Entrapment of Protein Using Electrosprayed Poly(D,L-lactide-*co*-glycolide) Microspheres with a Porous Structure for Sustained Release

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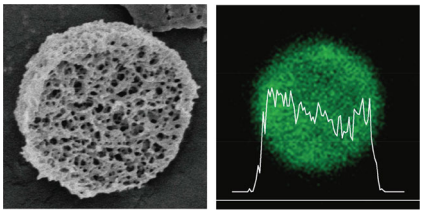
The entrapment of a protein in porous poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres is demonstrated through the closure of their outer surface pores for sustained delivery of the protein. The porous PLGA microspheres with less than 10 μm in size are prepared by electrospraying. Aqueous solutions containing fluorescein isothiocyanate-dextran or bovine serum albumin (BSA) are penetrated into the inner pores as a result of vacuum treatment, and the outer surface pores of the porous PLGA microspheres are then closed using a solvent (dimethyl sulfoxide) to ensure entrapment of the macromolecules. Confocal microscopy images confirm the presence of a large amount of the macromolecules inside the porous structure. Circular dichroism spectroscopy and release analysis reveal that BSA is entrapped without denaturation and released in a sustained manner for a period of over 2 months, respectively.



1. Introduction

Due to the easy denaturation of proteins as a result of exposure to heat, toxic solvents, and surfactants, as well as the low half-life time of proteins in the human body, the encapsulation of a protein in polymeric particles or matrices is considered a major challenge for their practical applications. Much attention has been paid to the encapsulation of proteins in biodegradable polymeric particles or matrices for various biomedical applications, such as drug delivery and tissue engineering.[1] The aims of protein encapsulation are to protect from degradation in the human body and release the protein in a sustained manner for a prolonged therapeutic effect.[2] A number of techniques based on emulsion, spray drying, ionic interaction, and phase separation have been reported for the stable encapsulation of proteins.[3–5] However, most of these methods have a range of shortcomings, such as low encapsulation efficiency, burst release, and denaturation of proteins during fabrication.[6,7]

The electrospray method has been introduced as an alternative approach to overcome these problems.[8–13] Xie et al.[9] fabricated poly(D,L-lactide-*co*-glycolide) (PLGA) microparticles containing bovine serum albumin (BSA) using a single electrospraying technique based on the water-in-oil emulsion system. The encapsulation of a protein in biodegradable microparticles has also been performed through coaxial electrospraying to avoid the procedure of primary emulsification because the primary emulsion causes denaturation and aggregation of the protein.[10,11] Wu et al.[12] demonstrated the fabrication of core–shell microspheres containing BSA using an amphiphilic polymer. In addition, Liu et al.[13] fabricated porous PLGA microspheres loaded with BSA through electrospraying combined with phase separation in liquid nitrogen. However, the development of an encapsulation procedure that avoids denaturation of the protein remains a challenging task.

Here, we describe the fabrication of PLGA microspheres with a porous structure using an electrospraying technique and the entrapment of a protein inside the porous

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PLGA microspheres through the closing of the surface pores using a mild organic solvent. We also demonstrated that the denaturation of the encapsulated and released BSA can be prevented during the encapsulation procedure and that the PLGA microspheres can release BSA sustainably with a slight initial burst. These results clearly indicate that our approach can potentially be employed for the encapsulation of proteins for a variety of pharmaceutical and biological applications.

2. Experimental Section

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA, lactide 75: glycolide 25, M_w = 66 000–107 000, Sigma–Aldrich) and dimethyl sulfoxide (DMSO, Sigma–Aldrich) were used to fabricate the porous PLGA microspheres. Albumin from bovine serum (BSA, Sigma–Aldrich) served as the model protein, and fluorescein isothiocyanate-dextran (FITC-dextran, M_w = 40 000, Sigma–Aldrich) was used as the probe. Phosphate-buffered saline (PBS, pH 7.4, Sigma–Aldrich) was used as the media in all of the experiments.

