

Stabilization and Controlled Release of Bovine Serum Albumin Encapsulated in Poly(D, L-lactide) and Poly(ethylene glycol) Microsphere Blends

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Purpose. The acidic microclimate in poly(D, L-lactide-co-glycolide) 50/50 microspheres has been previously demonstrated by our group as the primary instability source of encapsulated bovine serum albumin (BSA). The objectives of this study were to stabilize the encapsulated model protein, BSA, and to achieve continuous protein release by using a blend of: slowly degrading poly(D, L-lactide) (PLA), to reduce the production of acidic species during BSA release; and pore-forming poly(ethylene glycol) (PEG), to increase diffusion of BSA and polymer degradation products out of the polymer.

Methods. Microspheres were formulated from blends of PLA (Mw 145,000) and PEG (Mw 10,000 or 35,000) by using an anhydrous oil-in-oil emulsion and solvent extraction (O/O) method. The polymer blend composition and phase miscibility were examined by FT-IR and DSC, respectively. Microsphere surface morphology, water uptake, and BSA release kinetics were also investigated. The stability of BSA encapsulated in microspheres was examined by losses in protein solubility, SDS-PAGE, IEF, CD, and fluorescence spectroscopy.

Results. PEG was successfully incorporated in PLA microspheres and shown to possess partial miscibility with PLA. A protein loading level of 5% (w/w) was attained in PLA/PEG microspheres with a mean diameter of approximately 100 μm . When PEG content was less than 20% in the blend, incomplete release of BSA was observed with the formation of insoluble, and primarily non-covalent aggregates. When 20%–30% PEG was incorporated in the blend formulation, *in vitro* continuous protein release over 29 days was exhibited. Unreleased BSA in these formulations was water-soluble and structurally intact.

Conclusions. Stabilization and controlled release of BSA from PLA/PEG microspheres was achieved due to low acid and high water content in the blend formulation.

KEY WORDS: poly(D, L-Lactide); poly(ethylene glycol); bovine serum albumin; microspheres.

INTRODUCTION

Biodegradable polymers have been investigated extensively to provide controlled release from days to months for

therapeutic proteins and vaccine antigens (1). The drug release mechanism from poly(D, L lactide-co-glycolide) (PLGA) microspheres can be based on diffusion, polymer degradation, and or osmotically mediated events (1). A major limitation of this delivery system is the common formation of an acidic microclimate within the polymer device during release due to polymer degradation, which leads to destabilization of encapsulated acid-labile bio-macromolecules. Previous work in our lab demonstrated that the acidic microclimate and intermediate water content existing in PLGA 50/50 delivery systems were the major causes of instability of encapsulated bovine serum albumin (BSA) (2). By co-encapsulating poorly water-soluble basic additives such as $\text{Mg}(\text{OH})_2$, the acidic microclimate was neutralized and encapsulated BSA was stabilized for more than 1 month (2,3). Another two therapeutic proteins, recombinant human basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) also showed enhanced stability in the polymer following neutralization of the acidic microclimate (2).

In this study, instead of neutralizing the acidic microclimate, a strategy to avoid acid build-up was devised. A blend of: slowly degrading poly(D, L-lactide) (PLA), to reduce the production of acidic species during protein release; and water-soluble poly(ethylene glycol) (PEG), to increase diffusion of the protein and polymer degradation products, were used to modify the microsphere microclimate and protein release behavior. PLA has a much slower degradation rate than PLGA 50/50 due to its higher hydrophobicity and the steric hindrance for the water attack on the ester bond introduced by the methyl group of lactic acid (4). Likewise, PLA has been shown to produce acidic by-products and release monomers at a reduced rate relative to the glycolic-containing copolymers (5,6). For example, the monomer content released from PLA has been reported to be ~14-fold less than PLGA 50/50 over a 4-week incubation in buffered media (6). However, like PLGA 50/50, PLA will still cause an eventual acid build-up. In addition, its slow degradation may lead to slow and discontinuous release of proteins and its strong hydrophobicity has been suggested to possibly denature proteins (7). Therefore, the second component, relatively more hydrophilic PEG, is introduced into PLA to adjust the microsphere acidity, hydrophobicity, and permeability. PEG is nontoxic and soluble in numerous organic solvents and water. During release, PEG can be soluble in the release medium, resulting in the formation of swollen structure with high water content in the polymer blend. This swollen polymer structure is expected to increase exchange of polymer degradation products with the surrounding medium, minimizing the risk of acid-induced protein degradation. Moreover, before excessive PLA degradation occurs, aqueous pores formed by PEG dissolution are expected to increase diffusion of the encapsulated protein, providing continuous protein release.

