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# Effect of Methanol Treatment on Regenerated Silk Fibroin Microparticles Prepared by the Emulsification-Diffusion Technique

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**Abstract:** Silk Fibroin (SF) microparticles containing hollow structure were prepared by a water-in-oil emulsion solvent diffusion method without any surfactants. Aqueous SF solution and ethyl acetate were used as water and oil phases, respectively. Influences of SF concentration and post methanol treatment on microparticle characteristics were investigated. All microparticles contained open hollow structures. Microparticle sizes increased with the SF concentration. Conformation of SF microparticles determined from FTIR spectra changed from predominantly random coil to β-sheet form after methanol treatment. Particle surfaces of methanol-treated SF microparticles were rougher than those of non-treated. The SF concentrations and methanol treatment did not affect open hollow structure of the SF microparticles. Major advantages of this method are the elimination of surfactants and extractable pore templates. These hollow SF microparticles are expected to be potential used as biodegradable microcarriers of cell and protein drug, because their hollow structures should permit cell attachment and enzyme immobilization.

**Key words:** Biodegradable polymer, water-in-oil emulsion solvent diffusion method, molecular conformation, open hollow particles

## INTRODUCTION

Silk Fibroin (SF) is a fibrous protein which recognized as a biodegradable and biocompatible material created by the silkworm (Altman *et al*., 2003) and has recently been extensively investigated as a biomaterial such as matrix for cell culture substrate (Kim *et al*., 2005) and drug delivery systems (Hofmann *et al*., 2006; Wang *et al*., 2007). The most widely characterized SF is from the domesticated silkworm, *Bombyx mori*. The minimal inflammatory reactions *in vitro* and *in vivo* of SF film have been reported by Meinel *et al.* (2005). However, a method for preparing SF microparticles has been scarcely published. These methods are spray drying (Yeo *et al*., 2003) and water-in-oil (W/O) emulsion solvent evaporation (Srisuwan *et al*., 2009) methods.

In the current work, we have developed the simple W/O emulsion solvent diffusion method for fabricating SF microparticles before methanol treatment. Aqueous SF solution and ethyl acetate were used as water and oil phases, respectively. From this method, SF microspheres are formed after diffusion out of SF solvent (water) from emulsion droplets of SF solution to external continuous phase (ethyl acetate) of W/O emulsion before solidification. Influences of SF concentration and post methanol treatment on SF microparticle characteristics were investigated and discussed.

## MATERIALS AND METHODS

This research was conducted on January-July 2009 at Mahasarakham University, Mahasarakham, Thailand.

**Materials:** Aqueous Silk Fibroin (SF) solution of *B. mori* was prepared by a chemical de-gummed method before dissolution and dialysis, respectively. A de-gummd solution and a solvent were $Na_2CO_3$ solution and $CaCl_2$-ethanol-water (8-2-1 mole ratio) mixture, respectively (Srisuwan *et al*., 2008). The final SF concentration after dialysis was adjusted to 6 and 3% (w/v) with distilled water.

### Methods

**Preparation of SF microparticles:** Hollow SF microparticles were prepared in a one-step process by using a water-in-oil emulsion solvent diffusion technique. The typical procedure was explained as following. About 0.5 mL of SF solution was slowly added drop-wise into 100 mL of ethyl acetate with magnetic stirring at 900 rpm for 1 h. The beaker was covered with aluminium foil to

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prevent organic solvent evaporation during diffusion process. The SF microparticles were recovered by centrifugation before drying in a vacuum oven at room temperature for 48 h to remove residue organic solvent. The SF matrix can change from random coil to β-sheet form using alcohol treatment (Srisuwan *et al*., 2009). For methanol treatment, the SF microparticles were immersed into 90% (v/v) methanol solution for 1 h before centrifugation and drying in a vacuum oven at room temperature for 48 h.

**Characterization of SF microparticles:** Chemical structures of the SF particles were studied 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 using a KBr disk method (Srisuwan *et al*., 2009).

Morphology of SF microparticles was determined by Scanning Electron Microscopy (SEM) using a JEOL JSM-6460LV SEM. The microparticles were coated with gold for enhancing surface conductivity before scanning (Srisuwan *et al*., 2009).

Particle size distribution of SF microparticles was measured by sieving method. For this purpose, the microparticles were sieved into three particle size ranges of <80 μm, 80-150 μm and >150 μm before weighing (Srisuwan *et al*., 2009).