2.2. Fabrication of Porous PLGA Microspheres by Electrospraying

Porous PLGA microspheres were produced using a vertical electrospray setup (Figure S1, Supporting Information).[14] The PLGA solution in DMSO (5, 7, 10, and 15 wt%) was electrosprayed onto aluminum foil (collector plate) at a flow rate of 0.02 mL min^{-1} using a syringe pump (NE-1000, New Era Pump Systems Inc.). A high voltage of 15 kV was applied between the spinneret (24G needle) and the collector plate, which was located 20 cm from the spinneret. After evaporation of the solvent, the resulting porous microspheres were harvested from the aluminum foil using PBS and freeze-dried.

2.3. Protein Encapsulation Using the Porous PLGA Microspheres

The porous PLGA microspheres were soaked in PBS (5 mL) containing FITC-dextran or BSA (40 mg mL^{-1}) and then placed in a vacuum to allow the penetration of the BSA solution into the inner pores of the porous PLGA microspheres. After centrifugation, the supernatant was removed using a pipette and an aqueous solution (5 mL) containing DMSO (20 w/w%) was added. The mixture was then placed on an orbital shaker for 4 h to allow the closure of the surface pores. The resulting dispersion was placed on a filter paper to remove the excess aqueous solution containing DMSO and freeze-dried for storage because proteins are generally more stable in the solid state than the solution state.[15] A scanning electron microscope (SEM, S-4800, Hitachi High-Technologies, Co. Ltd., Japan) was used to characterize the morphologies of the porous PLGA microspheres before and after solvent treatment. The cross sections of the PLGA microspheres were observed by SEM after sectioning with a freezing microtome (Cryotome FSE, Thermo Scientific, Co. Ltd., USA). The average sizes of the porous PLGA microspheres were calculated from the SEM images using the ImageJ software (National Institutes of Health, USA) (n = 500). A laser scanning confocal microscope (LSM700, ZEISS, Germany) was used to observe the presence of FITC-dextran inside the PLGA microspheres. The ImageJ software was used to determine the fluorescence intensity profiles across the PLGA microspheres from the confocal microscopy images, which depict the distribution of FITC-dextran in the PLGA microspheres. To evaluate the degree of protein denaturation, BSA treated with aqueous DMSO at various ratios (0, 20, and 50 w/w%) for 4 h was analyzed using a circular dichroism spectroscope (CD, J-815, Jasco International, Co. Ltd., Japan).

2.4. Encapsulation Efficiency and Release Analysis

The encapsulation efficiency of BSA in the PLGA microspheres was measured according to the extraction method.[16] Dried PLGA microspheres (10 mg) containing BSA were dissolved in DCM (1 mL) and PBS (10 mL) was subsequently added, followed by vortexing for 1 h to selectively extract the BSA. Afterward, the mixture was centrifuged at 3000 rpm for 5 min to induce phase separation. The concentration of BSA in the water phase was measured using Bio-Rad Protein Assay reagent (Cat. #500-0006, Bio-Rad Laboratories Inc.). The aqueous BSA solution (10 μL) and the protein assay solution (200 μL) were added into PBS (800 μL), followed by vortexing for 2 min prior to analysis using a microplate spectrophotometer (EON, BioTek, USA) at 595 nm. The encapsulation efficiency was calculated as the ratio of the actual amount of BSA to the theoretical amount of BSA.

To determine the release profile, the PLGA microspheres containing BSA (10 mg mL^{-1}) were immersed in PBS (2 mL) at 37 °C and the amount of released BSA was measured using a microplate spectrophotometer at 595 nm with respect to time. All of the experiments were performed in triplicate. The experimental data are presented as the means ± standard deviation.

3. Results and Discussion

The procedure for fabricating the porous PLGA microspheres containing a protein includes three major steps: (1) production of porous PLGA microspheres by electrospraying; (2) penetration of a water-soluble protein into the inner pores by vacuum; and (3) closure of outer surface pores using a mild solvent. The features of this approach include the use of a nontoxic solvent, nonuse of a surfactant, and the lack of a change in pH during the encapsulation procedure, resulting in the non-denaturation of the protein.

