Preparation of Porous Silk Fibroin Microparticles by a Water-in-Oil Emulsification-Diffusion Method

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ABSTRACT: Porous silk fibroin (SF) microparticles were prepared by a simple one-step water-in-oil emulsification-diffusion method. Aqueous SF solution and organic phase solvent were used as water and oil phases, respectively. Four organic solvents with different water solubility were studied, i.e., ethyl acetate, diethyl ether, dichloromethane, and chloroform. Influences of organic phase solvent, water/oil volume ratio, and SF concentration on SF microparticle characteristics were investigated. It was found that shape of the SF microparticles depended upon the type of organic phase solvent. The SF microparticles with hollow or bowl-like shape were fabricated when organic solvents with higher water solubility, which are ethyl acetate and diethyl ether, were used as the continuous oil phase. While using organic solvents with lower water solubility, i.e., dichloromethane and chloroform resulted in the spherical shape SF microspheres. The water/oil volume ratios and the SF concentrations did not affect on the bowl-like and spherical shapes of SF microparticles. Results from scanning electron microscope shows that all the SF microparticle matrices were porous structures. Using ethyl acetate and diethyl ether as the oil phase gave larger SF microparticle sizes than using dichloromethane and chloroform. The influences of water/oil volume ratio and the SF concentration showed slightly effect on the sizes of SF microparticles. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 1127–1133, 2010

Key words: silk fibroin; microparticles; microspheres; porous structures

INTRODUCTION

In recent years, interest in hollow and porous particles has increased steadily due to their high surface area and high volume contain. They have many potential applications, such as delivery carriers for drugs, cosmetics, dyes and inks, catalytic supports, cell culture substrates, and selective separation.[1–9] For these purposes, the hollow and porous microparticles have been prepared from ceramics, polymers, metals and their composites. The bioresorbable polymers have been extensively studied in medical and pharmaceutical applications due to their biocompatibility and biodegradability. The hollow and porous microparticles of biodegradable polymers have been fabricated by several techniques such as water-in-oil-in-water emulsion solvent extraction/ evaporation,[10–12] oil-in-water emulsion core template extraction,[6] oil-in-water-in-oil emulsion hydration,[9] spray drying,[13] and electrospraying[14] methods. However, preparation of biocompatible and biodegradable porous silk fibroin (SF) microparticles with controllable hollow and spherical shapes has not been published.

SF of *Bombyx mori*, a natural protein polymer has attracted great attention because of its potential applications in tissue engineering,[15–18] enzyme immobilization,[19] and controlled release drug delivery.[20,21] The SF matrices have been found to be excellent cell adhesion and proliferation and enzyme immobilization materials.[17,22,23] Methods for preparing SF microspheres by spray drying[13,24] and lipid template[25] have been reported on a few previous occasions.

Herein, a one-step method for fabricating the controllable hollow and spherical shapes of porous SF microparticles is reported for the first time. This method is the use of water-in-oil (W/O) emulsification-diffusion concept. The continuous organic phase (oil phase) with different water solubility including ethyl acetate, diethyl ether, dichloromethane, and chloroform were used as an oil phase. The SF aqueous solution was used as a water phase. Influences of the organic phase types, W/O volume ratios and SF concentrations on SF microparticle characteristics were investigated.

TABLE I
Preparing Formulations, Yields, and Average Sizes of the Porous SF Microparticles

Sample no.	SF concentration (% w/v)	SF solution (mL)	Organic phase solvent[a]	Microparticle yield[b] (%)	Average particle size[c] (μm)
1	4.0	0.2	Ethyl acetate	85	134 ± 65
2	4.0	0.4	Ethyl acetate	80	141 ± 58
3	4.0	0.8	Ethyl acetate	76	148 ± 69
4	4.0	0.2	Diethyl ether	89	125 ± 72
5	4.0	0.4	Diethyl ether	85	131 ± 81
6	4.0	0.2	Dichloromethane	92	91 ± 35
7	4.0	0.05	Chloroform	95	48 ± 18
8	2.0	0.2	Ethyl acetate	88	105 ± 46
9	1.0	0.2	Ethyl acetate	90	101 ± 48

[a] 100 mL of each organic phase solvent was used.
[b] Measured from gravimetric method.
[c] Determined from several SEM images.

