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Silk Fibroin Spheres Crosslinked by Polyethylene Glycol Diglycidyl Ether for Drug Delivery

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Abstract

Silk fibroin (SF) has been widely investigated in biomedical applications due to its biodegradability and biocompatibility. The objective of this work was to prepare SF spheres for drug delivery application. Drug-loaded SF spheres with and without polyethylene glycol diglycidyl ether (PEGDE) crosslinking were prepared by a water-in-oil emulsion solvent diffusion method. Blue dextran was used as a water-soluble model drug. Effects of homogenizing speed and PEGDE ratio on characteristics and drug release behaviors of the SF spheres were determined. All of the SF spheres were nearly spherical in shape. The particle size of SF spheres decreased as the homogenizing speed increased. The PEGDE crosslinking can induce SF conformational transition from random coil to β-sheet form and increase drug loading efficiency. *In vitro* drug release content decreased as the particle size and PEGDE ratio increased. The results suggested that the desired drug release profiles of SF spheres can be tailored by adjusting the particle size and PEGDE ratio.

Keywords: Water-in-oil emulsification-diffusion; crosslinking; morphology; conformation; controlled release.

Introduction

Fibrous protein from silk cocoons, silk fibroin (SF) has been widely investigated for use in tissue engineering [1], enzyme immobilization [2, 3] and controlled-release drug delivery [4] due to its biocompatibility and biodegradability [5]. The SF from the domesticated silkworm, *Bombyx mori* is the most widely characterized. Regenerated

SF devices such as fibers [6, 7], films [8, 9] and particles [10, 11] have been prepared for these applications. Due to their well-defined model for degradation and drug release, biodegradable polymeric spheres are often proposed for use as controlled–release drug delivery systems. Spray drying, water-in-oil (W/O) emulsion solvent evaporation, lipid template and W/O emulsion solvent diffusion methods have been used to prepare the SF particles with drug entrapment [12-14].

Heat, alcohol and chemical crosslinked treatments have been used to control SF conformations, random coil (water soluble) and β-sheet (water insoluble) forms. For chemical crosslinking of SF, genipin [14] and polyethylene glycol diglycidyl ether (PEGDE) [15-17] have been used as crosslinkers. Water solubility of the SF matrix decreased significantly when the PEGDE ratio was increased. However, a suitable method for preparing the crosslinked SF spheres with controllable drug release profiles remains to be identified.

In this work, the W/O emulsion solvent diffusion method was developed for preparing SF spheres with and without PEGDE-crosslinking. SF aqueous solution and ethyl acetate were used as water and oil phases, respectively. The SF solution was crosslinked with PEGDE before SF sphere preparation. Influences of homogenizing speed and PEGDE-crosslinking on characteristics, drug loading efficiency and drug release behavior of SF spheres were determined.

Materials and Methods

Materials

Silk fibroin (SF) aqueous solution was prepared by chemical de-gumming to extract glue-like sericin proteins before dissolving. Briefly, silk cocoons of *B. mori* were de-gummed by boiling twice with 0.5% Na_2CO_3 solution at 90^{o}C for 60 min and then washed with distilled water before air drying at room temperature. De-gummed SF fibers were dissolved in a $CaCl_2$-ethanol-water (1/2/8 mole ratio) mixture at 85°C. This SF solution was dialyzed against distilled water for 3 days using a dialysis tube (molecular weight cut off = 7,000 Da). The distilled water was changed every day. The final SF concentration was adjusted to 1% (w/v) with distilled water. Ethyl acetate in analytical grade (Lab Scan) was used as a continuous oil phase. Tween80 (Sigma), blue dextran (BD, GE Healthcare) and polyethylene glycol diglycidyl ether (PEGDE, Aldrich) were used without further purification as an oil-soluble emulsifier, a water-soluble model drug and a crosslinker, respectively.