In general, three types of morphologies, namely spherical, sphere-on-string, and fibrous structures, were observed in electrospinning and these structures can be tuned by the process variables, including the type and concentration of polymer, solvent, applied voltage, and flow rate of the polymer solution.[17] Among these process

variables, the polymer concentration is one of the key factors that control the morphology. PLGA solutions with different concentrations of DMSO were first electrosprayed to investigate the effect of the polymer concentration on the morphology. Figure 1A shows SEM images of PLGA constructs electrospun with different PLGA concentrations. Spherical morphologies were observed at the PLGA concentrations of less than 7 wt% and the sphere-on-string structure began to form at the concentrations of 10 wt%. A more porous structure was observed at a lower PLGA concentration due to rapid solvent evaporation in the course of electrospraying.[18] The PLGA microspheres prepared with a PLGA concentration of 5 wt% were too brittle to support a highly porous structure and were thus difficult to handle in the subsequent experiments. Figure 1B shows a plot of the size distributions of the PLGA microspheres prepared with different PLGA concentrations. The

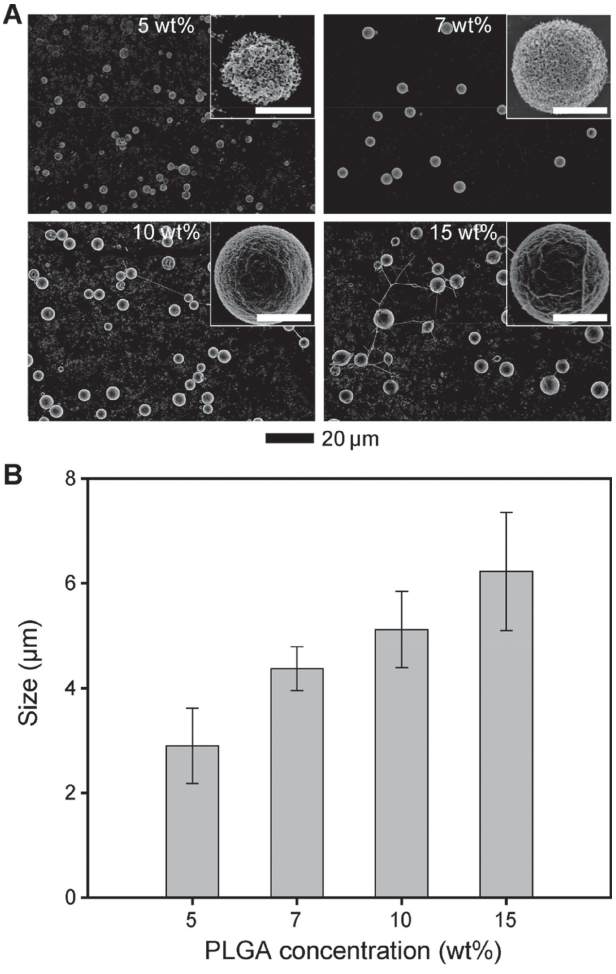

Figure 1. A) SEM images and B) average sizes of porous PLGA microspheres prepared with different PLGA concentrations (5, 7, 10, and 15 wt%) in electrospraying. The insets in (A) are high-magnification images of the surfaces of the porous PLGA microspheres, where the scale bars are 3 μm.

average size of the microspheres was found to increase from 2.90 ± 0.72 to 6.23 ± 1.13 μm with an increase in the polymer concentration. The coefficient of variance of the PLGA microspheres prepared with a PLGA concentration of 7 wt% was found to be 9.6%, suggesting a relative uniformity in size. Based on an analysis of their porosity, mechanical property, and size uniformity, the PLGA microspheres prepared with a PLGA concentration of 7 wt% were used for the subsequent protein encapsulation experiments. The electrospraying was conducted at 26 °C above the melting point of DMSO (19 °C), suggesting that there was no crystallization effect of DMSO on pore formation. Therefore, the pore development was attributed to the phase separation during solvent evaporation in electrospraying. If DMSO is not fully evaporated during electrospraying, it is impossible to obtain the PLGA microspheres in the form of powders. The large surface area of the electrosprayed microdroplets facilitated the fast evaporation of DMSO although the boiling point of DMSO is 189 °C. In addition, it might be expected that there was no residual DMSO because the porous PLGA microspheres were washed with PBS three times and freeze-dried prior to loading proteins.

