A Heterogeneously Structured Composite Based on Poly(lactic-co-glycolic acid) Microspheres and Poly(vinyl alcohol) Hydrogel Nanoparticles for Long-Term Protein Drug Delivery

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Purpose. To prepare a heterogeneously structured composite based on poly (lactic-co-glycolic acid) (PLGA) microspheres and poly(vinyl alcohol) (PVA) hydrogel nanoparticles for long-term protein drug delivery.

Methods. A heterogeneously structured composite in the form of PLGA microspheres containing PVA nanoparticles was prepared and named as PLGA-PVA composite microspheres. A model protein drug, bovine serum albumin (BSA), was encapsulated in the PVA nanoparticles first. The BSA-containing PVA nanoparticles was then loaded in the PLGA microspheres by using a phase separation method. The protein-containing PLGA-PVA composite microspheres were characterized with regard to morphology, size and size distribution, BSA loading efficiency, *in vitro* BSA release, and BSA stability.

Results. The protein-containing PLGA-PVA composite microspheres possessed spherical shape and nonporous surface. The PLGA-PVA composite microspheres had normal or Gaussian size distribution. The particle size ranged from 71.5 μm to 282.7 μm. The average diameter of the composite microspheres was 180 μm. The PLGA-PVA composite microspheres could release the protein (BSA) for two months. The protein stability study showed that BSA was protected during the composite microsphere preparation and stabilized inside the PLGA-PVA composite microspheres.

Conclusions. The protein-containing PLGA-PVA composite may be suitable for long-term protein drug delivery.

KEY WORDS: poly(lactic-co-glycolic acid); poly(vinyl alcohol); microsphere; nanoparticle; protein drug delivery.

INTRODUCTION

After 30 years of commercial use of lactic/glycolic acid polymers (PLGA) as medical suture (1), their biocompatibility, bioabsorbability, changeable biodegradability as well as good mechanical property have been well recognized. These beneficial properties have motivated the extended use of these polymers in many others including drug delivery (2–6). However, when the PLGA is used as drug carriers, some intrinsic chemical properties of these polymers such as hydrophobicity and acidity bring in a difficulty for this purpose, particularly for protein and peptide drug delivery. For example, instability of protein drugs in these polymer matrices has been observed (7,8). Another problem is fast or burst release of protein drugs from PLGA matrices, particularly from the PLGA microspheres.

Many attempts have been made to modify the physicochemical properties of the PLGA (9–14). One approach is chemical modification of PLGA. For example, the lactide and/or glycolide (which is (are) dimer(s) of lactic and/or glycolic acid) was/were copolymerized with hydrophilic monomers such as ethylene glycol to introduce hydrophilic segments to the polymer and/or modify crystallinity of the polymer (9,10). A series of branched terpolymers were obtained by grafting poly(lactide-co-glycolide) onto a core molecule such as glucose. The physicochemical property as well as degradation behavior of the modified PLGA was different from their parents' materials (12). Another approach is physical blending of PLGA with other polymers. For example, the PLGA has been blended with hydrophilic polymers, including poly(ethylene oxide-co-propylene oxide) (13), poly(ethylene-co- vinyl acetate) (11), and PVA (14).

We explored a different approach to modify the properties of the PLGA matrix by using hydrogels (15,16). Proteins are stabilized by this new approach. In this work, we combined PLGA and PVA in such a way that a heterogeneous structure was generated. The PVA was fabricated as nanoparticles while the PLGA was made as microspheres. The drug was loaded in the PVA nanoparticles first and then the drug-containing nanoparticles were encapsulated in the microspheres to form a heterogeneously structured polymeric composite. Bovine serum albumin (BSA) was used as a model protein drug. This composite combines the hydrophilicity of PVA and the hydrophobicity of PLGA. To the protein molecules, it is a totally hydrophilic matrix because the protein molecules exist inside the PVA nanoparticles so that there is no protein stability problem for the new system. To the physiological environment, it is still a hydrophobic PLGA system that possesses a slow biodegradation and long-term release characteristics.

