Korean J. Chem. Eng., **28**(1), 293-297 (2011)
DOI: 10.1007/s11814-010-0322-4

Silk fibroin microspheres prepared by the water-in-oil emulsion solvent diffusion method for protein delivery

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(*Received 17 February 2010 • accepted 11 May 2010*)

Abstract−Silk fibroin (SF) microspheres were prepared by the simple water-in-oil emulsion solvent diffusion method without any surfactants. Aqueous SF solution and dichloromethane were used as water and oil phases, respectively. Influence of water : oil phase ratios on SF microsphere characteristics was investigated. From FTIR spectra, the resulting SF microspheres showed predominantly random coil SF conformation. SF microspheres observed from SEM images were spherical with deflated surfaces in some cases. Particle sizes of the SF microspheres were in the range 45-92 μm. Finally, bovine serum albumin (BSA) was used as a model protein for entrapment within the SF microspheres. The BSA-loaded SF microspheres were larger than unloaded SF microspheres. *In vitro* release tests indicate that BSA release from the SF microspheres was influenced by BSA content.

Key words: Silk Fibroin, Microspheres, Water-in-oil Emulsion Solvent Diffusion Method, Bovine Serum Albumin, Protein Delivery

INTRODUCTION

Silk fibroin (SF) of *Bombyx mori* is a natural protein polymer with excellent biocompatibility and biodegradability properties [1]. This polymer has been widely investigated as a biomaterial for tissue engineering [2,3], wound dressing [4], enzyme immobilization [5,6] and drug delivery [7]. For drug delivery applications, SF microspheres are very interesting as they can enhance the drug distribution, solubility and stability of water soluble drugs. Methods for preparing SF microspheres by spray drying [8,9] and lipid template [10] have been reported on a few previous occasions.

Enzymes or proteins are the most important water-soluble drugs. It is well known that current methods to entrap proteins within a polymer matrix are limited due to their sensitivity to heat and alcohol treatments during processing or post treatment. Protein immobilization on SF matrix surfaces has therefore been investigated in recent years [11,12]. Interest in direct protein entrapment within SF microspheres has increased steadily because of the higher protein loading efficiency and stability that can be obtained. However, little research has been performed into the preparation of protein-loaded SF microspheres.

In this work, we present an alternative method for preparing SF microspheres with and without protein entrapment. This method is a water-in-oil (W/O) emulsion solvent diffusion method. Influence of W: O phase ratios on the SF microspheres was investigated and is discussed. Bovine serum albumin (BSA) was used as a model protein for entrapment within SF microspheres. *In vitro* BSA release from the SF microspheres was also determined.

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EXPERIMENTAL

1. Materials

Silk fibroin (SF) aqueous solution was prepared by chemical degumming and dissolving before dialysis methods, respectively. Briefly, cocoons from *B. mori* were degummed by boiling twice with 0.5% Na_2CO_3 solution at 90 °C for 60 min to remove sericin and then washed with distilled water before air drying at room temperature. Degummed SF fibers were dissolved in ternary system solvent, $CaCl_2$-ethanol-water (mole ratio=1 : 2 : 8), by stirring at 90-95 °C. The resulting SF solution was dialyzed using cellulose tube (molecular weight cut off=6,000-8,000 Da) against distilled water for three days. The final SF concentration was adjusted to 4% (w/v) with distilled water.

2. Preparation of SF Microspheres

SF microspheres were prepared by the water-in-oil (W/O) emulsion solvent diffusion method. This involved adding 0.05 mL of SF solution dropwise into different dichloromethane volumes with stirring at 800 rpm for 20 min. The W : O phase ratios of 0.2, 0.1,

Table 1. Preparatory conditions for SF microspheres with and without BSA entrapment (0.05 mL of 4% w/v SF solution was used for all conditions)

Sample no.	SF : BSA ratio (w/w)	CH_2Cl_2 (mL)	W : O phase ratio (%)	Particle size (mm)
1	4 : 0	25	0.2	45±21
2	4 : 0	50	0.1	52±12
3	4 : 0	100	0.05	66±13
4	4 : 0	200	0.025	92±33
5	4 : 1	25	0.2	61±20
6	2 : 1	25	0.2	75±25

0.05 and 0.025% (v/v) were investigated. The beaker was tightly closed with aluminum foil to prevent dichloromethane evaporation during microsphere formation. The SF microspheres were recovered by centrifugation before freeze drying. Conditions for preparation of the SF microspheres are reported in Table 1, as sample nos. 1-4.