The use of polymer blends has been an appealing approach for controlled drug delivery. PLGAs have been blended with various poloxamers and PEGs to improve protein delivery capacity and obtain controlled release profiles (8–12). Moreover, blends of PLGA with PEG fabricated by a water-in-oil-in-oil method showed improved ovalbumin loading and stability (12). However, in the above formulations,

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ABBREVIATIONS: BSA, bovine serum albumin; DTT, dithiothreitol; PLGA, Poly(D, L-lactide-co-glycolide); PLA, Poly(D, L-lactide); PEG, Poly(ethylene glycol); PBST, phosphate buffer saline containing 0.02% Tween 80®; GnCl, Guanidine-HCl; W/O/W, water-in-oil-in-water double emulsion-solvent evaporation; O/O, oil-in-oil single emulsion-solvent extraction.

incomplete release and aggregation of the protein from microsphere formulations were still observed.

The PLA-PEG microspheres studied here were prepared by an oil-in-oil emulsion and solvent extraction (O/O) method, instead of the most commonly used water-in-oil-in-water double emulsion and solvent evaporation (W/O/W) method. The former approach generally results in high protein entrapment levels and superior protein stability due to the absence of water (13,14). A model protein antigen, bovine serum albumin (BSA), was selected and encapsulated in the polymer blend. Acid-labile BSA has been used as a model protein to examine the acidic microclimate in PLGA microspheres (2). In addition, its structure and antigenicity are well documented (15,16). In this report, we describe the stabilization and slow release of BSA from PLA-PEG microspheres without any observed acid-induced instability.

MATERIALS AND METHODS

Chemicals

Poly(D, L-lactide) with inherent viscosity of 1.07 dl/g (Lot 112-15-1, Mw 145,000; polydispersity 1.50) in CHCl_3 was from BPI (Birmingham, AL). Poly(ethylene glycol) with molecular weight 10,000 (Lot 14904BU) and 35,000 (Lot 403392) was obtained from Aldrich Chem. Co. (Milwaukee, WI) and Fluka (Milwaukee, WI), respectively. Bovine serum albumin (A-3059, Lot 32H0463) was purchased from Sigma Chemical Co. (St. Louis, MO). Protein molecular weight and pI standards for electrophoresis were from Pharmacia LKB (Piscataway, NJ). All other biochemicals and chemicals were of analytical grade or purer and obtained from commercial suppliers.

Microsphere Preparation

The polymeric microspheres were prepared by an anhydrous O/O method. First, PLA and PEG at various weight ratios were co-dissolved in the acetonitrile at a total polymer concentration of 20% (w/v). Sieved BSA dry powder (< 20 μm) was suspended in acetonitrile-polymer solution and homogenized at 15,000 rpm in an ice bath. Then the antigen suspension was added drop-wise into the continuous phase (cottonseed oil containing 1.6% (W/V) span 85) stirred at 750 rpm with an overhead stirrer. After 5 h, petroleum ether (b.p. 50–110°C) was poured into the cottonseed oil bath to extract the remaining acetonitrile from the polymer. After an additional 15 min of stirring, the microspheres were filtered, washed with 250 ml of petroleum ether and lyophilized.

Microsphere Characterization

Morphology and Particle Size Determination

The microspheres were coated with gold-palladium by using Pelco Model 3 Sputter Coater 91000 (Ted Pella, Inc., Redding, CA). Surface morphology of the microspheres was examined by a Philips XL Scanning Electron Microscope (FEI, Deutschland Gmb H, Germany). Particle size was estimated by averaging diameters of 50 microspheres.

Polymer Composition Analysis by FTIR

The composition of microspheres prepared from different blends of PLA and PEG was analyzed by infrared spectroscopy. A Nicolet protégé 460 (Thermo Nicolet, Madison, WI) was used to obtain the spectra (32 scans per sample, over 600–4000 cm^{-1}) for the samples. A series of PLA and PEG physical mixtures with different weight ratios was used to make a calibration curve. Samples were dissolved in chloroform and casted into a sodium chloride cell. The composition of the microparticles was estimated by comparing peak height ratios corresponding to the carbonyl (C = O) band of PLA at 1757 cm^{-1} and the CH_2 band at 2876 cm^{-1} due to the PEG component, and assuming a negligible content of span 85 surfactant in microspheres.

Polymer Phase Behavior Analysis by DSC

Samples (3–5 mg) were loaded into aluminum pans and DSC thermograms were recorded by a Perkin-Elmer DSC 7 Differential Scanning Calorimeter (Norwalk, CT). Nitrogen gas was the sweeping gas and the heating rate was 20°C/min.

