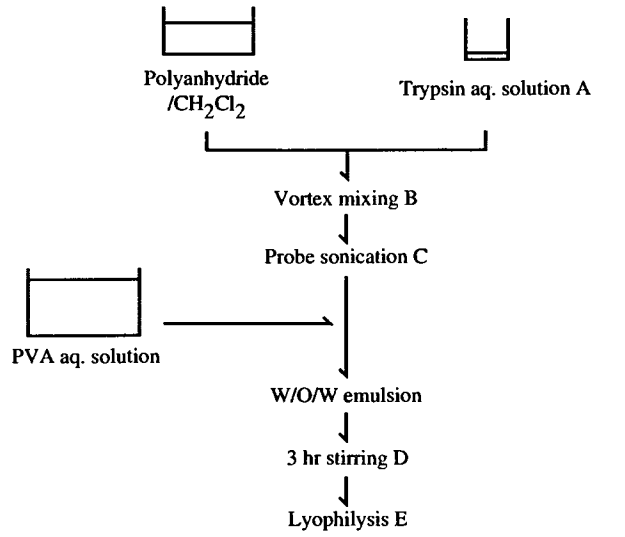


Fig. 5. (A) Drug release (circles) and SA release (triangles) profiles of P(FAD-SA) 25/75 microspheres in 0.1 M phosphate buffer at 37°C: (○, △) BSA-loaded and (●, ▲) AO-loaded microspheres. M_w = 42,900; 2% drug loading. (B) Release of drug (circles) and SA (triangles) from PSA microspheres in 0.1 *M* phosphate buffer at 37°C: (○, △) BSA-loaded and (●, ▲) AO-loaded microspheres. M_w = 42,900; 2% drug loading. (C) Release of drug (circles) and CPP (triangles) from P(CPP-SA) 20/80 microspheres in 0.1 *M* phosphate buffer at 37°C: (○, △) BSA-loaded and (●, ▲) AO-loaded microspheres. M_w = 42,900; 2% drug loading.

protein microencapsulation. This microencapsulation procedure is reproducible with respect to yield and size distribution. The recovery of microspheres is very high (85–90%) and the trapping efficiency of protein was around 80%, irrespective of the type of protein used. P(FAD-SA) microspheres were spherical, with a smooth outer surface, and more than 90% of the microspheres had diameters in the range 50–125 mm (Figs. 1 and 3A). This size distribution was greatly dependent on the sonication conditions for the preparation of the inner emulsion. No change in the size distribution was observed by altering the exposure period of sonication in the range from 10 to 30 sec. However, exposure

Table II. Activity Loss of Trypsin in Each Step of Microsphere Preparation



Step		Activity remaining (%)
Original trypsin	A	100.0
After vortex mixing	B	100.0
After probe sonication	C	58.9
After 3 hr of stirring	D	56.0
After lyophilization	E	59.2

periods of less than 10 sec did not lead to the preparation of microspheres of an injectable size (<150 mm). In this microsphere preparation, we tried to minimize the degradation of polyanhydrides by their contact to water and sonication exposure during the process because the polymers possess a water-labile linkage and sonication enhances polymer degradation (21) and loss of protein activity (Tables II and III). GPC studies demonstrated no loss of the polymer molecular

Table III. Activity Loss of Trypsin During Microsphere Preparation

Preparation conditions		Microsphere	
Period of sonication (sec)	Stabilizer (wt %)	Trapping efficiency (%)	Activity remaining (%)[a]
10	None	83.2	80.5
20	None	81.5	67.3
30	None	82.3	58.9
30	BSA (5)	85.6	58.6
30	BSA (10)	82.7	58.1
30	Glycine (7.5)	80.5	59.8
30	Glycine (15)	81.6	57.2
30	Sucrose (4)	83.2	58.7
30	Sucrose (8)	84.5	56.6
30	BSA (10) + glycine (15) + sucrose (8)	82.1	60.0

[a] Percentage of specific activity of trypsin to that of original trypsin solution.

weight due to exposing the polymer to an aqueous phase for 4 hr (the maximum time for the whole process of microsphere preparation) or ultrasound for a short time period, e.g., 30 sec. Moreover, the encapsulation of trypsin or heparinase inside P(FAD-SA) microspheres was effective in reducing their biological activity loss (Fig. 2A). This may be explained in terms of protein hydration. It is possible that the microspheres become hydrated very slowly, preventing protein–water interactions or protein–protein interactions. This may lead to the prevention of protein denaturation by encapsulation at 37°C. However, depending on the stability of the protein, it may be necessary to understand the mechanisms of protein inactivation and to use this information to design more stable formulations (22,23).