3. Preparation of BSA-loaded SF Microspheres

BSA-loaded SF microspheres were also prepared by the same method as described above. BSA model protein was dissolved in the SF solution before fabricating the SF microspheres. The SF : BSA ratios of 4 : 1 and 2 : 1 (w/w) were investigated. Preparatory formulations of the BSA-loaded SF microspheres are also reported in Table 1, as sample nos. 5 and 6. BSA microspheres were also fabricated for comparison. For this purpose, 0.05 mL of BSA solution (4% w/v) was added dropwise into 25 mL of dichloromethane with stirring at 800 rpm for 20 min. The beaker was tightly closed with aluminum foil to prevent dichloromethane evaporation. The BSA microspheres were collected by centrifugation before freeze drying.

4. Characterization of SF Microspheres

Chemical structure of the SF microspheres with and without BSA loading was determined by Fourier transform infrared (FTIR) spectroscopy using a Perkin-Elmer Spectrum GX FTIR spectrometer with air as the reference. A resolution of 4 cm^{-1} and 32 scans were used in this work. FTIR spectra were collected using a KBr disk method.

Morphology and size of the SF microspheres were investigated by scanning electron microscopy (SEM) using a JEOL JSM-6460LV SEM. The SF microspheres were coated with gold for enhancing conductivity before scan. Particle sizes and size distributions were determined from several SEM images counting at least 50 microspheres using the smile view program, version 1.02.

5. *In vitro* BSA Release Test

For the BSA release test, approximately 0.01 g of BSA microspheres or BSA-loaded SF microspheres was incubated in 1 mL of phosphate buffer solution (PBS) pH 7.4 at 37 ºC. At each time, the release medium was collected and replaced by fresh PBS medium. The amount of BSA in released medium was measured by the Lowry protein assay using a calibration standard curve. The fresh PBS was replaced at each collected time. Pure SF microspheres were used as a control for BSA release study. Actual amount of the released BSA was calculated by subtracting with dissolution of pure SF microspheres (a control group) at the same incubation time.

RESULTS AND DISCUSSION

1. Preparation of SF Microspheres

The W/O emulsion solvent diffusion method is a single step for preparing surfactant-free SF microspheres without heat and alcohol treatment. The solubility of water in dichloromethane is approximately 0.24% (v/v) (CAS No. 75-09-2). This suggests that the water should completely diffuse out from dispersed emulsion droplets of SF solution to continuous external phase, dichloromethane when the W : O phase ratio is less than 0.24 % (v/v). Therefore, W : O phase ratios of 0.2, 0.1, 0.05 and 0.025% (v/v) were chosen to prepare the SF microspheres in this work (Table 1).

It was found that the SF microspheres can solidify and suspend in the dichloromethane phase after the emulsification-diffusion process. Yields of the SF microspheres measured by gravimetric method were higher than 90%. It should be noted that this method is a very simple, fast and suitable for preparing surfactant-free protein-loaded SF microspheres.