## RESULTS AND DISCUSSION

**FTIR analysis:** Usually, positions of amide bands from FTIR spectra were used to determine random coil and β-sheet conformations of SF matrices. Figure 1a and b show FTIR spectra of SF microparticles before and after methanol treatment, respectively prepared from 6% (w/v) SF concentration. The absorption bands of FTIR spectrum of non-treated SF microparticles in Fig. 1a at 1655 $cm^{-1}$ (amide I) and 1559 $cm^{-1}$ (amide II) were assigned to predominantly random coil conformation (Park *et al*., 1999; Srisuwan *et al*., 2009). The amide bands of FTIR spectrum of methanol-treated SF microparticles in Fig. 1b were shifted to lower wave number, 1623 and 1530 $cm^{-1}$ for amide I and II bands, respectively. This indicates that conformation of methanol-treated SF microparticles was predominantly β-sheet form (Kweon *et al*., 2001). In addition, amide III bands of the non-treated and methanol-treated SF microparticles was 1232 and 1235 $cm^{-1}$, respectively indicated their conformations were predominantly random coil to β-sheet forms, respectively (Srisuwan *et al*., 2009).

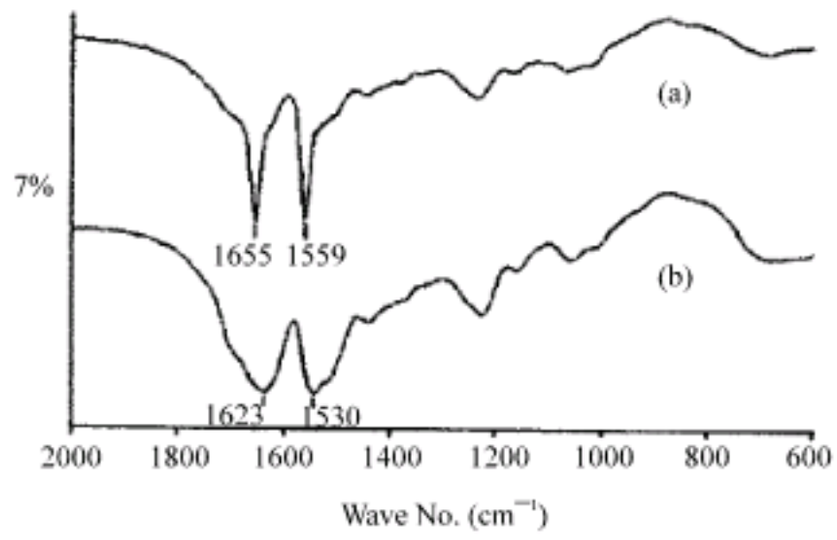


Fig. 1: FTIR spectra of hollow SF microparticles prepared from 6% (w/v) SF solution: (a) before and (b) after methanol treatment

The FTIR spectra of SF microparticles with and without methanol treatment prepared from 3% (w/v) showed with the same evidence.

**Morphology study:** Microparticle morphology was determined from SEM micrographs as shown in Fig. 2a and b. It is surprised that the almost non-treated SF microparticles prepared from the W/O emulsion solvent diffusion method with the both 6 and 3% w/v SF solutions showed open hollow structures. The SF microparticles consisted of deflated surfaces were found as a minor fraction. The open hollow microparticles contained also deflated surfaces, as shown in Fig. 3. Moreover, the both outer and inner hollow structures of SF microparticles were rough surfaces, as shown in Fig. 4.

Figure 5a and b show the SEM micrographs of the methanol-treated SF microparticles. It was found that the methanol-treated SF microparticles showed also open hollow structure. In addition, surfaces of the methanol-treated SF microparticles were clearly rougher than the non-treated, as shown in Fig. 6.

**Particle sizes:** Particle size distributions of methanol-treated SF microparticles were measured by the sieving method in three size ranges of < 80, 80-150 and > 150 μm. The results are presented in Fig. 7. It can be observed that the main size ranges were > 150 and 80-150 μm for the methanol-treated SF microparticles prepared using 6 and 3% w/v SF solutions, respectively.