Figure 2 shows SEM images of the surfaces and cross sections of the porous PLGA microspheres prepared with a PLGA concentration of 7 wt% before and after closure of the outer surface pores. The pristine PLGA microspheres had surface pores with diameters of 62.7 ± 8.5 nm. Note that the inner pores were larger than the surface pores, as is often observed in the emulsification and solvent evaporation method.[19,20] This can be explained as follows. The surface of electrosprayed microdroplets is first solidified due to the rapid evaporation at the interface of the oil and air, determining the overall size of the microspheres. The PLGA molecules inside the microdroplets continue to move to the surface and precipitate during the electrospraying process, resulting in a relatively dense surface and a porous inside. This feature can be advantageous for the entrapment of proteins because the small surface pore facilitates pore closure and the large porous inside allows the confinement of a large amount of protein. A mixture of PBS and DMSO was used to close the outer surface pores. DMSO was selected as a solvent for PLGA due to its relatively low effect on protein denaturation.[21] In addition, DMSO is commonly used in the pharmaceutical industry and is classified as a class 3 solvent.[22] At DMSO ratios higher than 30 w/w%, the porous PLGA microspheres tended to be fused together, forming large aggregates. Therefore, the ratio of DMSO in PBS was maintained at 20 w/w%. At this ratio, all of the porous PLGA microspheres were individually well dispersed without aggregation. As shown in Figure 2C,D, most of the surface pores disappeared while the inner porous structure remained well. It was hypothesized that the pore closure

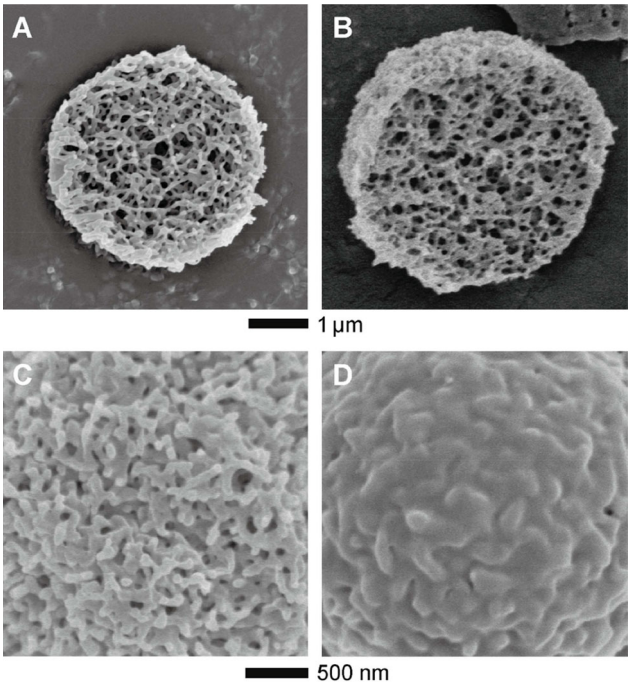

Figure 2. SEM images of porous PLGA microspheres prepared with a PLGA concentration of 7 wt% A,C) prior to and B,D) after treatment with a solvent mixture. The cross sections are shown in (A) and (B), and the outer surfaces are shown in (C) and (D).

at the outer surface may allow the sustained release of the encapsulated proteins and the maintenance of the biological activity and structural stability of the proteins for a long period.