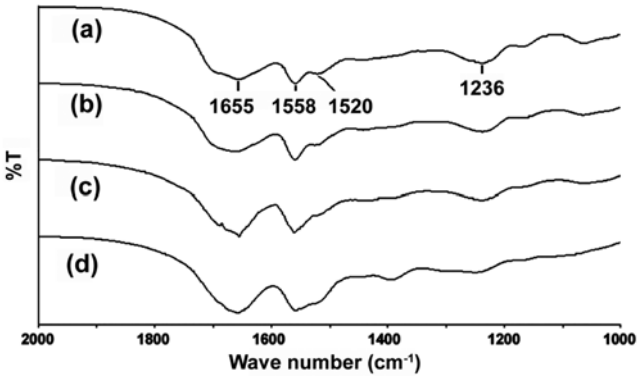

Fig. 1. FTIR spectra of SF microspheres of sample nos. (a) 1, (b) 5, (c) 6 and (d) BSA microspheres.

2. FTIR Spectra

SF conformation of the SF microspheres was determined by position of amide bands in their FTIR spectra. Fig. 1 shows the FTIR spectra of SF microspheres, BSA-loaded SF microspheres and BSA microspheres. The absorption bands of the SF microspheres in Fig. 1(a) at 1,655 cm^{-1} (amide I, C=O stretching), 1,558 cm^{-1} (amide II, N-H bending) and 1,236 cm^{-1} (amide III, C-N stretching) were assigned to random coil conformation [13,14]. Thus, the SF microspheres showed predominantly random coil form. The band shoulders at 1,701 and 1,520 cm^{-1} can be assigned to β-sheet structure of amide I and II, respectively [13,14]. This suggests that the SF microsphere matrix consisted of both the random coil and β-sheet forms. In addition, the FTIR spectra of the SF microspheres with W : O phase ratios of 0.1, 0.05 and 0.025% (v/v) showed similar conformations.

The FTIR spectrum of BSA microspheres in Fig. 1(d) also shows amide I and II bands. From the FTIR spectra of BSA-loaded SF microspheres in Figs. 1(b) and (c), it can be seen that the BSA entrapment did not affect the shifting of amide band or conformational transitions of the SF microsphere matrices. However, the intensity of amide III band of SF at 1,236 cm^{-1} was significantly decreased when the BSA was entrapped and increased, suggesting that the BSA was successfully entrapped in the SF microspheres.

3. Morphology of SF Microspheres

Particle morphology was determined from their SEM images. All SF microspheres were nearly spherical and smooth of surface as shown in Figs. 2 and 3. Some deflated surfaces occurred due to dehydration during the diffusion process. The observation of highly deflated SF microspheres prepared by the spray drying method has been reported previously [8,9]. This may be due to rapid evaporation of water during the spray drying process. The morphological results indicate that the W/O emulsion solvent diffusion is a suitable method for preparing SF microspheres.

The morphology of the BSA-loaded SF microspheres was investigated from their SEM images. They were also nearly spherical shapes with some deflated surfaces similar to the BSA-unloaded SF microspheres. The results suggest that the BSA entrapment did