Preparation of drug-loaded SF spheres

SF spheres containing BD model drug were prepared by a water-in-oil (W/O) emulsion solvent diffusion method. Aqueous SF solution and 0.5% (w/v) Tween80 in ethyl acetate were used as water and oil phases, respectively. The SF/BD ratio was kept constant at 9/1 (w/w). The BD was directly dissolved in the SF solution before preparing SF spheres. One mL of SF and BD solution was added-drop wise to 200 mL of ethyl acetate under homogenizing for 2 min. before magnetic stirring at 900 rpm

for 2 h. After emulsification-diffusion process, the resultant drug-loaded SF spheres suspended in ethyl acetate were collected by centrifugation before rinsing with fresh ethyl acetate twice and drying in a vacuum oven at room temperature overnight. For SF crosslinking, the SF solution was crosslinked with PEGDE for 24 h before SF sphere preparation. Influences of homogenizing speed (5,000, 10,000, 15,000 and 20,000 rpm) and PEGDE-crosslinking (5, 10, and 20% w/w PEGDE) on characteristics and drug release behaviors of the SF spheres were determined.

Characterization of drug-loaded SF spheres

Morphology of the SF spheres was determined by scanning electron microscopy (SEM) using a JEOL JSM-6460LV SEM. The spheres were sputter coated with gold for enhanced conductivity before scan. Average particle sizes and standard deviation (SD) were measured from particle diameter and determined from several SEM images of each sample by manually counting a minimum of 150 particles using smile view software (version 1.02, JEOL Ltd.).

Chemical structure and SF conformation of the SF spheres were investigated 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 chosen. FTIR spectra were obtained from a KBr disk method.

The SF spheres could not be completely dissolved in phosphate buffer solution. Actual drug loading content (DLC_{actual}) of the SF spheres was determined by subtracting the amount of coated BD from the total loaded BD. For this purpose, the SF spheres were suspended in phosphate buffer solution at 37 °C with gentle shaking for 5 min to dissolve the coated BD from the sphere surfaces. The coated BD-free SF spheres were separated from the supernatant by centrifugation at 10,000 rpm for 10 min. The amount of BD in clear supernatant was measured by UV-Vis spectrophotometer at λ_{max} = 620 nm. %DLC was calculated form Equation (1). Weight of entrapped BD is weight of total loaded BD – weight of coated BD. The %DLC value is the average of three different determinations. Feed drug loading content (DLC_{feed}) was calculated from Equation (2). Drug loading efficiency, ratio of DLC_{actual} and DLC_{feed} was calculated from Equation (3).

$\%DLC_{actual}$ =

$$[\text{Weight of entrapped BD}/\text{Weight of drug-loaded SF spheres}] \times 100 \quad (1)$$

$$\%DLC_{feed} = [\text{Weight of feed BD}/\text{Weight of feed BD and SF}] \times 100 \quad (2)$$

$$\%DLE = [\%DLC_{actual}/\%DLC_{feed}] \times 100 \quad (3)$$

***In vitro* drug release test**

In vitro drug release test was performed in 1.5 mL of 0.1 M phosphate buffer solution pH 7.4 at 37 °C under shaking at 100 rpm. The 15 mg of each SF sphere sample was used for drug release testing. At each interval time, 1.0 mL of released drug solution was collected after centrifugation at 10,000 rpm for 5 min. One mL of fresh phosphate

buffer solution was added. BD concentration was measured by UV-vis spectrophotometry at λ_{max} = 620 nm. Each sample was run in triplicate. The %drug release was plotted with time.

Results

Morphology and Average Particle Sizes

Morphology of drug-loaded SF spheres was determined from SEM images as shown in Figures 1 and 2 for the SF spheres prepared using different homogenizing speeds and PEGDE ratios, respectively. It can be seen that they were nearly spherical in shape with smooth surface.