These PLGA-PVA composite microspheres were characterized with regard to morphology, size and size distribution, BSA loading efficiency, *in vitro* BSA release, and BSA stability.

MATERIALS AND METHODS

Materials

PVA (average molecular weight: 70,000–100,000) was purchased from Sigma Chemical Co. (St. Louis, MO). PLGA (molar ratio of d,l-lactic to glycolic acid: 75:25, inherent viscosity: 0.58 dl/g in $CHCl_3$ at 30°C) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Bovine serum albumin (BSA, MW: 68,000) was from Boehringer Mannheim Corp. (Indianapolis, IN). Methylene chloride was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Silicon oil (350 cs) was from Dow Corning Corporation (Midland, MI). Acetone was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, California). Trifluoroacetic acid (TFA, anhydride,

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protein sequencing grade), and acetonitrile (HPLC grade) were also purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of PVA Nanoparticles

The preparation of BSA-containing PVA nanoparticles was previously reported (17). Briefly, PVA (500 mg) was dissolved in 3 ml of phosphate buffer (20 mM, pH 7.4) by heating to a temperature near 100°C for 15 min, while BSA (125 mg) was dissolved in 2 ml of phosphate buffer at room temperature. After PVA solution was cooled down to room temperature, above two solutions were mixed thoroughly to form a 10% PVA solution containing 2.5% of BSA. This aqueous solution was added to 100 ml of silicone oil, and the mixture was homogenized (10,000 rpm) for 5 min to form a water-in-oil emulsion. The emulsion was then frozen at −20 °C for 20 hrs and then allowed to thaw for 4 hrs. Three such freezing-thawing cycles were performed for each sample, which resulted in conversion of the emulsion to a suspension. After three such cycles, the suspension was mixed with large quantity of acetone. The new mixture formed two layers: acetone layer containing PVA nanoparticles (top layer) and the silicone oil layer (bottom layer). The acetone layer was collected and filtered. The filtrant or the PVA nanoparticles were vacuum dried, and kept refrigerated for the preparation of the PLGA-PVA composite microspheres.

Preparation of the Heterogeneously Structured PLGA-PVA Composite Microspheres

The PLGA-PVA composite microspheres were prepared by using a phase separation method. The procedure is described below. The PLGA (0.8 g) was dissolved in 10 g of methylene chloride. The PVA nanoparticles (200 mg) containing 40 mg of BSA were suspended in the PLGA solution by using a homogenizer (Model M 122, Biospec Products Co., Barhesville, OK). The suspension was stirred at 200 rpm in a three-neck flask. Silicon oil (7 g) was then progressively added to the suspension at a speed of 1 g/min to form an oil-in-oil emulsion. When the PVA nanoparticle-containing PLGA droplets reached the desired size (around 150 μm), the emulsion was transferred with stirring to a quenching tank containing 2 L heptane. The quenching tank was stirred for 3 hrs to harden the PVA nanoparticle-laden PLGA microspheres. Finally, the composite microspheres were washed with heptane, collected by filtration, and dried *in vacuo*.

Morphology Observation

The surface morphology and internal structure of the PLGA-PVA composite microspheres were observed by using a scanning electronic microscope (SEM). The cross section of the PLGA-PVA composite was viewed by using the SEM after freeze-fractured and then gold coated. Polaroid pictures were taken during the observation.

Size and Size Distribution Measurement

The PLGA-PVA composite microspheres (10 mg) were evenly dispersed in 1% Tween 80 solution (10 ml). The suspension was then evaluated using a particle sizer (Accusizer, Model 770, Santa Barbara, CA). Average size and volume weighted size distribution of the PLGA-PVA composite microspheres were obtained from this study.