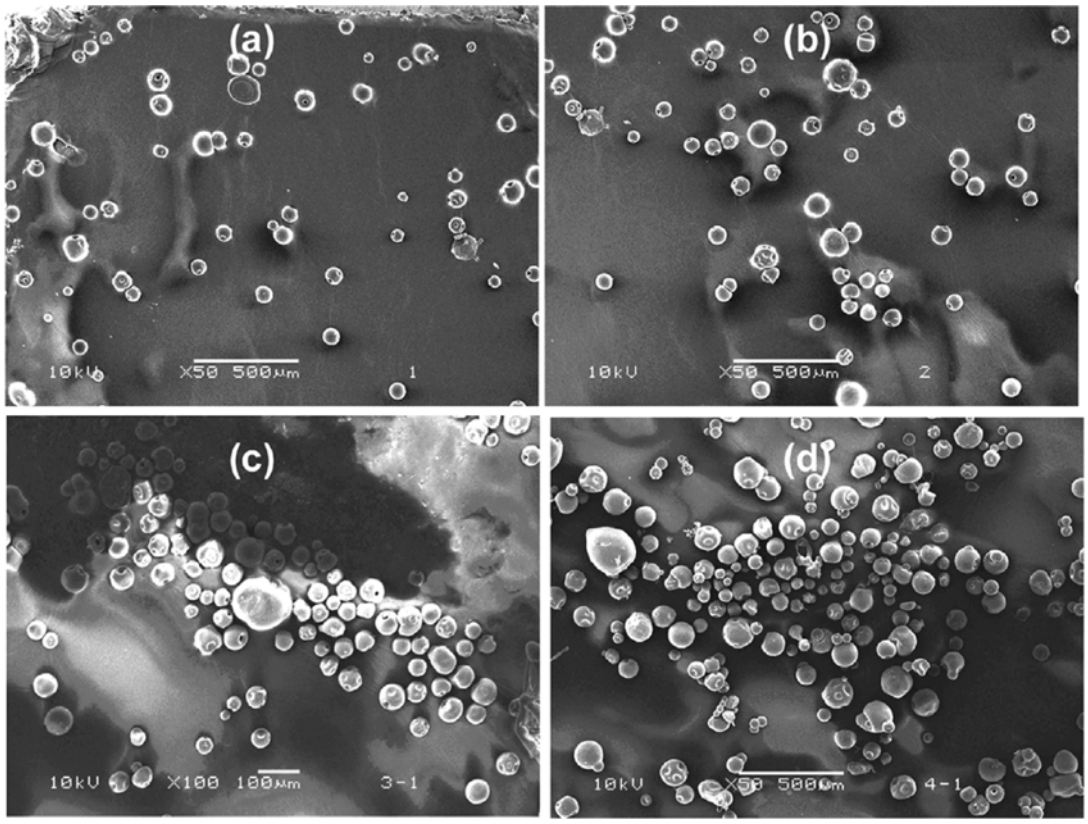

Fig. 2. SEM image of SF microspheres of sample nos. (a) 1, (b) 2, (c) 3 and (d) 4. All bars=500 μm, except (c) =100 μm.