EXPERIMENTAL

Materials

SF aqueous solution was prepared by a chemical degummed method before dissolving and dialysis, respectively. Briefly, SF cocoons from *B. mori* were degummed by boiling twice with 0.5% (w/w) Na_2CO_3 solution at 100°C for 1 h to remove sericin and then washed with distilled water. They were then dried at room temperature. Degummed SF fibers were dissolved in a ternary solvent system of $CaCl_2$–ethanol–water (1 : 2 : 8 in mole ratio) at 95°C for 5 h with stirring. The resulting SF solution was dialyzed to remove $CaCl_2$ and ethanol using cellulose tubular membrane (molecular weight cut off = 6,000–8,000 Da) in distilled water for 3 days. The fresh distilled water was changed every day. The final SF concentrations after dialysis and filtration were adjusted to 4, 2, and 1% (w/v) with distilled water. Ethyl acetate, diethyl ether, dichloromethane, and chloroform in analytical grade were used as received for organic phase solvents.

Preparation of SF microparticles

The surfactant-free SF microparticles were prepared using the water-in-oil (W/O) emulsification-diffusion method. An appropriate amount of SF solution was slowly added drop-wise into 100 mL of organic solvent with magnetic stirring at 900 rpm for 30 min, using an IKA Yellowline RST digital magnetic stirring apparatus. The beaker was covered with aluminum foil to prevent evaporation of organic phase solvent during emulsification and diffusion processes. The SF microparticles were recovered by centrifugation before drying in a vacuum oven at room temperature for a week. The volumes and concentrations of SF solutions and types of organic phase solvents used in this research are summarized in Table I.

Characterization of SF microparticles

Chemical structures of the SF microparticles were investigated by Fourier transform infrared (FTIR) spectroscopy using a Perkin-Elmer Spectrum GX FTIR spectrometer with air as the reference. The resolution of 4 cm^{-1} and 32 scans were chosen in this work. FTIR spectra were obtained from a KBr disk method.

Morphology and average size of microparticles were examined by scanning electron microscopy (SEM) using a JEOL JSM-6460LV SEM. The microparticles were coated with gold for enhancing conductivity before scanning. The average microparticle sizes were determined from several SEM images by counting a minimum of 100 particles using smile view software (version 1.02).

RESULTS AND DISCUSSION

In this work, the simple one-step water-in-oil (W/O) emulsification-diffusion method without any surfactant for preparing SF microparticles was disclosed. The SF aqueous solution and different organic phase solvents (ethyl acetate, diethyl ether, dichloromethane, and chloroform) were used as the water and the oil phases, respectively. The maximum water solubility in ethyl acetate, diethyl ether, dichloromethane, and chloroform are 3.30% (CAS No. 141-78-6), 1.26% (CAS No. 60-29-7), 0.24% (CAS No. 75-09-2), and 0.06% (CAS No. 67-66-3) by volume, respectively. It can be postulated that if the water/oil volume ratio of W/O emulsion was less than the maximum value of water solubility in each organic phase solvent, the SF particles could be solidified and formed after almost the water diffused out from the emulsion droplets of W phase (SF aqueous solution) to the O phase (continuous organic phase). It was found in our cases that the SF microparticles were successfully prepared using these organic phase
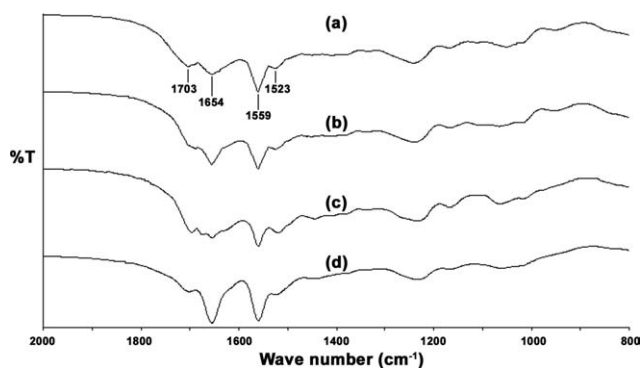
Figure 1 FTIR spectra of porous SF microparticles of sample numbers (a) 1, (b) 4, (c) 6, and (d) 7.

solvents. Table I summarized preparation formulation, yields, and average size of SF microparticles. It shows that the percent yields of the SF microparticles slightly decreased with the increasing W/O volume ratio and SF concentration.