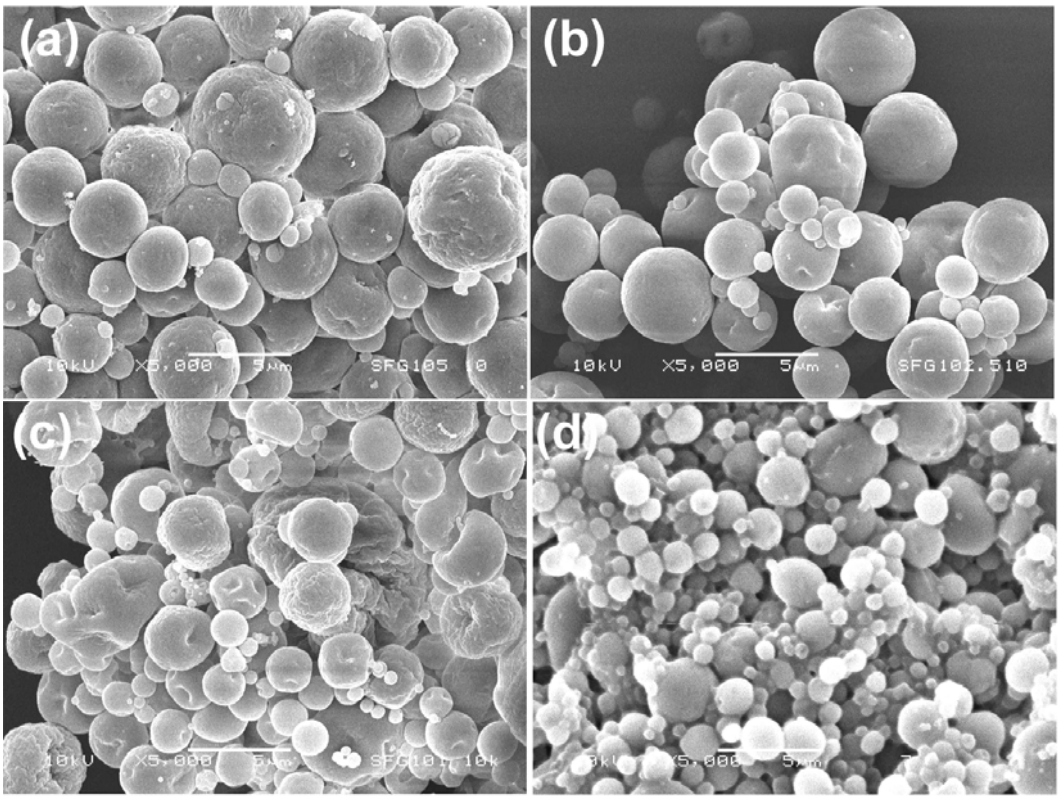

Figure 1: SEM images of 20% PEGDE-crosslinked drug-loaded SF spheres prepared with homogenizing speeds of (a) 5,000, (b) 10,000, (c) 15,000 and (d) 20,000 rpm for 24 h crosslinking time. All bars = 5 μm.