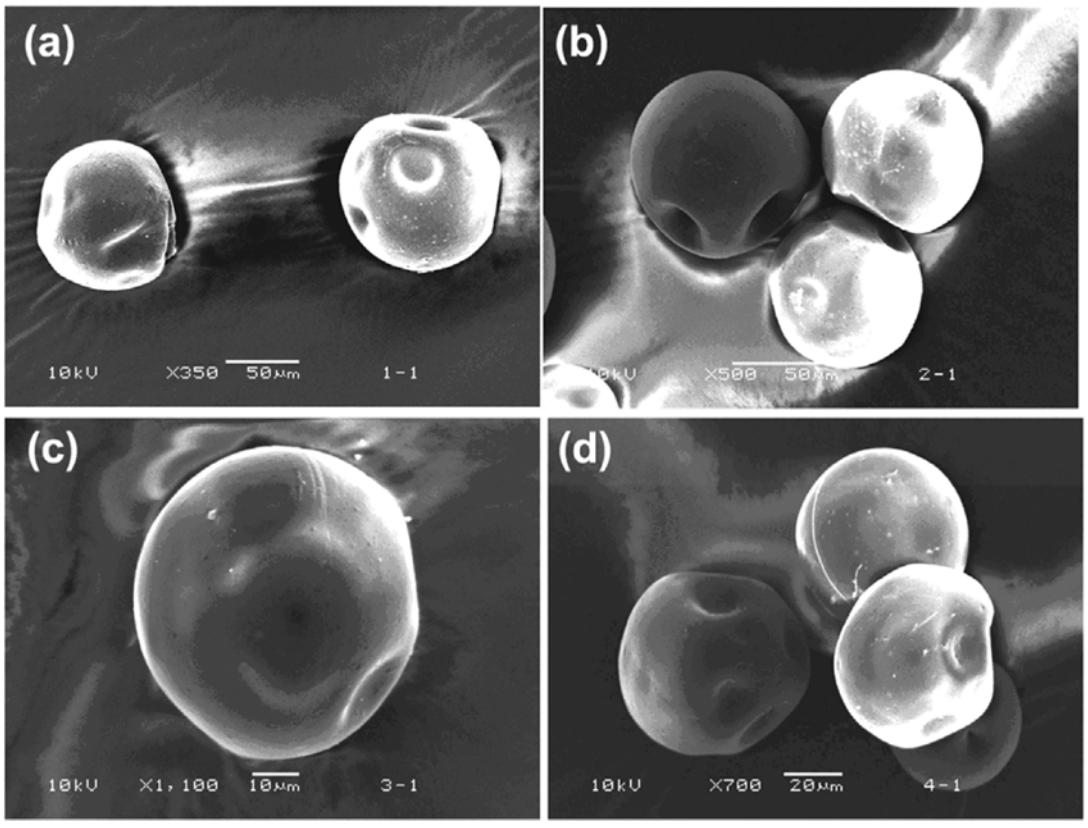

Fig. 3. Expanded SEM images of SF microspheres of sample nos. (a) 1, (b) 2, (c) 3 and (d) 4. Bars=50, 50, 10 and 20 μm for (a), (b), (c) and (d), respectively.

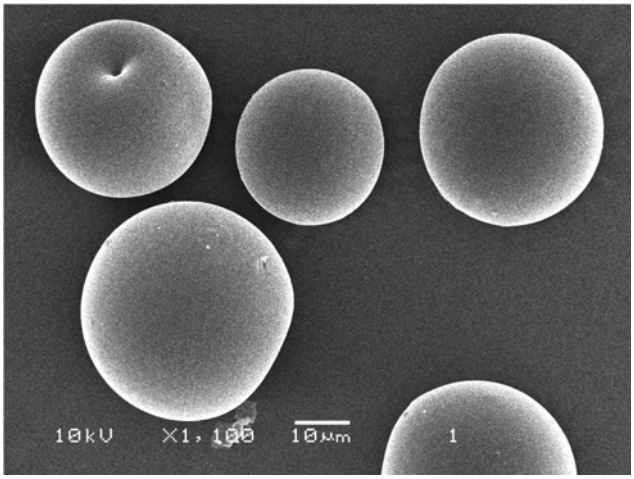

Fig. 4. SEM image of BSA microspheres. Bar=10 μm.

not affect SF microparticle shape. It can be concluded that the preparatory conditions of SF microspheres used in this work are appropriate for producing BSA-loaded and unloaded SF microspheres. In addition, the BSA microspheres with approximately 95% yield were also spherical with smooth surfaces, as shown in Fig. 4.

4. Particle Sizes of SF Microspheres

Particle sizes and size distributions of the SF microspheres with and without BSA entrapment were directly measured from several SEM images. The calculated results of SF microsphere sizes are summarized in Table 1. These were in the range of 45-92 mm. The SF microspheres were slightly larger with the dichloromethane volume. This may be explained by the larger dichloromethane volume inducing faster solvent diffusion and rapid microsphere solidification during the emulsification process. The larger SF microspheres were formed by rapid solidification of the larger emulsion droplets. Therefore, larger SF microspheres were obtained with increasing dichloromethane volume (or decreasing W : O phase ratio). Li and Yang [15] have been prepared ethyl cellulose microspheres by the O/W emulsion solvent diffusion/evaporation method. The oil and water phases were dichloromethane/methanol/acetone ternary mixture and aqueous chitosan solution, respectively. The larger ethyl cellulose microspheres were obtained due to methanol and acetone diffusing out fast.

The size of the SF microspheres slightly increased when the quantity of entrapped BSA was increased (Table 1). The particle size results indicate that the SF microsphere size is directly related to BSA content. This suggests that the W/O emulsion solvent diffusion method is appropriate for preparing protein-loaded SF microspheres. This is due to the poor solubility of both SF and BSA in the continuous dichloromethane phase. The increasing microsphere size after BSA entrapment suggests the high BSA entrapment efficiency of W/O emulsion solvent diffusion.