Determination of Microsphere Loading

The amount of antigen encapsulated in microspheres was determined by recovering the protein from the microspheres. First, acetone was added to the microspheres to dissolve the polymer. The mixture was vortexed, centrifuged, and then supernatant was removed. After the removal of polymer was repeated three times, the remaining protein pellet was air dried and reconstituted in phosphate buffer saline pH 7.4 containing 0.02% Tween 80® (PBST) and protein content was determined by the Coomassie Plus method (Pierce Chem Co., Rockford, IL)

Evaluation of Model Antigen Release from Microspheres

Samples of 10 mg microspheres were suspended in 1 ml PBST. The suspension was incubated at 37°C under mild agitation. At pre-selected intervals, release media were removed for determination and replaced with fresh buffer. The amount of protein released was assayed by the Coomassie Plus method (Pierce). At the end of release, microspheres were collected and remaining soluble protein in the microspheres was analyzed as described in the section "Determination of Microsphere Loading." Any insoluble protein aggregates were collected by centrifugation and reconstituted in denaturing agent (8 M Urea or 6 M Guanidine-HCl (GnCl)). Determination of any aggregates soluble in denaturing agent gave the amount of non-covalently bonded aggregates. With the further addition of reducing agent (10 mM DTT + 1 mM EDTA), any disulfide-bonded aggregates were dissolved. The total dissolved portion in denaturing and reducing agents gave the total amount of non-covalent and disulfide-bonded aggregates.

pH Change in the Release Medium During Release

The pH of the release medium was monitored by a Corning 430 pH meter (Corning, NY) at each sampling interval.

Water Uptake of Microspheres

After incubation at 97% relative humidity and 37°C, microspheres were taken out and weighed immediately. The water uptake of microspheres was estimated by:

$$\text{Water uptake (\%)} = (W_1 - W_2) / W_2 \times 100\%$$

Where W_1 and W_2 are the weights of the hydrated microspheres and microspheres before incubation, respectively. No corrections were made for inter-particle water content in W_1 or the water content within lyophilized microspheres in W_2 .

Structural Analysis of Encapsulated BSA and GnCl-induced Unfolding of BSA

At the end of release period, the integrity of remaining BSA in the polymer was determined by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) analysis, which were performed on a Pharmacia PhastSystem (Pharmacia Biotech, Uppsala, Sweden) according to the file no. 110 and 100 in the PhastSystem™ User Manual, respectively. In both analyses, Coomassie staining file no. 200 was used. The secondary structure of antigen samples was determined by measuring circular dichroic (CD) spectra. The spectra were taken with a J-500A Jasco spectropolarimeter (Hachioji, Japan) at room temperature. The tertiary structure of protein samples was analyzed by measuring the intrinsic fluorescence emission spectra. Fluorescence emission spectra (300–500 nm for BSA) were obtained on a Perkin-Elmer LS50B luminescence spectrometer (Norwalk, CT) scanned at 240 nm/min. The excitation wavelength for BSA was set to 295 nm. Details of these procedures were as described previously (17).

For GnCl unfolding of BSA, 100 µg/ml of BSA stock solution in the presence or absence of 5-fold weight excess PEG 10,000 or PEG 35,000 were prepared in 30 mM MOPS buffer (pH 7.0). BSA stock solution was diluted in various concentrations of GnCl to reach the final protein concentration of 6.25 µg/ml. The fluorescence of BSA at 345 nm was determined when the protein was excited at 280 nm by using Fluoromax-2 fluorometer (Instrument S. A., Inc., NJ). The unfolding fraction of the protein, α , was calculated by:

$$\alpha = \frac{F_N - F_i}{F_N - F_U}$$

where F_N and F_U are the fluorescence of the native (in absence of GnCl) and fully unfolded states (in concentrated GnCl), respectively; and F_i is the protein fluorescence at the i th concentration of GnCl.

RESULTS AND DISCUSSION

Microsphere Composition and Phase Behavior Analysis

In Fig. 1, the IR spectra of blank microspheres prepared from pure PEG, pure PLA (i.v. = 1.07 dl/g), and a PLA/PEG blend are displayed. A broad band at 2876 cm^{-1} and a peak at 1757 cm^{-1} were assigned to the CH_2 stretching on the PEG unit and the carbonyl group ($\text{C}=\text{O}$) of PLA, respectively. Both characteristic peaks for CH_2 and $\text{C}=\text{O}$ appeared in the IR spectrum of the blend of PLA and PEG. By estimating the PEG content in the blend with a calibration curve generated

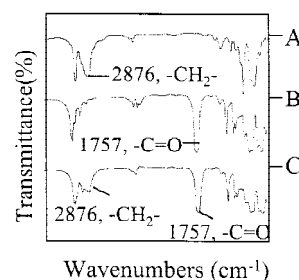


Fig. 1. IR spectra of (A) PEG 35,000 microspheres, (B) PLA (i.v. = 1.07 dl/g) microspheres, and (C) PLA/PEG 35,000 (80/20) microspheres.

from PLA and PEG physical mixtures with different weight ratios, complete incorporation of PEG in PLA matrix by the O/O encapsulation method was indicated (data not shown).