BSA Loading Efficiency Determination

The BSA loading efficiency was determined by using a method previously reported by Uchida *et al.*(8) with minor modification. The PLGA-PVA composite microspheres (10 mg) were accurately weighed and dissolved in 3 ml acetonitrile in a test tube. After the PLGA was completely dissolved, the test tube was centrifuged at 2621 × g using an ultracentrifuge (Model J-6M, Beckman Instruments Inc., Palo Alto, CA). The supernatant was discarded. The above procedure was repeated. One millimeter of PBS was then added. The test tube was incubated in a 37°C water bath (shaking speed: 30 rpm) for 24 hours to completely extract the BSA from the PVA hydrogel nanoparticles. After extraction, the PBS was filtered through a 0.22 μm Teflon syringe filter. The clear filtrate was analyzed by using a high performance liquid chromatography (HPLC) equipped with a photo diode array (PDA) detector (Thermo Separation Products, Inc., Riviera Beach, FL). A Delta-pak C_{18} column (Waters Corporation, Milford, MA) was used and maintained at 27°C. The mobile phase was a mixture of solution A (0.1% TFA in H_2O) and solution B (CH_3CN:H_2O = 90:10, containing 0.1% TFA) at a ratio of 100:0 initially, and programmed for gradient elution linearly over 13 min to the ratio of 30:70 of solution A to solution B. The detecting wavelength was 220 nm and the flow rate was 1 ml/min. The loading efficiency was calculated using the following equation:

$$\text{Loading efficiency of BSA (\%)} = (L_A/L_T) \times 100$$

where L_A is the actual loading of BSA in the PLGA-PVA composite microspheres, which has been determined above experimentally, while L_T is the theoretical loading of BSA in the PLGA-PVA composite microspheres calculated from the feeding amount during the preparation. The above experiments have been performed in triplicate.

In Vitro Release Study

The *in vitro* release experiment was conducted in triplicate. The PLGA-PVA composite microspheres (30 mg) were suspended in 1 ml phosphate buffered saline (PBS, 20 mM, pH 7.4) in a glass test tube. The test tube was incubated in a 37°C water bath shaking at the speed of 30 rpm. At predetermined time intervals, 0.5 ml of incubating medium was withdrawn and the same amount of fresh PBS was added. The incubating medium was filtered through a 0.22 μm syringe filter and then analyzed by using the same HPLC system and the same operating conditions as described in the BSA loading efficiency determination experiment. The cumulative percentage of BSA released was calculated using the following equation:

$$\text{Cumulative percentage of BSA released (\%)} = (M_t/M_\infty) \times 100$$

where M_t is the amount of BSA released at time t and M_∞ is the total amount of BSA released at time infinity, which is the actual loading of BSA determined in the BSA loading efficiency determination experiment.

BSA Stability Analysis

The PLGA-PVA composite microspheres (20 mg) was incubated in 2 ml of distilled water overnight in a water bath

at 37°C. After incubation, 1 ml supernatant was withdrawn and filtered through a 0.22 μm syringe filter. The clear filtrate was analyzed by using a size exclusion HPLC system equipped with the same PDA detector as described above. A size exclusion chromatography column (Alltech Macrosphere GPC column, 7 μm, 250 × 4.6 mm, Alltech Associates, Inc., Deerfield, IL) with 300 Å pore size was used and maintained at 40°C. The mobile phase was a pH 7.0 buffer solution containing 0.05 M KH_2PO_4 and 0.15 M Na_2SO_4. The flow rate was 0.8 ml/min. The detecting wavelength was 280 nm. A pure BSA solution was used as the reference.

RESULTS

Microscopic Characteristics

Figure 1 is SEM micrographs of the PLGA-PVA composite microspheres showing their shape, size, and surface morphology. Figure 1a shows a population of the PLGA-PVA composite microspheres while Fig. 1b is an enlargement of a PLGA-PVA composite microspheres. The micrograph presents a typical population of the composite microspheres because the sample is randomly picked up from the bulk microspheres. One can see from the micrograph that the PLGA-PVA composite microspheres possess spherical shape and nonporous surface. PVA nanoparticles can be seen on the surface of the PLGA microspheres. No obvious aggregation has been observed. Figure 2 is SEM micrographs of the fractured PLGA-PVA composite microspheres showing their internal structure and distribution of PVA nanoparticles inside the PLGA microspheres. Figure 2a demonstrates a group of the fractured PLGA-PVA composite microspheres, while Fig. 2b gives us an enlarged view of a fractured composite microspheres. As one can see, the PVA nanoparticles are evenly distributed in the PLGA microsphere matrix.