5. BSA Release from SF Microspheres

In vitro BSA release was investigated in PBS (pH 7.4) at 37 °C for 48 h. BSA release profiles of the BSA and the BSA-loaded SF microspheres are shown in Fig. 5. The BSA microspheres fabricated by the W/O emulsion solvent diffusion method showed rapid dissolution suggesting that the BSA did not denature during microsphere formation.

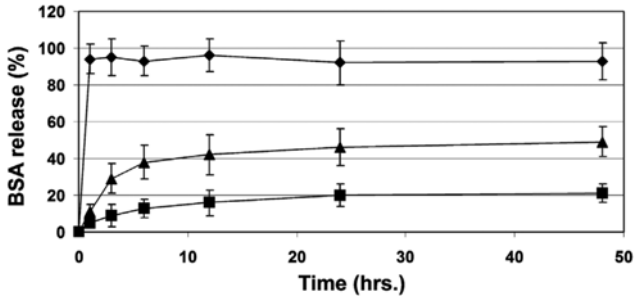

Fig. 5. BSA release profiles of BSA microspheres (◆) and BSA-loaded SF microspheres with SF : BSA ratios of (■) 4 : 1 and (▲) 2 : 1 (w/w).

Both sample nos. 5 and 6 showed release separation in a burst release effect in the first 6 h, followed by a second phase of slower BSA release. FTIR results show that the SF microsphere matrices contained predominantly SF random coil (water-soluble) form. It would be expected that the burst release effect occurred due to rapid BSA release from the dissolution of entrapped BSA on the microsphere surfaces, and from the random coil SF phase of microsphere matrix. However, the burst release effect and release rate of BSA from the SF microspheres increased as the BSA content increased. It can be concluded that the rate of burst release is strongly dependent upon the BSA ratio. Thus BSA release rate from the SF microspheres can be adjusted by varying the BSA content.

The drug release kinetics of these SF microspheres can be explained by the following equation [16]:

$$\frac{Q_t}{Q_o} = kt^n$$

Where Q_t is the amount of drug released at time t, Q_o is the amount of the total drug in the microspheres, k is the zero-order release constant, and n is the release exponent, which describes the release kinetic. The n is between 0.5 and 1 for diffusion-erosion controlled drug release system. The drug release is mainly controlled by diffusion when n is close to 0.5. The erosion type is the main determination factor when n is close to 1. It can seen that the n of 4 : 1 and 2 : 1 (w/w) BSA-loaded SF microspheres were 0.369 and 0.348, respectively, close to 0.5, which suggests the diffusion controlled release kinetic may play an important role in the release behavior of these SF microspheres.

CONCLUSIONS

SF microspheres which were nearly spherical were successfully prepared by the W/O emulsion solvent diffusion method. The SF aqueous solution and dichloromethane were used as W and O phases, respectively. Different W : O phase ratios were used to prepare the SF microspheres. The microsphere sizes slightly increased as the W : O phase ratio decreased. The BSA-loaded SE microspheres with SF : BSA ratios of 4 : 1 and 2 : 1 (w/w) were also successfully fabricated by directly dissolving BSA in the SF solution before microsphere preparation using the same method. The microsphere sizes and BSA release rates were increased when the BSA content was increased.

The W/O emulsion solvent diffusion method is a simple and rapid

method for preparing protein-loaded SF microspheres. The use of emulsifiers and heat/alcohol treatments is not necessary. Therefore, the technique described is potentially very useful for drug delivery applications, especially with proteins and water-soluble bioactive molecules.

ACKNOWLEDGEMENTS

The authors thank the National Metal and Materials Technology Center (MTEC), National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand (MT-B-52-BMD-68-180-G) and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand for financial support. Appreciation is also expressed to Dr. Tim Cushnie, Faculty of Medicine, Mahasarakham University for helping to improve the manuscript.

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