In Table I, the DSC thermograms of prepared blank microspheres are shown. PLA exhibited a T_g of roughly 53.5°C and PEG showed a T_m of 71°C. In the PLA and PEG microsphere blend, down shifts in both T_g and T_m of 7–11°C and 8–10°C, respectively, were observed, indicating partial miscibility between PLA and PEG.

Microsphere Morphology

As seen in Fig. 2, after preparation, microspheres with different weight ratios of PLA and PEG had spherical and smooth surfaces. An average size of ~100 µm was recorded for these microsphere preparations. After 35 days of incubation, microspheres prepared from 100% PLA remained intact with a smooth surface. With the blend of PEG, the microsphere structure still remained intact, but a small amount of pores appeared on the PLA/PEG microspheres surface. With higher PEG blend, more pores became visible. In addition, the microsphere surface showed indentations, which may have occurred during drying of the particles before analysis. The SEM images suggested that the incorporation of PEG into PLA created more channels in the microspheres, which may have increased the permeability to the encapsulated protein and polymer degradation products. In addition, the microsphere surface likely consisted of a PLA-rich phase, whereas the interior of microspheres was likely PEG-rich. Otherwise, more pores created by PEG solubilization would be expected on the microsphere surface. The PLA-rich surface phenomenon is possibly due to the higher hydrophobicity and longer chain of PLA, which could have caused selective PLA precipitation at the surface during the O/O micro-

Table I. The Effect of PLA and PEG Contents on Polymer Thermo-Properties of the Polymer Blend

Microspheres compositions	Wt % of PEG 35 kD	T_g (°C)	T_m (°C)
PEG ^a	100	—	71.1
PLA ^a	0	53.5	—
PLA/PEG ^a	10	44.5	62
PLA/PEG ^a	20	42.3	63.1
PLA/PEG ^a	30	46.5	61.4

^a All the examined microspheres were blank microspheres. Total polymer concentration (w/v) was 20%.

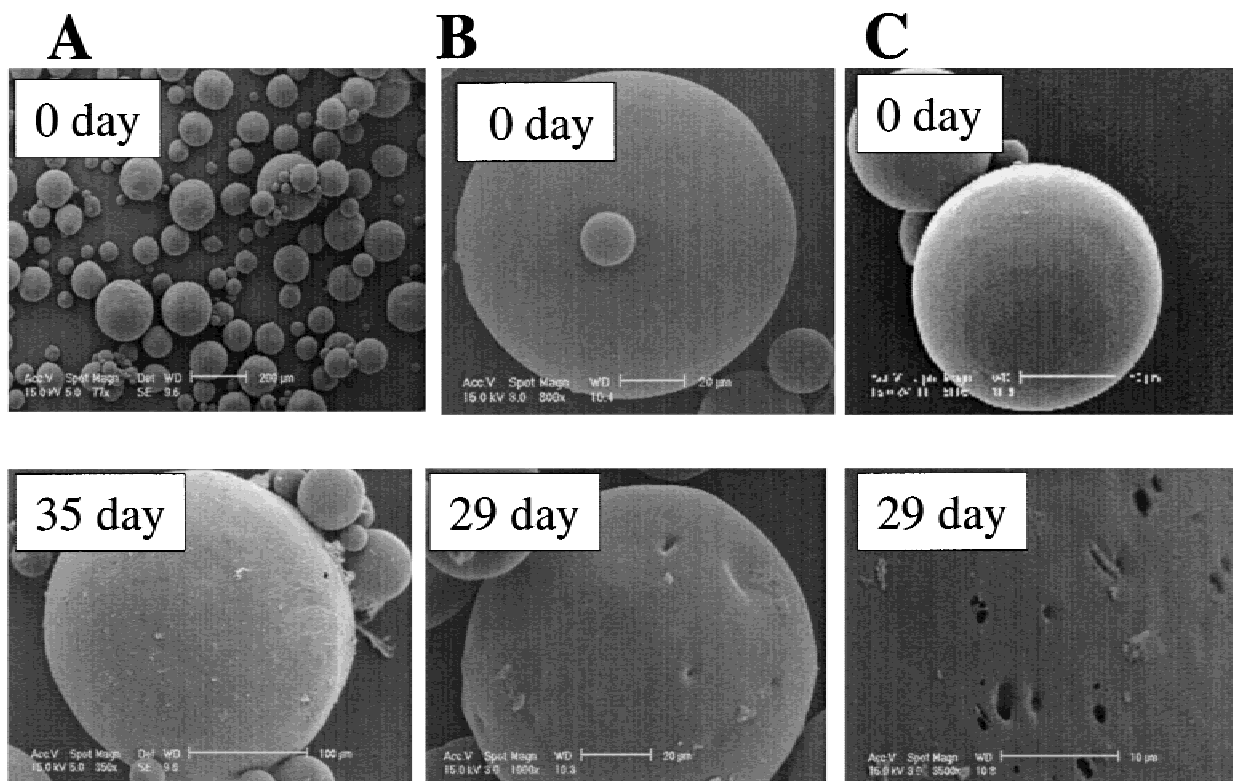


Fig. 2. The SEM images of (A) PLA (i.v. = 1.07 dl/g) microspheres, (B) PLA/PEG 35,000 (90/10) microspheres, and (C) PLA/PEG 35,000 (80/20) microspheres before and after incubation in the release medium.

sphere preparation. Further surface analysis would be required for a definitive conclusion.