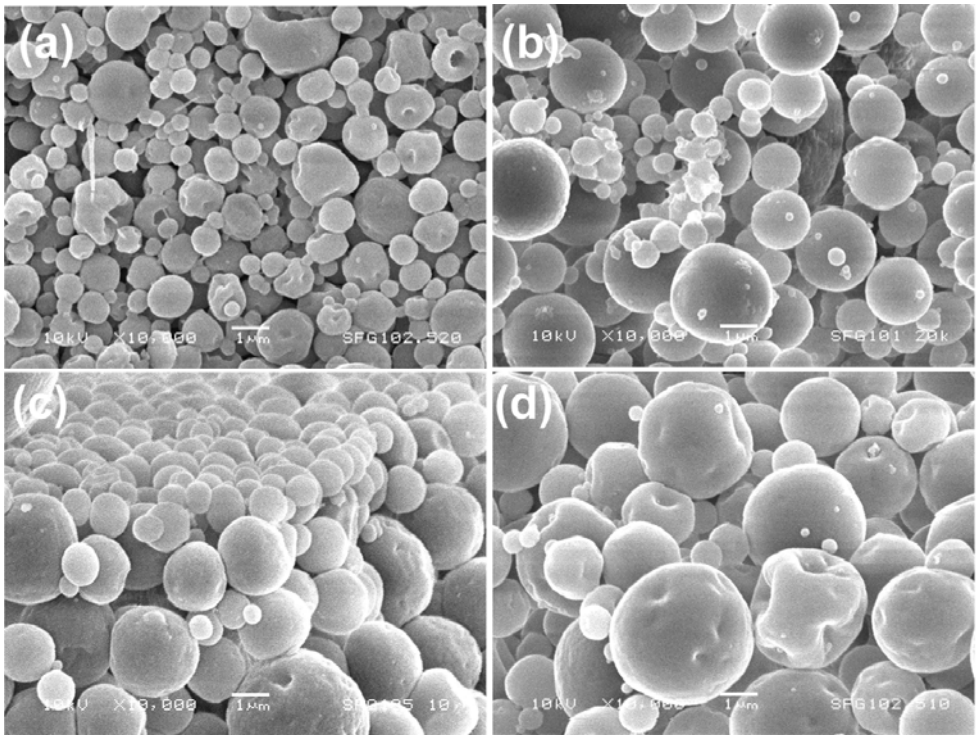

Figure 2: SEM images of drug-loaded SF spheres (a) without crosslinking and crosslinked with PEGDE ratios of (b) 5, (c) 10 and (d) 20%. All bars = 1 μm.

Average particle sizes calculated from at least 150 particle diameters of each sample are summarized in Table 1. The particles prepared with different PEGDE ratios were similar in size. The average particle sizes of the SF spheres prepared using homogenizer speeds in range 5,000 – 15,000 rpm are in range 2.5 – 3.2 μm. However, the average particle size significantly decreased as the homogenizing speed increased up to 20,000 rpm.

Table 1: Average particle sizes of drug-loaded SF spheres.

Homoginizing speed (rpm)	PEGDE ratio (%w/w)	Average particle size (μm)
5,000	20	3.2 ± 0.8
10,000	20	3.1 ± 0.9
15,000	20	2.5 ± 1.1
20,000	20	1.4 ± 0.7
20,000	0	1.6 ± 0.7
20,000	5	1.5 ± 0.9
20,000	10	1.7 ± 0.8

FTIR analysis

The absorption bands of the non-crosslinked SF spheres containing BD model drug in Figure 3(a) at 1685 cm^{-1} (amide I, C=O stretching), 1544 cm^{-1} (amide II, N-H bending) and 1237 cm^{-1} (amide III, C-N stretching) were assigned to random coil form [18, 19]. As shown in Figure 3, the amide I and II bands shifted to lower wave number, while the amide III band shifted to higher wave number when the SF was crosslinked with PEGDE and the PEGDE ratio was increased. This indicates the β-sheet content increased.

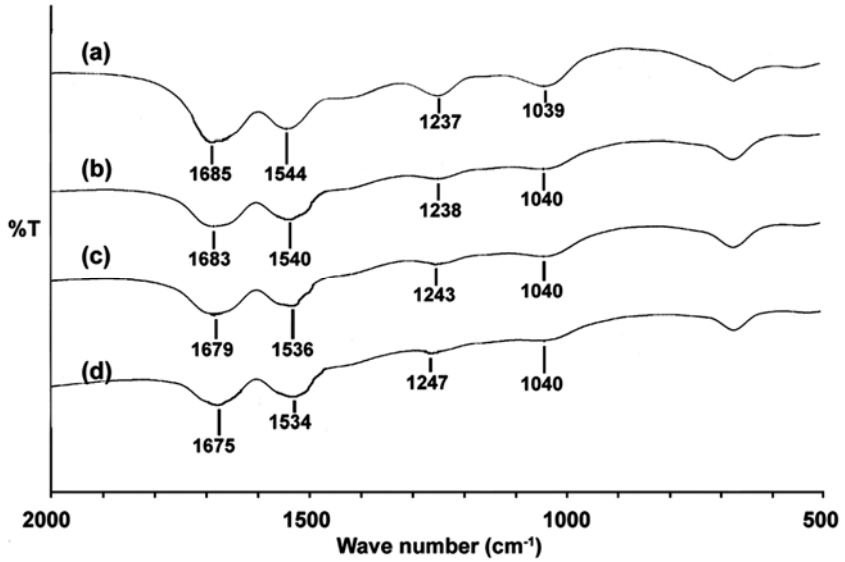

Figure 3: FTIR spectra of drug-loaded SF spheres (a) without crosslinking and crosslinked with PEGDE ratios of (b) 5, (c) 10 and (d) 20%.