Size and Size Distribution

The PLGA-PVA composite microspheres possess a normal or Gaussian size distribution as shown in Fig. 3. The size of the PLGA-PVA composite microspheres ranges from 71.5 μm

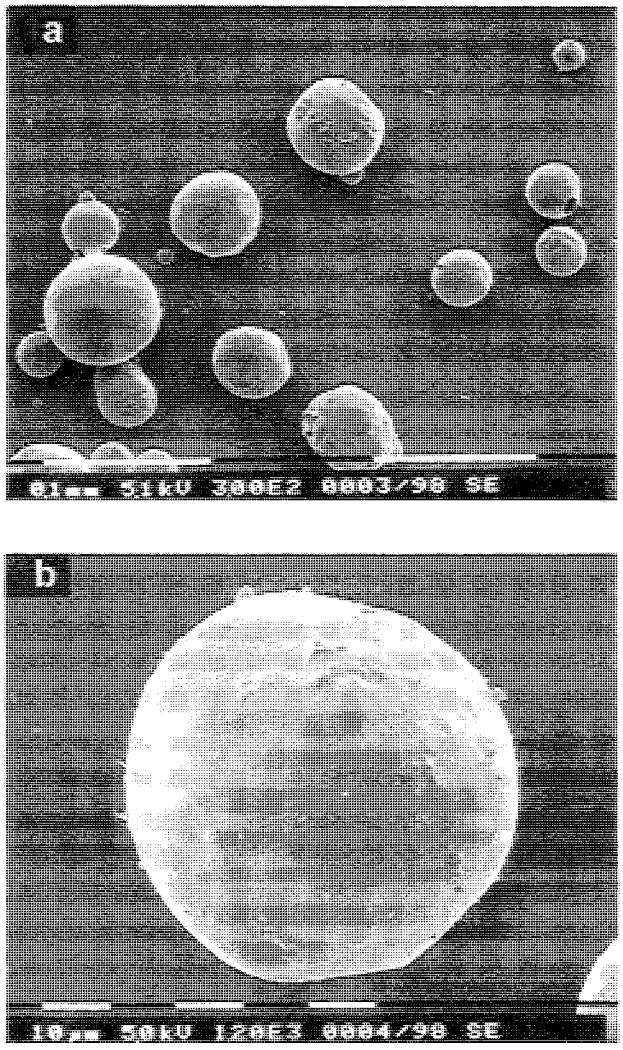

Fig. 1. Scanning electronic micrographs of the PLGA-PVA composite microspheres showing their shape, size, and surface morphology. (a) a population of the composite microspheres and (b) an enlargement of a composite microsphere.