To confirm the encapsulation, FITC-dextran was first entrapped inside the porous PLGA microspheres, which were prepared as described above. Figure 3A shows confocal microscopy images of the porous PLGA microspheres containing FITC-dextran before and after treatment with a solvent mixture. The porous PLGA microspheres prior to treatment with a solvent mixture exhibited low fluorescence, which was attributed to the physical adsorption of FITC-dextran at the surface of the inner and outer pores. In contrast, a large amount of FITC-dextran was found throughout the PLGA microspheres treated with a solvent mixture. The white lines in each confocal image indicate the fluorescence intensity profile of FITC-dextran along the center of each of the types of PLGA microspheres. Figure 3B shows the quantitative fluorescence intensity of the PLGA microspheres containing FITC-dextran before and after closure of the surface pores. The fluorescence intensity of the PLGA microspheres treated with a solvent mixture is sevenfold higher than that obtained with the untreated PLGA microspheres, confirming the presence of a large amount of FITC-dextran inside the PLGA microspheres.

As a next step, BSA (M_w = 66 kDa) was chosen for the demonstration of protein encapsulation due to its wide

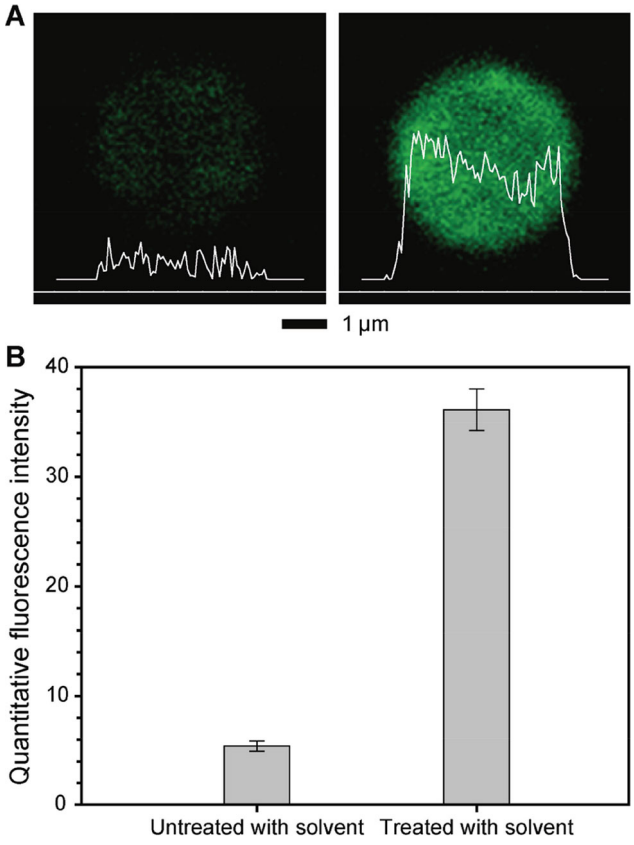

Figure 3. A) Confocal microscopy images of PLGA microspheres containing FITC-dextran (left) untreated and (right) treated with a solvent mixture, and B) plot of the quantitative fluorescence intensity. The fluorescence intensity profiles (white curves in A) were analyzed along the line through the center of the PLGA microspheres. Both samples were evaluated after washing with PBS three times.

use as a model protein.[23] It is well known that proteins have their own 3D structure for biological activities. The denaturation of a protein is the most critical issue associated with protein encapsulation. Therefore, we investigated the effect of DMSO on protein denaturation using CD spectroscopy. Figure 4A shows the CD spectra of BSA exposed to mixtures of PBS and DMSO at different ratios. BSA is generally characterized by a α-helix fingerprint in the CD spectrum at 208 and 222 nm. There was no significant difference in between the CD spectra of BSA even at the presence of 50 w/w% DMSO, compared with pristine BSA in PBS. We also evaluated the CD spectra of the encapsulated and released BSA from the PLGA microspheres, confirm the stability of its original conformation (Figure S2 for BSA and Figure S3 for lysozyme, Supporting Information).