FTIR spectra

The chemical structure of the SF microparticles was investigated from the FTIR spectra as shown in Figure 1. It is well known that the amide band positions in the FTIR spectrum can be used to indicate the SF molecular conformation. It was found that the FTIR spectra of all SF microparticles prepared from all organic phase solvents showed predominantly amide I

and II bands at 1654 and 1559 cm^{-1}, respectively. These amide band positions were assigned to predominantly random coil conformation of SF matrices similar to the case of SF films prepared by drying of aqueous SF solution at room temperature.[26] Results suggested that the W/O emulsification-diffusion process did not completely induce SF conformational transition from random coil to β-sheet forms. However, the amide bands of β-sheet SF characters were also detected as shoulder bands at 1703 cm^{-1} (amide I) and 1523 cm^{-1} (amide II) for all SF microparticles.[26,27] The results indicated that the random coil and β-sheet forms coexisted in the SF microparticle matrices with predominantly random coil conformation.

A few methods have been used to change the SF conformation from random coil (water-soluble) to β-sheet (water-insoluble) form such as heat treatment,[27] alcohol treatment,[28] and cross linking reaction.[29] The selection of each method strongly depend upon its applications. For cell culture application, the heat and alcohol treatments can be used, while the cross linking reaction is suitable for drug delivery application.

Microparticle morphology

Morphology of the SF microparticles was characterized from their SEM images, as shown in Figure 2. It was found that the hollow or bowl-like shape of SF microparticles with some deflated surfaces were
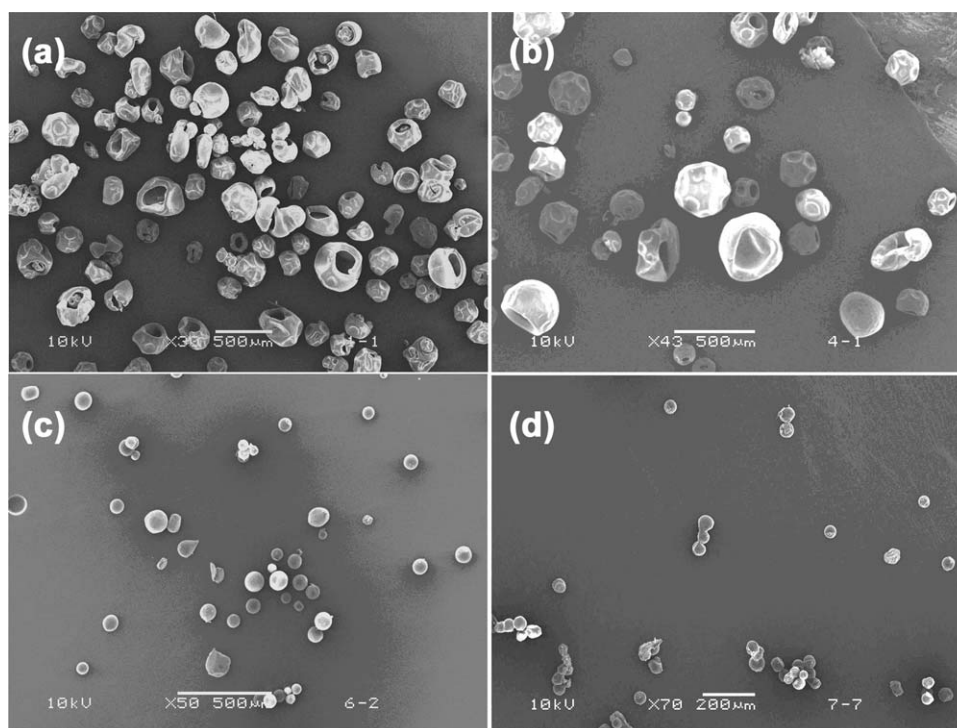