All of the drug-loaded 20% PEGDE-crosslinked SF spheres prepared using homogenizing speeds in range of 5,000 – 20,000 rpm exhibited predominant β-sheet form. In addition, the BD model drug exhibits absorption band of saccharide groups at 1039 - 1040 cm^{-1}. The FTIR spectra of all of the drug-loaded SF spheres showed BD absorption band suggested the BD was entrapped in the SF spheres.

Drug Loading Content

The DLC_{feed} of all SF spheres is 9.09 %w/w. The DLC_{actual} and the DLE are summarized in Table 2. The DLC_{actual} values of the SF spheres prepared using different homogenizing speeds and PEGDE ratios are in the range of 6.5 – 6.7% and 5.2 – 6.6%, respectively. The DLC_{actual} slightly increased as the PEGDE ratio increased but did not homogenizing speed.

Table 2: Drug loading of drug-loaded SF spheres.

Homoginizing speed (rpm)	PEGDE ratio (%w/w)	DLC_{actual} (%w/w)	DLE (%w/w)
5,000	20	6.6	72.6
10,000	20	6.7	73.7
15,000	20	6.5	71.5
20,000	20	6.6	72.6
20,000	-	5.2	57.2
20,000	5	5.7	62.7
20,000	10	6.1	67.1

Drug Release

In vitro drug release profiles from the SF spheres are shown in Figures 4 and 5 for the SF spheres prepared with different homogenizing speeds and PEGDE ratios, respectively. The BD was selected as a model of water-soluble macromolecular drug to investigate the effects of homogenizing speed and PEGDE crosslinking on the drug release behavior from the SF spheres. All of the SF spheres clearly showed initial burst release effect within the first 12 h of releasing time followed by further slower release of BD. The SF spheres prepared using homogenizing speeds of 5,000, 10,000, 15,000 and 20,000 rpm exhibited 32%, 35%, 45% and 50% drug release at 72 h, respectively. The smaller SF spheres induced faster drug release. As shown in Figure 5, the non-crosslinked SF spheres had the highest drug release (66%) at 72 h. The drug release of the PEGDE-crosslinked SF spheres were slower than the non-crosslinked. The drug release contents decreased steadily as the PEGDE ratio increased.

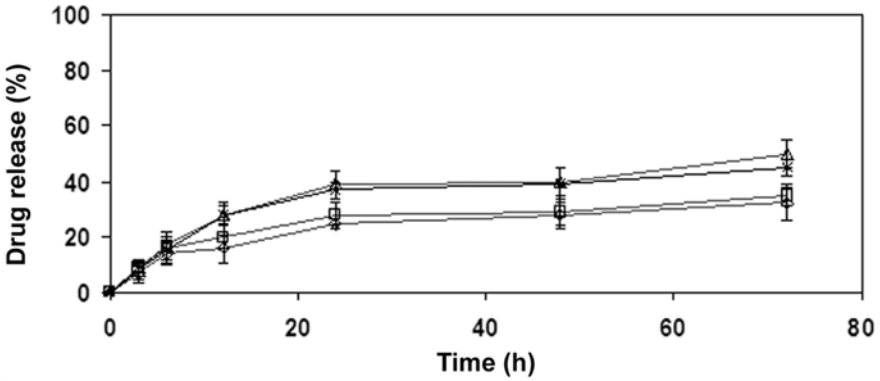

Figure 4: Drug release profiles of 20% PEGDE-crosslinked drug-loaded SF spheres prepared with homogenizing speeds of (◊) 5,000, (□) 10,000, (×) 15,000 and (Δ) 20,000 rpm for 24 h crosslinking time.