Release Kinetics and Stability of BSA in the PLA/PEG Microspheres

To investigate the effect of PEG in the PLA/PEG microspheres, microspheres with different weight ratios of PEG 10,000 to PLA were prepared and the BSA controlled release was monitored in PBST at 37°C. Theoretical BSA loading of all these formulations was 5% and encapsulation efficiency was invariably between 90% and 100%. As seen in Fig. 3A, when PEG content was less than 10% of polymer weight, similar release kinetics of BSA from microspheres was observed and less than 45% of BSA was released after a 4-week incubation. When PEG content was raised to 20%, the total releasable amount of protein was significantly increased to 75%. The effect of PEG molecular weight on protein release was also evaluated. As seen in Fig. 3B, BSA had almost identical release kinetics in microspheres irrespective of whether PEG 10,000 and PEG 35,000 was used (the weight ratio of PEG:PLA was 20:80). When PEG 35,000 content was increased to 30% in PLA/PEG microspheres, a higher burst release of BSA was observed.

The residual BSA remaining in these devices after the 4-week release interval (45 days for formulation without PEG, i.e., formulation *o*) was analyzed and listed in Table II. For the formulation *o*, of the original encapsulated protein, 15% was still water-soluble and 25% of BSA had become water-insoluble aggregates in the residue. Most of the aggregates were soluble in a denaturing solvent (6 M urea), indi-

cating their non-covalent character. When 5% of PEG was incorporated in PLA (formulation *a*), soluble BSA remaining in microspheres was increased to 30%, and the non-covalent aggregates were 41% of the original encapsulated BSA. When PEG content was increased to 10% (formulation *b*), 36% of the protein formed insoluble aggregates. Besides non-covalent aggregates, a small fraction of disulfide-bonded aggregates (soluble in 10 mM DTT) was also formed. However, no insoluble BSA aggregates were observed in formulations containing 20% PEG or more.

The integrity of the soluble BSA recovered from the polymer (29-day incubation) was further examined by SDS-PAGE. As seen in Fig. 4A, some faint peptide fragments were observed in lanes 6 and 7 (formulations *a* and *b*), indicating mild peptide bond hydrolysis occurred during incubation. In contrast, soluble BSA recovered from formulations containing more than 20% PEG showed a very similar band with standard BSA and no degradation product bands were noticeable. Soluble BSA recovered from formulations *c*, *d*, and *e* was further examined by IEF. No pI alterations in BSA were observed in these samples. Likewise, secondary and tertiary structure of BSA was virtually identical to the standard BSA control. Hence, the structure of BSA in formulations *c*, *d*, and *e* was retained within the polymer for nearly one month.

Mechanisms of BSA Stabilization in the PLA/PEG Microspheres

One-month continuous release of stable BSA from microspheres was achieved when PEG content in the PLA/PEG blends was above 20% or above. As identified previously, an

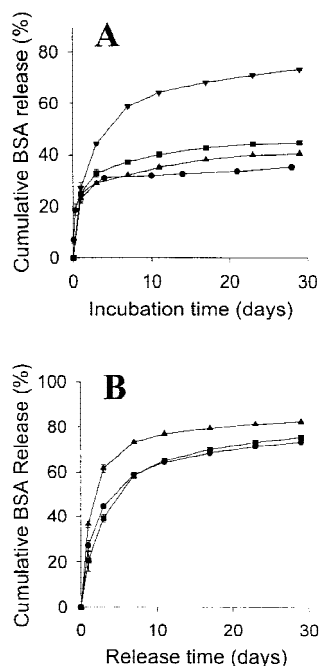


Fig. 3. The effect of PEG content and molecular weight in the PLA/PEG blend on the release kinetics of BSA. (A) PEG 10,000 content was 0% (●), 5% (■), 10% (▲), and 20% (▼); (B) PEG molecular weight and content were 20% PEG 10,000 (■) and 20% PEG 35,000 (●), 30% PEG 35,000 (▲). (average \pm SD, $n = 3$)

acidic microclimate and intermediate moisture levels are the two major factors that cause non-covalent aggregation and peptide-hydrolysis of BSA in PLGA 50/50 microspheres. Does the blend of PEG with PLA improve the microclimate as we designed, i.e., by avoiding the acidic microclimate and increasing the water content to stabilize BSA encapsulated in microspheres?