Figure 2 SEM images of porous SF microparticles of sample numbers (a) 1, (b) 4, (c) 6, and (d) 7. Bars = 500 and 200 μm for (a)–(c) and (d), respectively.
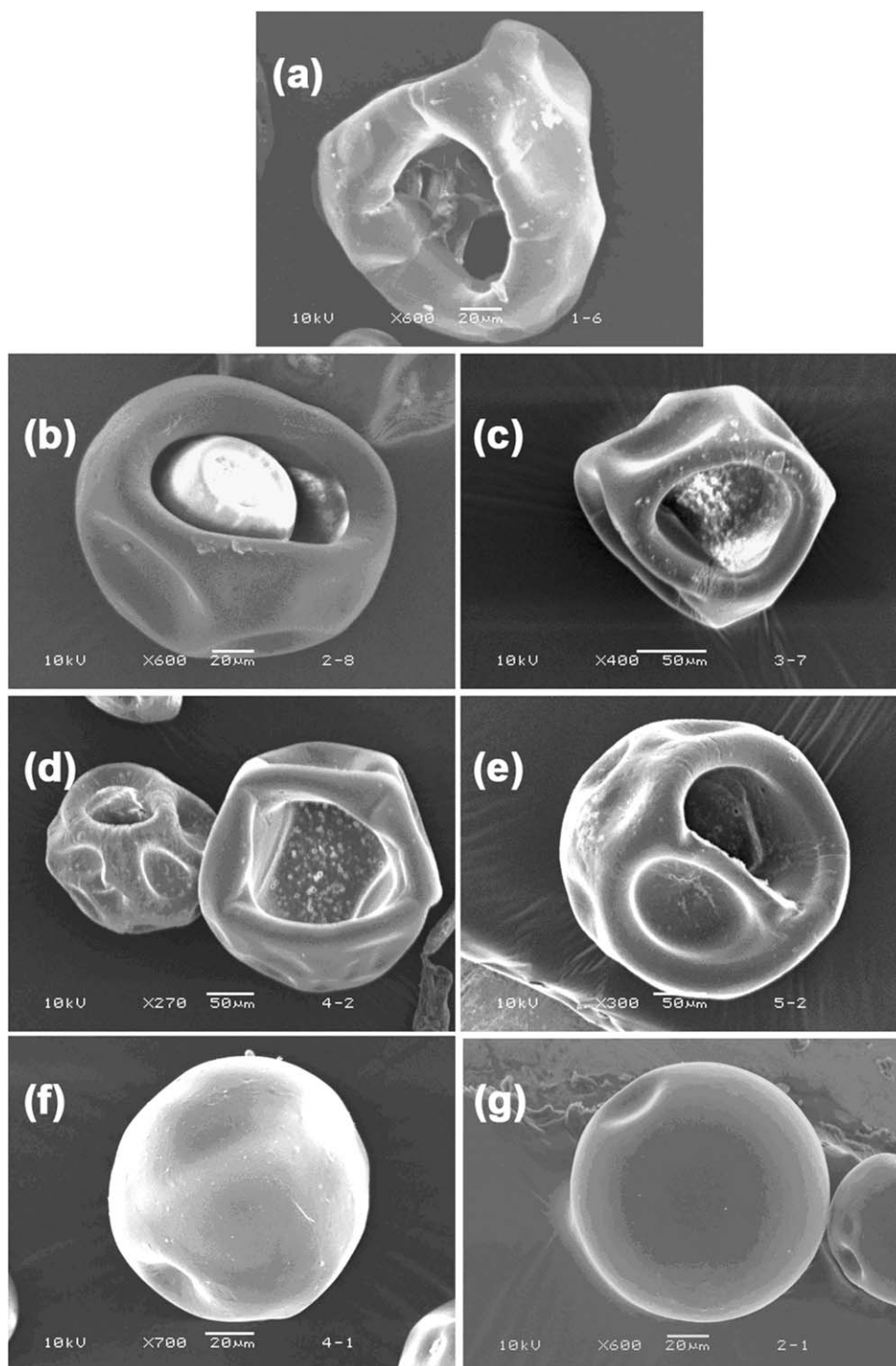
Figure 3 Expanded SEM images of porous SF microparticles of sample numbers (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, and (g) 7. Bars = 20 um for (a), (b), (f) and (g) and 50 um for (c), (d) and (e).