In this study, we first disclosed the simple W/O emulsion solvent diffusion method for preparing surfactant-free SF microparticles as compare with previous works (Yeo *et al*., 2003; Srisuwan *et al*., 2009). The SF solution and ethyl acetate were used as water and oil phases, respectively. The water solubility in ethyl

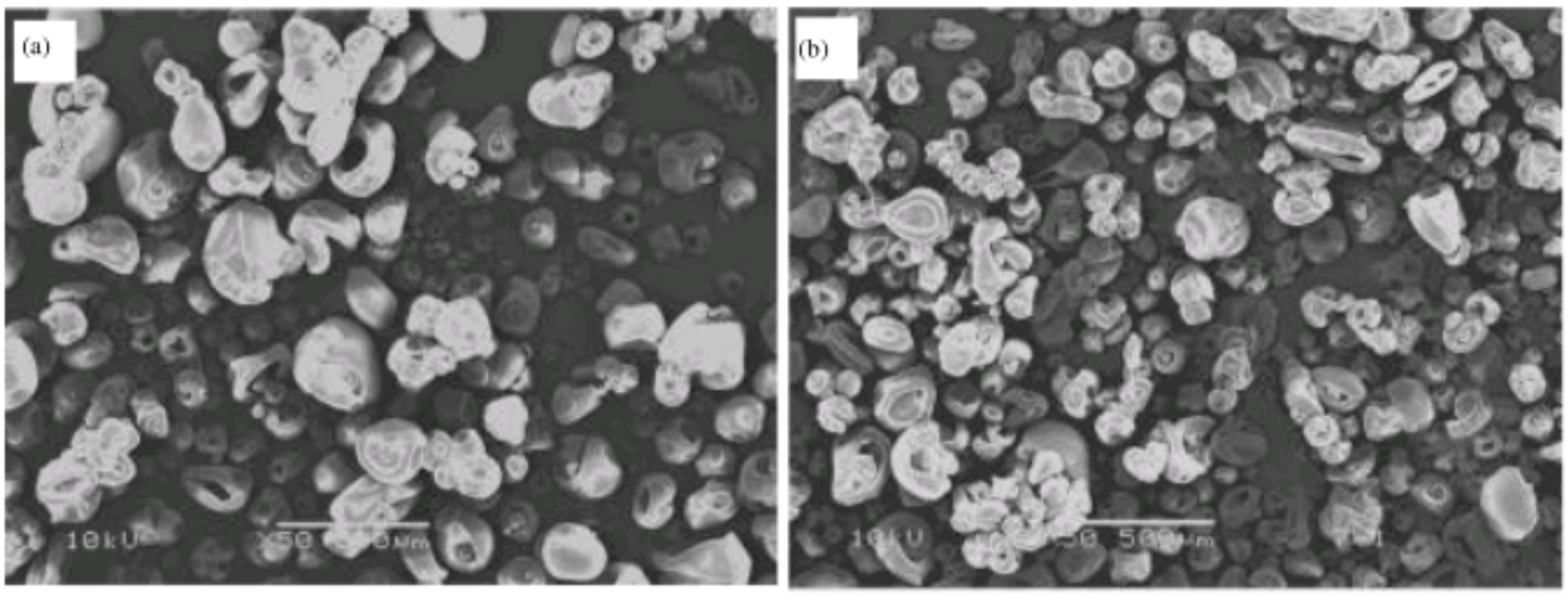


Fig. 2: SEM micrographs of non-treated hollow SF microparticles prepared from SF concentrations of (a) 6 and (b) 3% w/v, all bar scales = 500 μm