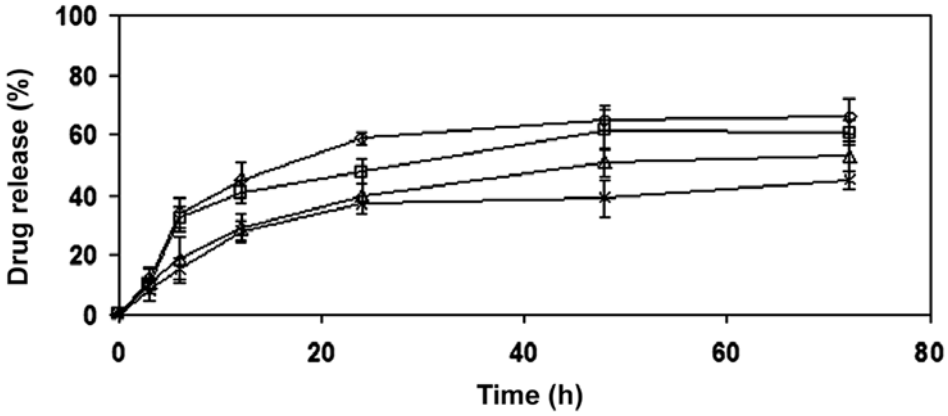

Figure 5. Drug release profiles of drug-loaded SF spheres (◊) without crosslinking and crosslinked with PEGDE ratios of (□) 5, (Δ) 10 and (×) 20% prepared using homogenizing speed of 20,000 rpm and 24 h crosslinking time.

Discussion

SF spheres were obtained and solidified after diffusion out of water from W/O emulsion droplets to external continuous oil phase, ethyl acetate [13, 14]. Oil soluble emulsifier dissolved in continuous oil phase can induce spherical in shape of SF particles [14]. The morphology results in Figures 1 and 2 suggested the homogenizing speed and PEGDE crosslinking did not affect the morphology of SF spheres. It was proposed that the drug particulate carriers with spherical shape enhanced more consistent drug release rate than the irregular shape. From Table 1, the PEGDE ratio did not affect particle size. However, the particle size decreased as the homogenizing speed increased. This is due to the faster speed induced smaller emulsion droplets before particle solidification.

The SF in aqueous solution is a random coil (water-soluble) form [12]. The SF conformation can be changed from predominant random coil to β-sheet (water-insoluble) form by chemical crosslinking [14, 15]. FTIR analysis is widely used to identify SF conformation [13-15]. The BD entrapment did not induce SF conformational changes from random coil to β-sheet form [Figure 3(a)]. The extent of β-sheet form of SF spheres increased as the PEGDE ratio increased [Figure 3(b) – 3(d)] according to the literature [15].

The DLE values in Table 2 indicated the efficiency of the technique to entrap the drug within the SF spheres were in range 57.2 – 73.7% corresponded to the DLC_{actual}. The results suggested that some BD can diffuse out from the emulsion droplets to the ethyl acetate continuous phase during the emulsification-diffusion process [20]. It was found that the DLC_{actual} did not depend upon the homogenizing speed. The increasing of DLC_{actual} with the PEGDE ratio indicated that the network structured SF obtained by PEGDE crosslinking was high effective for entrapment BD molecules.

The drug release content decreased when the PEGDE ratio was increased. This may be due to the swelling and surface erosion of polymer particle matrix can be

reduced by crosslinking. The network structure of crosslinked SF matrix decreased its swelling and surface erosion.

The mechanism of drug release from a biodegradable polymer matrix consisted of two main processes, matrix swelling-controlled (initial burst drug release) and matrix erosion-controlled (slow drug release) systems [21]. The drug release behavior from the SF spheres may include both drug diffusion and surface erosion mechanisms [20]. The drug release in the first 12 h releasing time is then due to the drug concentration gradient by swelling of SF spheres. The slow drug release may occur by surface erosion of the SF matrix. The drug release pattern of the SF spheres depended upon the particle size and chemical crosslinking. The drug release results suggested that the PEGDE-crosslinked SF spheres showed potential for use in controlled release drug delivery applications. The drug concentrations in blood could be maintained in range therapeutic levels for longer periods of time than the pure drug. So the drug administration frequency could be reduced.

Conclusions

The drug-loaded SF spheres with and without PEGDE-crosslinking can be prepared by the W/O emulsion solvent diffusion method. These spheres were nearly spherical in shape. The average particle size decreased as the homogenizing speed increased. The increasing β-sheet form and drug loading efficiency of SF sphere matrix was induced by PEGDE-crosslinking and increasing PEGDE ratio. The drug release from these SF spheres may be tailored by adjusting the homogenizing speed and PEGDE ratio.

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