obtained when using ethyl acetate (sample no. 1) and diethyl ether (sample no. 4) as the organic phase solvents, as illustrated in Figure 2(a,b), respectively. Whereas, the SF microparticles prepared using dichloromethane (sample no. 6) and chloroform (sample no. 7) as the organic phase solvents were nearly spherical in shapes with some deflated surfa-

ces, as shown in Figure 2(c,d), respectively. The microparticle morphology was better seen from their expanded SEM images in Figure 3. This result was seen similarly as in the case of the erythrocyte-like shape of insulin microparticles prepared by spray-drying method.[30] The solid shell forms because the drying started at the outside of the droplets.
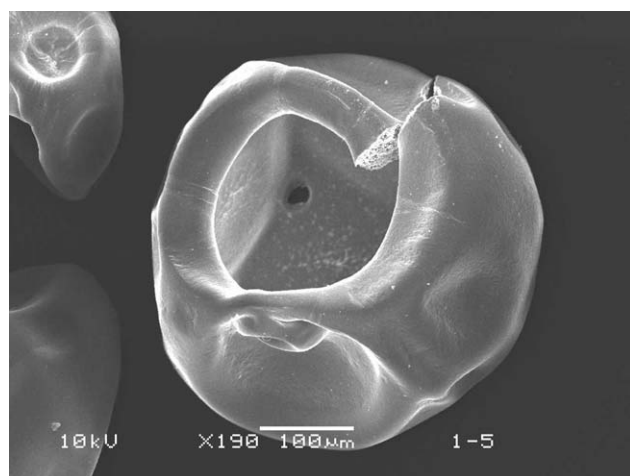
Figure 4 SEM image of internal matrix of hollow SF microparticle of sample number 1. Bar = 100 μm.

The miscibility between aqueous and organic phases of emulsion was directly related to the solvent and nonsolvent diffusion rates.[31] Diffusion rate of water from the emulsion droplets of SF aqueous solution (W phase) to the continuous organic solvent phase (O phase) of the W/O emulsion depends on water solubility in each organic phase solvent. The water diffusion rate increased with the increased water solubility of organic phase solvent. It can be estimated that faster water diffusion rate, indicated by higher water solubility of organic phase solvent, induced faster SF droplet solidification. The water solubility values of ethyl acetate and diethyl ether are higher than those of dichloromethane and chloroform. Therefore, the water molecules should be expulsed faster from the emulsion droplets of SF solution to the continuous ethyl acetate and diethyl ether phases than in the case of dichloromethane and chloroform phases, resulted in rapid solidification of SF matrices forming observed hollow or bowl-like in shapes of the SF microparticles as shown in Figure 3. Whereas, the SF microparticles prepared using dichloromethane and chloroform as the organic phase solvents were nearly spherical in shapes due to slower water diffusion rate. Moreover, it may be proposed that the lower interfacial tension between aqueous and ethyl acetate or diethyl ether phases, resulting from their higher water solubility also induced the hollow shape of the SF microparticles. This may be due to possible penetration or dissolution of organic molecules of organic phase solvent into the emulsion droplets of SF solution occurred simultaneously before complete solidification of the SF microparticles. Then, the irregular surfaces or hollow shapes of the SF microparticles were obtained when the organic phase solvents with high water solubility were used. In addition, the W/O volume ratio did not have significant effect

on the hollow structures of SF microparticles which prepared using ethyl acetate and diethyl ether as the organic phase solvents, as shown in Figure 3(a–e) for the sample numbers 1–5, respectively.