To address this question, we first examined the pH change of the release medium when the PLA/PEG formulations were incubated at 37°C and PBST (pH 7.4). Unlike PLGA 50/50, which caused a dramatic pH drop in the release medium after a 4-week incubation (3), a relatively neutral pH was retained in the release medium for both PLA and PLA/PEG formulations. However, a slightly lower pH in the release medium incubated with PLA/PEG formulation relative

to that in PLA was observed (~ 0.1 – 0.2 pH unit difference). This result indicated that some acidic degradation products were able to diffuse out of polymer device through the water channels formed by PEG in PLA/PEG formulation. In addition, by using a previously reported method (for hydronium activity a_{H^+} determination of polymer solution in the mixture of ACN and water) (18), the $p_{\text{a}_{\text{H}^+}}$ inside formulation *d* before and after 30-day incubation was determined as 6.5 and 5.4, respectively (data not shown), suggesting a very small accumulation of acid in the polymer. In contrast, PLGA 50/50 microspheres were reported to reach $p_{\text{a}_{\text{H}^+}} \sim 3$ after a similar incubation time (19). These results demonstrated that acid build-up was largely reduced in the PLA/PEG blend formulation.

The water content difference in formulations during release was compared by performing a water uptake kinetic study of microspheres at 97% [RH]. Under controlled humidity, microspheres will adsorb water vapor and potential water uptake of different formulations during release can be predicted and compared. As seen in Fig. 5B, PEG 35,000 showed a strong water uptake. On the second day, the water content in PEG 35,000 blank microspheres was almost 120% of the dry microsphere weight, whereas PLA blank microspheres showed insignificant water uptake. Upon blending PEG in the formulation, the water uptake rate was significantly increased compared to PLA blank microspheres. The higher the PEG content, the higher the increase in water uptake. Microspheres containing 20% PEG had almost twice the amount of water relative to those with 10% of PEG in the humid environment. Therefore, when microspheres are incubated in the release medium, higher water content in the PLA/PEG blend than in PLA is expected. The presence of 5% BSA did not increase water uptake rate significantly in the blend formulation. The water uptake in the blend was likely overwhelmed by the strong water sorption by PEG.

The above results demonstrated that a less acidic and more hydrophilic microenvironment was achieved in the PLA/PEG blend. Maintenance of a relatively neutral microclimate in PLA/PEG blend formulation can be attributed to the following. First, few acidic species were produced during early incubation due to the slow degradation of PLA. The rate constant of PLA degradation at 37°C in water has been reported to be roughly 0.012 day^{-1} , much slower than PLGA

Table II. The Effect of PEG Molecular Weight and Content on BSA Aggregation in O/O Microspheres After a 29-day Release Study in PBST at 37°C

Form. Code.	MW of PEG in blend ^a	Wt % of PEG	Released BSA %	Soluble BSA % ^b	Non-covalent aggregates % ^c	Non-covalent and disulfide bonded aggregates % ^d	Recovery %
o	—	0	36 \pm 1 ^e	15 \pm 1 ^e	22 \pm 1 ^e	25 \pm 2 ^e	76 \pm 2 ^e
a	10 kD	5	44.6 \pm 0.3	30 \pm 2	41 \pm 2	41 \pm 7	116 \pm 7
b	10 kD	10	40.4 \pm 0.3	39 \pm 2	26 \pm 4	36 \pm 4	115 \pm 4
c	10 kD	20	73.2 \pm 0.3	37 \pm 4	—	—	110 \pm 4
d	35 kD	20	75.5 \pm 0.3	30.2 \pm 0.2	—	—	106 \pm 1
e	35 kD	30	82.3 \pm 0.2	30 \pm 2	—	—	112 \pm 2

^a Theoretical protein loading was 5%; PLA (i.v. = 1.07 dl/g) was used in the formulation and the total polymer concentration (w/v) was 20%.

^b Soluble in PBST.

^c Soluble in denaturing agents (8 M Urea).

^d Soluble in combined denaturing and reducing agents (8 M Urea and 10 mM DTT).

^e The BSA release study from the PLA specimen was performed over 45 days and the residue was recovered after this time.