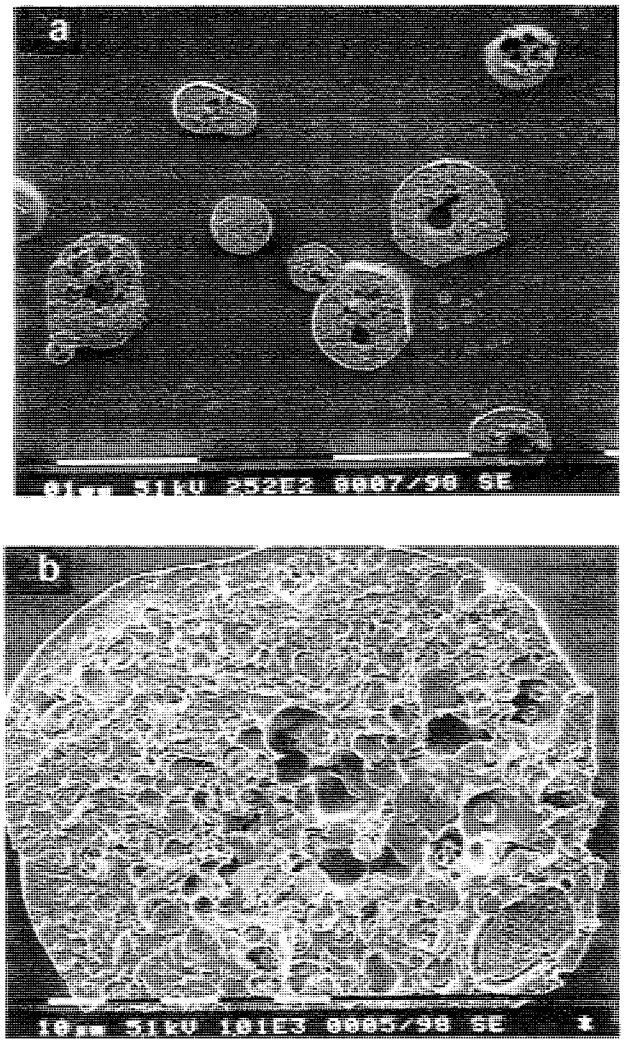

Fig. 2. Scanning electronic micrographs of the fractured PLGA-PVA composite microspheres showing the internal structure and distribution of the PVA nanoparticles inside the PLGA microspheres. (a) a group of the fractured composite microspheres and (b) an enlarged view of a fractured composite microsphere.

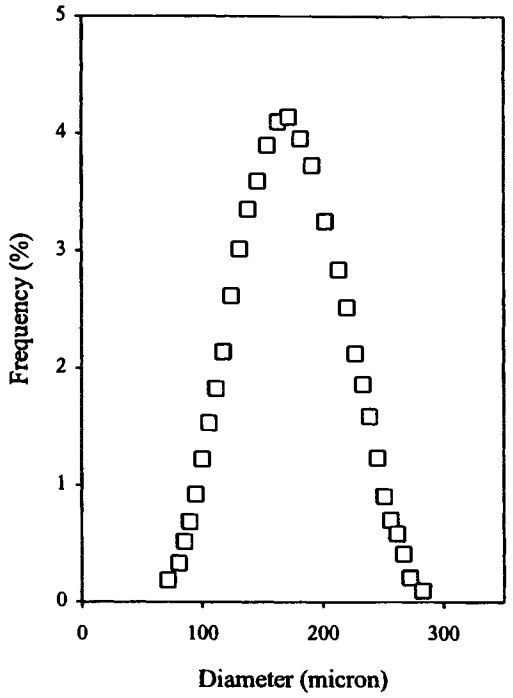

Fig. 3. A size distribution curve of the PLGA-PVA composite microspheres.

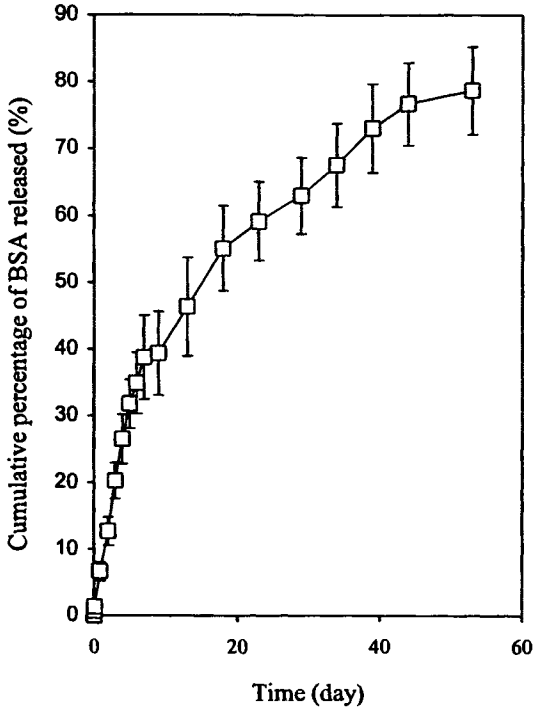

Fig. 4. *In vitro* BSA release from the PLGA-PVA composite microspheres as a function of time.

to 282.7 μm. The volume-weighted average diameter of the composite microspheres is 180 μm.