As proteins have a high molecular weight and are water soluble, while polyanhydrides are soluble only in the organic phase, we have searched for preparation methods that combine both organic and water phases. The double-emulsion method was chosen. Various proteins were released from injectable microspheres at a near-constant rate without any large initial burst in release (Fig. 4C); release profiles were dependent on the polymer which constitutes the microspheres (Fig. 4D). In addition, no difference in release profiles of proteins was observed for P(FAD-SA) microspheres which were prepared by different periods of ultrasound exposure ranging from 10 to 30 sec (data not shown).

Changes in the biological activity of proteins should be carefully considered during microsphere preparation because the microencapsulation includes many processes which may affect their activity, e.g., exposure of the proteins to organic phase, ultrasound, and freeze-drying. Protein activity studies demonstrated that trypsin's enzymatic activity was lost mainly by the sonication process during microsphere preparation, while the contact of trypsin to the methylene chloride phase as well as freeze-drying had no major effect on trypsin activity (Table II). Table III demonstrated that a shorter period of ultrasound exposure reduced trypsin's activity loss. This suggests that the best way to prevent activity loss of proteins is to minimize their exposure to ultrasound. However, the sonication process is critical for the preparation of these microspheres. It has been known that ultrasound has an effect on the physicochemical properties of macromolecules in solutions due to a number of factors, such as temperature, mixing, and cavitation (24). The degradation of proteins in aqueous solution was reported to be associated with the presence of cavitation (25). However, it is possible that the sensitivity to ultrasound exposure is different among proteins. Thus, current studies are directed toward a more detailed examination of mechanisms of activity loss of various proteins by ultrasound exposure.

The degradation profiles of protein-loaded polyanhydride microspheres was similar to that of the microspheres containing low molecular weight dyes (11). Complete loss of sebacic acid from the microspheres was observed within about 90 hr, irrespective of the type of protein encapsulated (Fig. 2C). The microspheres were degraded to form oily, poorly water-soluble FAD monomers (Figs. 2D and 3). These findings demonstrate that the type of drug encapsulated has no effect on the microspheres' degradation profiles based on SA release.

Although a close correlation between AO and sebacic acid (SA) release was observed for P(FAD-SA) microspheres, BSA release lagged behind SA release (Fig. 5A). However, no difference in release profiles between AO and BSA was observed for microspheres prepared from both PSA and P(CPP-SA), which did not have FAD components in the polymer (Figs. 5B and C). It is therefore likely that an interaction of BSA and FAD monomers may cause continued protein release even after the P(FAD-SA) microsphere matrix has completely degraded. A similar phenomenon has been found for the release of AO and gelatin from P(FAD-SA) microspheres. It was demonstrated that the delayed release resulted from the specific interaction between gelatin and FAD monomers produced with microsphere degradation, while there was no interaction between AO and the FAD monomers (11). BSA has a crystalline structure (26) and DSC showed an endotherm related to the melting of the crystalline region of unencapsulated BSA. However, the endotherm of powdered BSA produced via the process of microsphere preparation was broader. This may be explained by a structural change of BSA which may be induced by its exposure to methylene chloride or ultrasound. It is possible, however, that the crystalline region of BSA is not completely destroyed. Interestingly, the addition of FAD to the methylene chloride phase led to the complete disappearance of the BSA-related endotherm. This result demonstrates the possibility that there is an interaction between BSA and FAD monomers. It has been reported that serum albumin interacts with fatty acids and related compounds (27). Similar results were obtained for proteins other than BSA (data not shown). In addition, the procedure of microsphere preparation using BSA aqueous solution and FAD monomers/methylene chloride solution provided tiny solid masses with poor water solubility. These findings suggest that the interaction of proteins with FAD monomers permits formation of water-insoluble protein aggregates which slowly dissolve and diffuse out of the matrix, leading to delayed protein release from P(FAD-SA) microspheres.

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