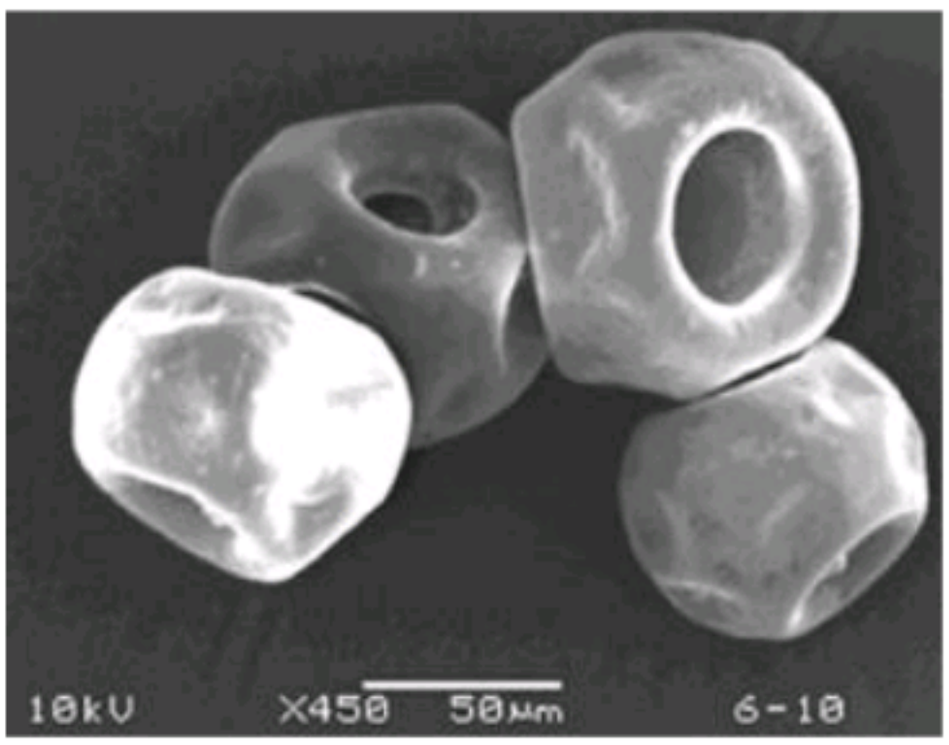


Fig. 3: Expanded SEM micrograph of methanol treated hollow SF microparticles prepared from 6% (w/v) SF solution, bar scale = 50 μm

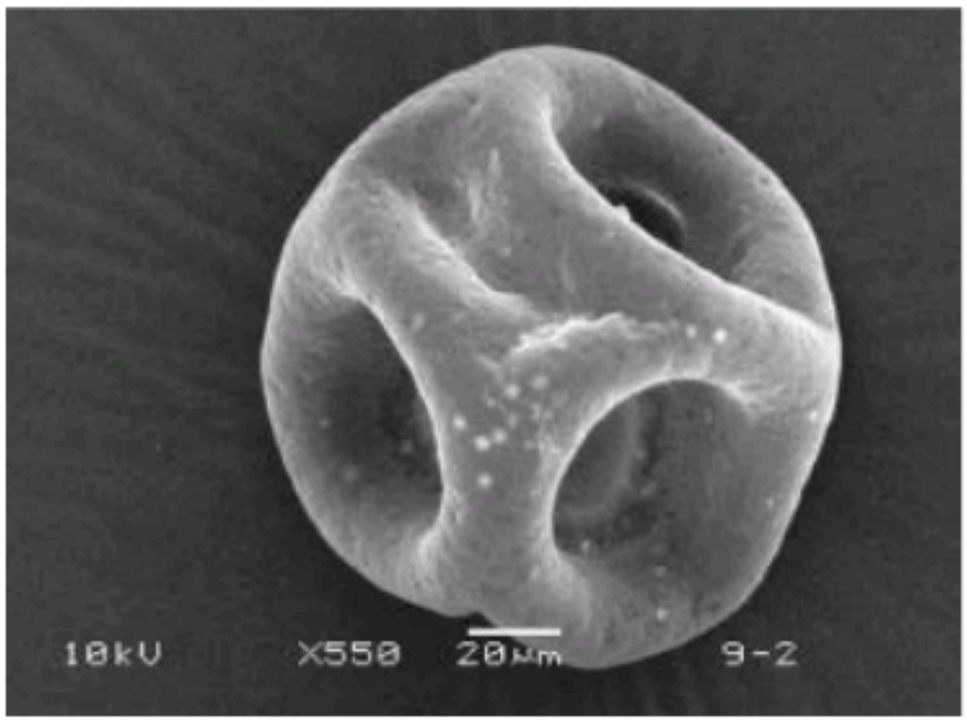


Fig. 4: Expanded SEM micrograph of non-methanol treated SF microparticles, bar scale = 20 μm