BSA Loading Efficiency

The loading efficiency of BSA in the PLGA-PVA composite microspheres was 82.31 ± 10.97%. This result was calculated from three measurements.

In Vitro BSA Release

Figure 4 shows the cumulative percentage of BSA released as a function of time. The BSA release from the PLGA-PVA composite microspheres can be prolonged to nearly two months. The BSA *in vitro* release follows a triphasic release pattern. The first 7 days of BSA release makes the first phase. The second release phase includes theperiod between 9 and 44 days. The release rate declines after 44 days, which produces the third release phase.

BSA Stability

The BSA stability in the PLGA-PVA composite microspheres can be examined by determining the BSA integrity change using a size exclusion chromatography (SEC). Figure 5 shows SEC chromatograms of two different BSA samples. Chromatogram A is from a BSA solution, which shows a peak corresponding to the native BSA. Meanwhile, chromatogram B shows the peak of the BSA released from the PLGA-PVA composite microspheres.

DISCUSSION

The PLGA-PVA composite microspheres had a heterogeneous structure in which the PVA nanoparticles were dispersed in the PLGA matrices as revealed by the SEM studies. The PVA nanoparticles were completely encapsulated in each individual PLGA microsphere.

During preparation, the PVA nanoparticles should be dispersed in the PLGA solution to their ultimate size. The presence of clumps of aggregated PVA nanoparticles may result in defectiveness of the internal structure of the PLGA-PVA composite microspheres. These defective PLGA-PVA composite microspheres may release drug very rapidly due to the lack of drug

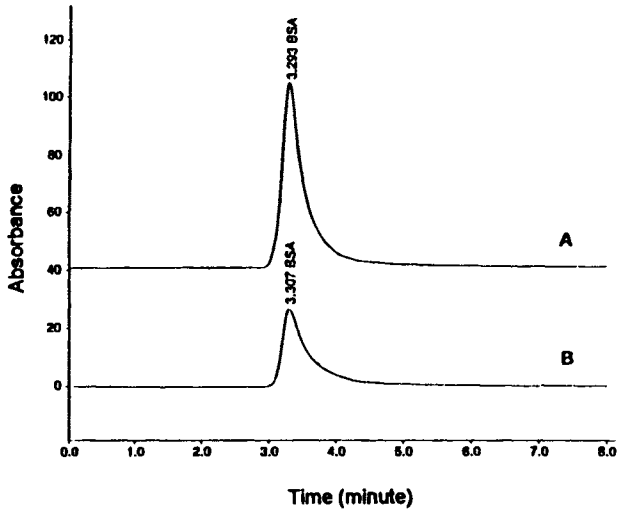

Fig. 5. Comparison of the SEC chromatograms of the BSA released from the PLGA-PVA composite microspheres with the native BSA. (Chromatogram (A) the pure BSA solution; chromatogram (B) the BSA released from the composite microspheres).

release rate controlling PLGA matrices. Although there was no dispersion agent added, the PVA nanoparticles could be well dispersed into the PLGA solution due to the use of a high-speed homogenizer and the viscous nature of the PLGA solution.

The microspherical drug delivery systems are mostly used for parenteral drug administration, such as intramuscular and subcutaneous injection (18–20). The microspheres should be small enough to be able to pass through clinically-used syringe needles to avoid possible myotoxicity (20). In addition, different particle sizes may result in different drug release characteristics because of the variation of surface area and diffusion length. Therefore, the size and size distribution are of importance for microspherical drug delivery systems.