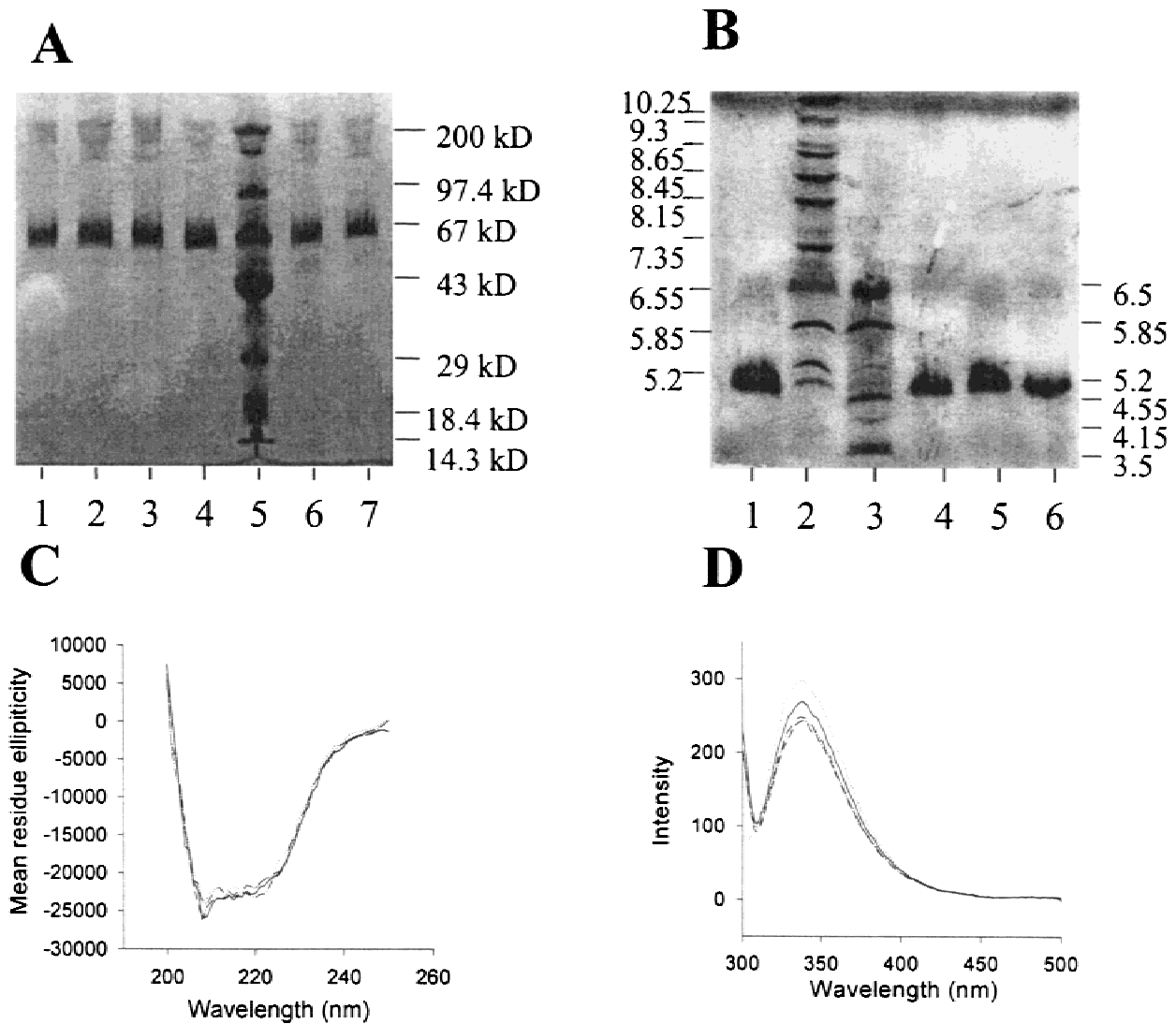


Fig. 4. BSA is stabilized in PLA-PEG microsphere blends with PEG content $\geq 20\%$. All BSA analysis was on protein recovered from the following polymer samples after 29 days of release. (A) Reducing SDS-PAGE. Lane 1: PLA/PEG35,000 (70/30); Lane 2: PLA/PEG35,000 (80/20); Lane 3: PLA/PEG10,000 (80/20); Lane 4: standard BSA; Lane 5: molecular weight marker; Lane 6: PLA/PEG10,000 (95/5); and Lane 7: PLA/PEG10,000 (90/10). (B) IEF. Lane 1: PLA/PEG10,000 (80/20); Lane 2: high pI marker; Lane 3: low pI marker; Lane 4: standard BSA; Lane 5: PLA/PEG35,000 (80/20); and Lane 6: PLA/PEG35,000 (70/30). (C) CD spectra. BSA standard (●●●), PLA/PEG35,000 (80/20) (●—●); PLA/PEG35,000 (70/30) (●●—●●); and PLA/PEG10,000 (80/20) (—). (D) Fluorescence spectra. BSA standard (●●●), PLA/PEG35,000 (80/20) (●—●); PLA/PEG35,000 (70/30) (●●—●●); and PLA/PEG10,000 (80/20) (—).

50:50, 75:25, 85:15 with rate constants of 0.103, 0.055, and 0.026 day^{-1} , respectively (4). In addition, prior to hydration, the polymer acid content was determined as 21 and 4.2 nmol/mg for the PLGA 50/50 used in our previous study and PLA used here, respectively (5). Therefore, the total amount of acidic species in PLA should be less than PLGA either during encapsulation or during hydration. Second, the blend of PEG with PLA significantly increased the water content in the formulation, which is expected to dilute the acidic species even further. Third, the dissolution of PEG in the release medium may create more water channels, thereby increasing the diffusion for acidic species out of the polymer and for buffering species into the PLA matrix.