The deflated surfaces of both hollow and spherical-like SF microparticles may be proposed that the microparticle matrices contained voids or porous structures. This was confirmed by SEM images of internal matrices of SF microparticles, as shown in Figures 4 and 5 for the sample numbers 1 and 7, respectively. It was reported that the porous microparticles of water-insoluble polymer could be prepared by $water_1$-in-oil-in-$water_2$ ($W_1/O/W_2$) double emulsion method.[32,33] Voids were formed due to diffusion out of W_1 to W_2 phase. It would be therefore expected in our case that the porous structures of SF microparticles occurred from diffusion out of water from emulsion droplets to continuous organic solvent phase.

Figure 6 shows the hollow SF microparticles prepared with different SF concentrations using ethyl acetate as the organic phase solvent. It was found that all the SF microparticles still contained hollow in shapes, suggesting that their morphology does not depend on the SF concentrations.

Particle sizes

Average sizes of the SF microparticles were measured from several SEM images instead of light scattering analysis because of the partial swelling and dissolution of SF microparticles in the water suspension medium. The results of particle sizes are also summarized in Table I. The average sizes of SF microparticles increased as the increasing of the water solubility of organic phase solvent (sample nos. 1, 4, and 6). The larger SF microparticles for ethyl acetate and diethyl ether were attributed to the
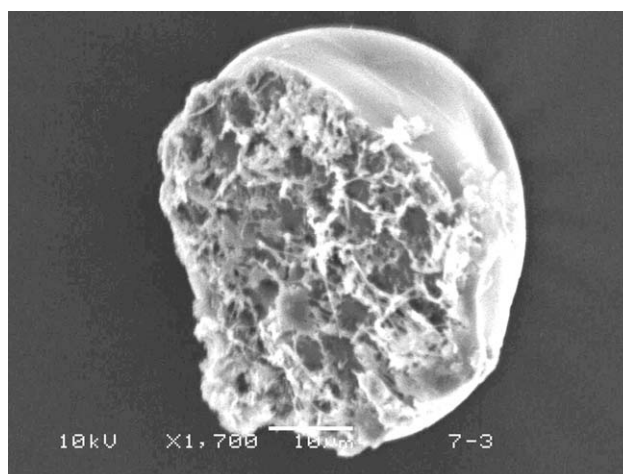