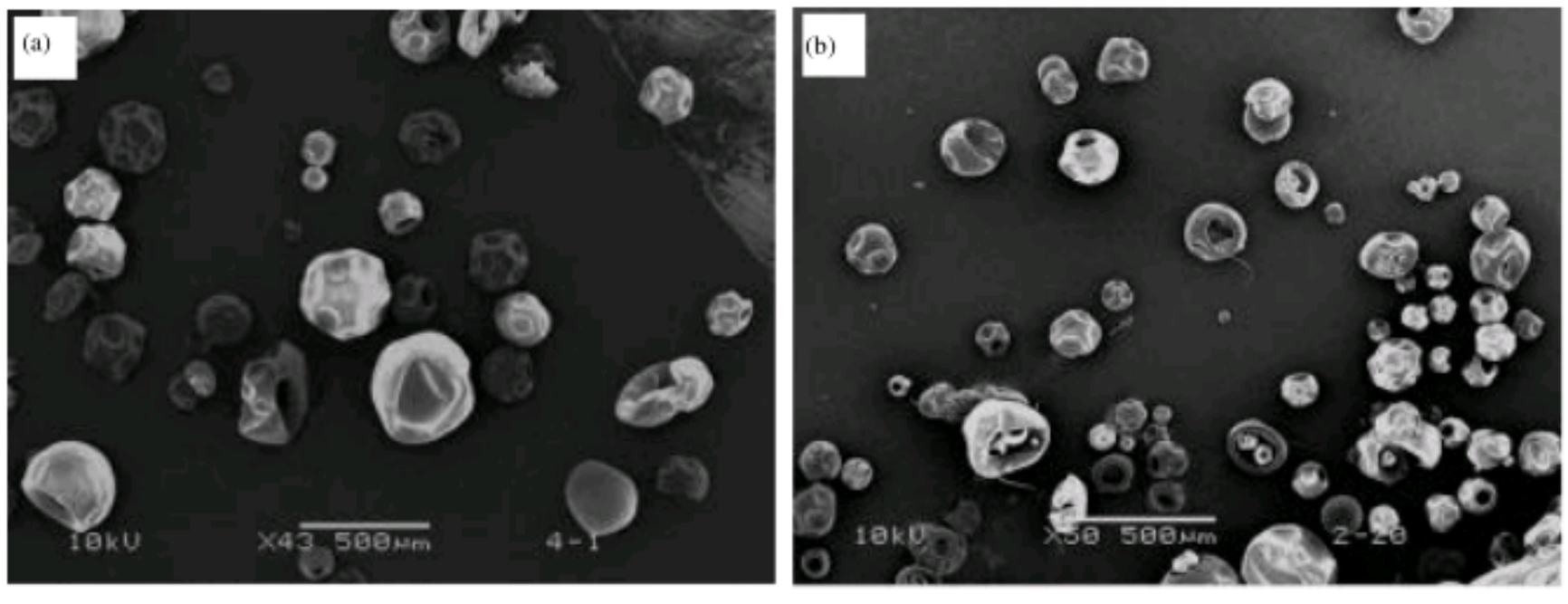


Fig. 5: SEM micrographs of methanol-treated hollow SF microparticles prepared from SF concentrations of (a) 6 and (b) 3% w/v, all bar scales = 500 µm


Fig. 6: Expanded SEM micrograph of methanol treated SF microparticles, bar scale = 20 µm

acetate is approximately 3.30% by volume. Therefore, it is postulated that if the SF solution/ethyl acetate volume ratio of W/O emulsion was less than the 3.30%, then SF particles would solidify after almost water molecules diffused out from emulsion droplets to the external ethyl acetate phase. However, SF microparticle yields decreased with increasing SF solution/ethyl acetate volume ratio because the particle aggregates stuck at the inner wall and bottom of beaker. The approximate 90% yield was obtained when the SF solution/ethyl acetate volume ratio of 0.5% was used.

**FTIR analysis:** From positions of amide I, II and III bands of FTIR spectrum in Fig. 1a of the non-treated SF microparticles prepared from the W/O emulsification-diffusion method showed predominantly random coil form suggested that ethyl acetate used as external continuous phase did not induce the transitional conformation of the SF microparticles from random coil to β-sheet forms.

Methanol treatment has performed in order to obtain the SF with β-sheet conformation or water-insoluble form. The transition of SF from random coil to β-sheet form can perform by various method including alcohol, acetone, heat and water vapor treatments. This β-sheet SF matrix form is more suitable for biomedical and pharmaceutical applications than the water soluble (random coil) form due to its contact with aqueous body fluids.

The β-sheet form of SF microparticles was clearly observed from shifting to lower wave number of the amide I and II bands and to higher wave number of the amide III band of its FTIR spectrum, as shown in Fig. 1b (Hino *et al*., 2003). The results suggested that the conformation of SF microparticles was changed from predominantly random coil to β-sheet form after methanol treatment.