By the above three mechanisms, a less acidic microclimate will be formed in the PLA/PEG blend. When PEG content is 10% or less in the blend formulations, non-covalent

aggregates and peptide fragments of BSA were still observed. This is likely due to regional acidity in the polymer. In certain regions, slowly produced polymer degradation products could not be diluted or diffuse out of the polymer because of insufficient water channels, resulting in regionally low pH. With increasing amount of PEG in the blend, a more neutral microclimate was gradually attained. Although slight pH decreases within the polymer was still detected in the blend containing 20% PEG, it was not significant enough to cause non-covalent aggregation and peptide-hydrolysis of BSA.

The stabilization of BSA in the PLA/PEG microspheres can also be attributed in part to the increased water content in the formulation. It was reported that the aggregation of BSA at acidic pH (pH = 2) exhibited a pronounced bell-shape with maximum aggregation at roughly 100g water/100 g dry protein. When water content was increased to 500%–1000%,

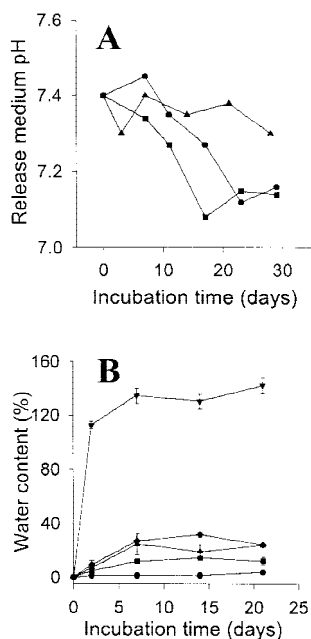


Fig. 5. Release medium pH and water uptake kinetics from PLA/PEG microspheres. (A) The pH change of release medium in PLA (▲), PLA/PEG 35,000 (80/20) (■) and PLA/PEG 10,000 (80/20) (●) microspheres containing 5% BSA when incubated at pH 7.4 PBST and 37°C; (B) Water uptake kinetics of microspheres after incubation at 97% R.H. and 37°C. blank PLA microspheres (●), blank PLA/PEG 35,000 (90/10) microspheres (■), blank PLA/PEG 35,000 (80/20) microspheres (▲), 5% BSA/PLA/PEG 35,000 (80/20) microspheres (◆) and blank PEG microspheres (▼).

aggregation of BSA at pH 2 was declined sharply (20). In the blend formulation containing 20% of PEG, when incubated at 97% R.H. for 1 week, the water content in the microspheres was ~25%. Assuming all the water is available for BSA (BSA loading is 5%) in microspheres, the water content of BSA is ~500%. During release, the water content in microspheres was expected to be higher than 500%. Thus, in addition to the minimal acid content, the aggregation of BSA was minimized by the high amount of imbibed water in the microenvironment.

A final consideration is that PEG may interact favorably with the hydrophobic side chains of protein molecules exposed upon unfolding (21). It was reported that PEG of low Mw 1000 and 4000 interacts favorably with such regions of human serum albumin, leading to a stabilization of the unfolded state (21). To test any potential interaction of high Mw PEG with BSA, the GnCl unfolding curve of BSA with the addition of PEG 10,000 and PEG 35,000 (the weight ratio of BSA to PEG was 1:5) was determined by fluorescence spectroscopy. As seen in Fig. 6, similar unfolding curves were observed in these three preparations. These data suggest that the conformational stability of BSA was, therefore, unaffected by the addition of PEG 10,000 and PEG 35,000, as demonstrated by CD and fluorescence spectroscopy previously.

CONCLUSIONS

Based on the fact that acidic microclimate and intermediate moisture levels were the major factors that cause BSA

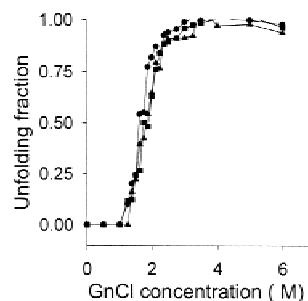


Fig. 6. No effect of PEG on the GnCl-induced unfolding of BSA monitored by fluorescence spectroscopy at room temperature. No PEG (●), PEG 10,000 (■) and PEG 35,000 (▲). BSA and PEG concentrations were 6.25 and 31.25 $\mu\text{g}/\text{ml}$, respectively.

instability, a PLA/PEG blend formulation with low acid and high water content was devised. By using the PLA/PEG blend, a one-month continuous release of BSA was achieved with the absence of insoluble aggregates and peptide hydrolysis. This formulation approach may be generally applicable for encapsulation of other acid-labile proteins and vaccine antigens.

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