Figure 5 SEM image of internal matrix of SF microsphere of sample number 7. Bar = 10 μm.
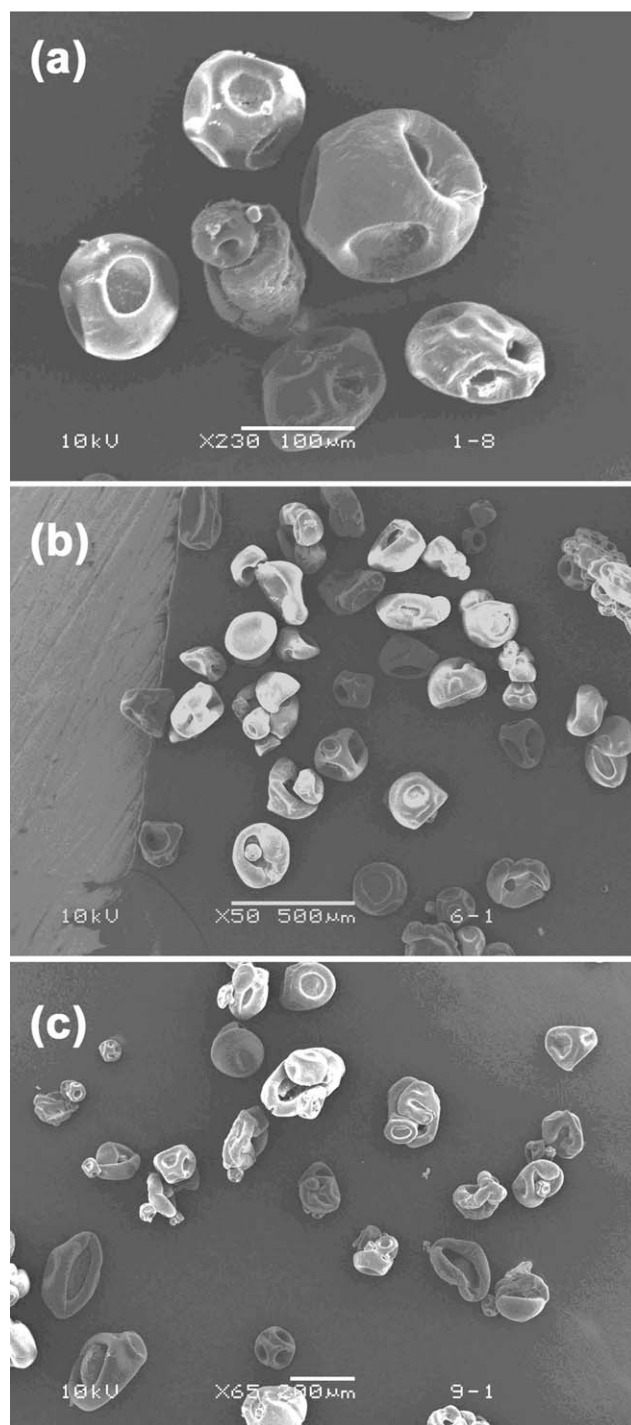
Figure 6 SEM images of porous SF microparticles of sample numbers (a) 1, (b) 8, and (c) 9. Bars = 100, 500, and 200 μm for (a), (b), and (c), respectively.

faster water diffusion from the emulsion droplets of SF aqueous solution to the external organic phase solvent. The high water solubility promoted fast water diffusion, hence rapid solidification which resulted in large particle size. This result means that fast water diffusion rate solidifies SF to form microparticles quickly. For the same organic phase solvent, effect on the average sizes of SF microparticles

were found when the volume and the concentration of SF aqueous solution phase were increased (sample nos. 1–5 and 8–9).

It can be noted that the effect of interfacial tension between emulsion aqueous droplets and continuous organic phases is inversely proportional to the average SF microparticle sizes. This may be due to high water solubility (or low interfacial tension) of the organic phase solvent induced rapid solidification of the emulsion droplets due to fast water diffusion out from the SF droplets. Then the larger SF microparticles were obtained for ethyl acetate and diethyl ether because the emulsion droplets rapidly solidified after dropping to the continuous organic phase.

The hollow or bowl-like shape of porous SF microparticles prepared by W/O emulsion solvent diffusion technique was first reported in this work. The ethyl acetate or diethyl ether was found as a suitable organic phase solvent for such a purpose. Moreover, this technique was successfully used to prepare the spherical-like SF microparticles, when dichloromethane or chloroform was employed as an organic phase solvent. Our technique is simple and carried out in mild condition. It can be an alternative method to prepare porous SF microspheres, normally prepared by spray-drying[24] and extractable lipid template[25] methods which require high temperature for solvent evaporation and multi-step procedure, respectively.

CONCLUSIONS

The controllable hollow and spherical-like shapes of porous SF microparticles were successfully prepared by a simple and rapid one-step W/O emulsification-diffusion method without any surfactants. The water solubility in organic phase solvent was an important factor affecting the shapes of porous SF microparticles. The SF microparticle matrices presented predominantly random coil conformation from FTIR results. The morphology of porous SF microparticles showed the hollow or bowl-like shapes using ethyl acetate and diethyl ether as the external organic phase, while the porous SF microparticles with spherical shape were achieved using dichloromethane and chloroform. The particle size did not significantly increase as volume and concentration of SF solution increased. It is believed that these surfactant-free porous SF microparticles fabricated by the W/O emulsification-diffusion method will find potential applications in microcapsules of artificial cell and controlled-release drug delivery systems, especially proteins and other water-soluble active molecules.

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