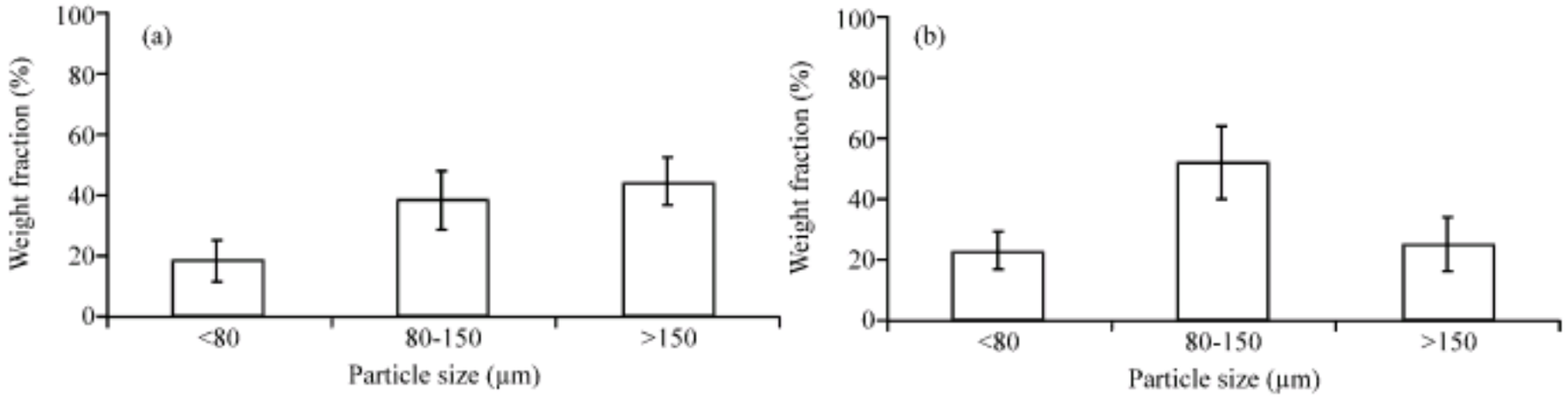


Fig. 7: Particle size distributions of methanol-treated SF microparticles prepared from SF concentrations of (a) 6 and (b) 3% w/v

**Morphology study:** Open hollow structure and deflated of surface of non-treated SF microparticles may be induced by particle shrinkage during solidification process because the SF matrix contained high inter- and intramolecular interactions, especially hydrogen bonding. However, the hollow structures of SF microparticles did not change for both microparticles prepared from 6 and 3% SF solutions after methanol treatment (Fig. 5). But surfaces of methanol-treated SF microparticles were clearly rougher than those non-treated (Fig. 6). This may be due to the surface shrinkage when the SF conformation changed from random coil to β-sheet form corresponding to FTIR results. These SF microparticles with open hollow structure and rougher surface are very suitable for applications in cell attachment and enzyme immobilization because of their high surface area.

**Particle sizes:** As would be expected that the SF microparticle sizes decreased as SF concentration decreased (Fig. 7). This can be explained that during the emulsification process, lower SF concentration produced smaller W/O emulsion droplets before solvent diffusion and solidification. The results showed that the SF microparticle sizes depended upon the SF concentration. In addition, the microparticle sizes fabricated in this paper (80-150 μm) were larger than the SF microparticles prepared by spray-drying method (2-10 μm) (Yeo *et al*., 2003). These larger microparticles are more suitable for cell microcarriers than those of smaller.

Finally, it should be noted that the W/O emulsion solvent diffusion method is a simple and fast method for preparing SF microparticles before methanol treatment. It is possible in large-scale production for use as novel biomaterial devices.

## CONCLUSION

The open hollow SF microparticles were successfully prepared by the W/O emulsion solvent diffusion technique. The surfactant and extractable porogen can be neglected for this technique. The conformational transition change of SF microparticles from random coil form to β-sheet conformation was induced by the methanol treatment. However, the SF concentrations and the methanol treatment did not influence on the open hollow structures of SF microparticles. It is believed that these open hollow SF microparticles with free from surfactant and porogen will find potential applications in microcapsules of artificial cell and immobilized enzyme.

## ACKNOWLEDGMENTS

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