The size of the PLGA-PVA composite microspheres ranged from 71.5 μm to 282.7 μm. Generally speaking, a microsphere larger than 200 μm will have difficulty passing through a commonly used 21 gauge syringe needle that has an internal diameter of 500 μm. When several over-sized microspheres enter a needle together, they may block the needle. The over-sized PLGA-PVA composite microspheres could be eliminated by either increasing the stirring rate during preparation or using sieves to remove them after preparation.

The microencapsulation method has significant influence on the loading efficiency of the encapsulated drugs. Two commonly used preparation methods for PLGA microspheres are phase separation method and multiple emulsion or water-in-oil-in-water emulsion method (3,5,8). For water soluble drugs, including proteins, the multiple emulsion method usually has low loading efficiency because aqueous phases are involved (19). During preparation, proteins are leaked to the external aqueous phase. In contrast, the phase separation method has much higher loading efficiency than the multiple emulsion method. For the preparation of the PLGA-PVA composite microspheres, the loading efficiency of BSA was 82.31 ± 10.97%. The reason for this high loading efficiency was that only organic liquids were involved in the phase separation method (e.g., methylene chloride, silicone oil, and heptane). BSA as well as PVA nanoparticles were not soluble in these organic liquids. When phase separation of the PLGA was induced by the addition of silicone oil, complete encapsulation of the BSA-containing PVA nanoparticles by the PLGA could be achieved. This special encapsulation mechanism yields a relatively high loading efficiency.

High loading efficiency can assure the drug content to reach the designed level. This is particularly important for a prolonged-release drug delivery system because the drug loading in such a system can affect the drug release duration as well as release rate. It was found that drug release from d,l-PLA microspheres increases exponentially with respect to increase in the drug loading (2).

The PLGA-PVA composite microspheres have prolonged BSA release characteristic. As can be seen in Fig. 4, seventy-eight percent of BSA is released after 53 days. The release of BSA from the PLGA-PVA composite microspheres follows a triphasic release pattern. In each release phase, the BSA has a relatively constant release rate. The BSA release rate progressively decreases from one phase to the other, i.e., the first release phase has the highest release rate while the third release phase has the lowest release rate. The elucidation of drug release mechanism and kinetics from PLGA microspheres is practically difficult. The presence of PVA nanoparticles complicates the system even further. The release of BSA from the PLGA-PVA composite microspheres cannot be described by a single transport process such as drug diffusion. It may involve water diffusing in, drug diffusing out, PLGA degradation, and PVA nanoparticle swelling.

The instability of protein drugs in PLGA microspheres has been demonstrated by many researchers (7,8,15,16). Because organic solvents (methylene chloride, silicone oil, and heptane) are used in the microencapsulation process, the structural change of protein molecules may occur. The structural change of protein molecules may include unfolding, aggregation, and/or degradation (22). Therefore, the stability of BSA in the PLGA-PVA composite microspheres becomes a concern.

In this work, we used SEC to examine the BSA integrity or structural change. As one can see in Fig. 5, both chromatograms A and B have only one peak. These two peaks have the same retention time, which is an evidence that the BSA released form the PLGA-PVA composite microspheres has the same size as the native BSA molecules. This result tells us that the BSA released from the PLGA-PVA composite microspheres is neither degraded nor aggregated, which is the most significant advantage of the PLGA-PVA composite microspheres.

CONCLUSIONS

The PLGA-PVA composite microspheres had been successfully prepared. The BSA-containing PVA nanoparticles were encapsulated and evenly distributed in the PLGA microsphere matrix by using the phase separation method. The PLGA-PVA composite microspheres possessed spherical shape and nonporous surface. The composite microspheres had a normal or Gaussian size distribution. The particle size ranged from 71.5 μm to 282.7 μm. The average diameter of the composite microspheres was 180 μm. The PLGA-PVA composite microspheres can release the protein (BSA) for two months. The protein stability study showed that BSA was protected during the PLGA microsphere preparation and stabilized in the PLGA microsphere matrix.

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