The encapsulation efficiency of BSA in the PLGA microspheres treated with a solvent mixture was found to be 89.7%, which was much higher than the 52.3% for the PLGA microspheres untreated with a solvent

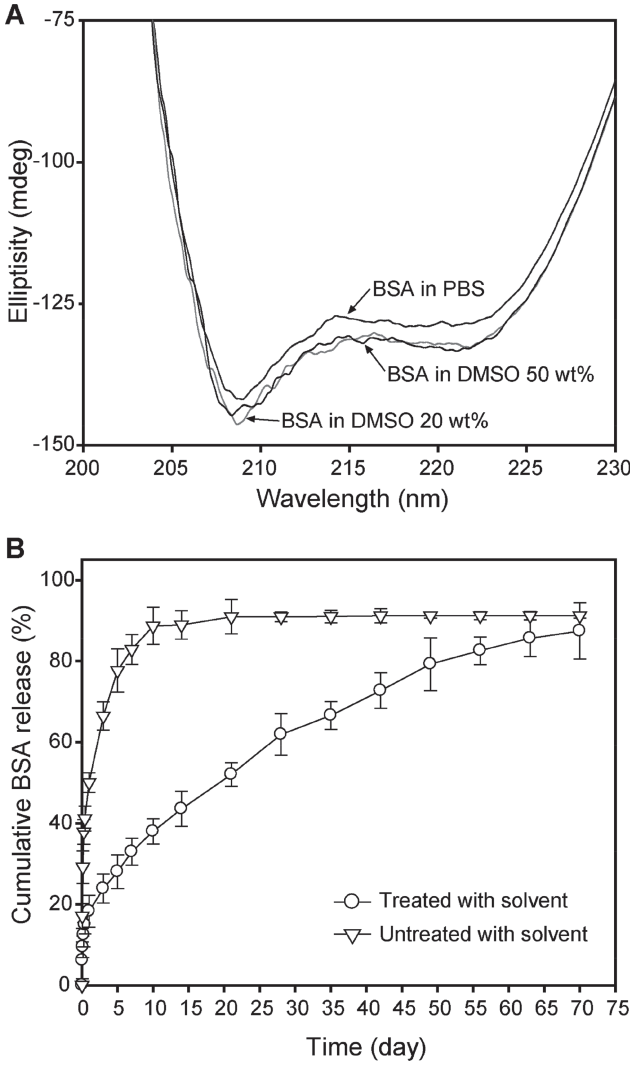

Figure 4. A) CD spectra of BSA exposed to mixtures of PBS and DMSO at different ratios (0, 20, and 50 wt%) for 4 h and B) release profiles of BSA from porous PLGA microspheres containing BSA treated and untreated with a solvent mixture. The cumulative release of BSA was defined as the ratio of the released amount of BSA at a given time divided by the initial amount of BSA that was encapsulated.

mixture. Figure 4B shows the release profiles of BSA from the porous PLGA microspheres over time. The analysis of the PLGA microspheres untreated with a solvent mixture revealed that ≈50% of the total BSA was released within 1 d, and a plateau was obtained after 10 d. In contrast, it was found that BSA was released in a sustained manner with a slight burst at the initial stage from the PLGA microspheres treated with a solvent mixture, achieving a nearly zero-order release profile. The release of BSA was prolonged over 60 d. Increases in the amount of encapsulated protein and the release period are likely possible by using a higher concentration of BSA solution.

4. Conclusions

We have successfully demonstrated a technique for the encapsulation without significant denaturation of BSA in porous PLGA microspheres and its nearly zero-order release from the microspheres. The porous PLGA microspheres with a micro-scale diameter are suitable for parenteral injection or oral administration. This strategy has a number of attractive features: a simple fabrication procedure, the absence of the use of a toxic solvent, heat, or surfactant, the encapsulation of a large amount of protein, and the sustained release of the protein. In addition, this encapsulation technique can be extended to a variety of other water-soluble macromolecules. We believe that this procedure is not limited to proteins. Any other water-soluble molecules can be entrapped inside the porous PLGA microspheres. Our next goal is the development of a strategy for the long-term release of insulin for diabetes therapy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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