Effect of Ca^{2+} ions on Swelling Behavior of Silk Fibroin Hydrogel

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Abstract
Assembly of silk fibroin protein as a 3-dimentional hydrogel was demonstrated by taking advantage of the difference in phase transition behavior of the protein and water. Effect of Ca^{2+} ions remained in the fibroin protein solution after the preparation of protein solution on the formation of hydrogel was investigated as a function of dialysis time, including 1, 2 and 3 days. The swelling behavior of the hydrogel was reported. The information from this study may be useful for medical applications of silk fibroin hydrogel.

Keyword: Silk fibroin protein; Hydrogel; Swelling behavior

1. Introduction

Silk is a protein polymer that is spun into a fiber by silkworm and spider. Silk from silkworm has been used in textile industry for thousands of years and as biomedical suture for centuries, but only in recent decades its potential as biomaterial has been studied extensively [1-2]. Silk from *B. mori* consists primarily of two protein components, fibroin and sericin [1-3]. Silk fibroin (SF) is the structural protein of silk fibers and sericin is glue that binds and covers the fibroin fibers. SF molecule consists of heavy and light chain of polypeptides with molecular weights of ~350 kDa and ~25 kDa, respectively. SF is the protein with the dominated amino acids of glycine, alanine, and serine, which form antiparallel β-sheets [4-6].

It is well known that SF has excellent mechanical properties, biocompatibility with a variety of cells, susceptibility to proteolytic degradation and low inflammatory response. Thus, this protein has been extensively studied for biomaterials and scaffolds for tissue engineering. Another important application of SF in biomaterial applications is to form hydrogels.

Hydrogel is a 3-dimensional polymeric network fabricated from polymers such as alginates, chitosan and collagen [6]. It can stabilize through physical or chemical crosslinking, passing from an amorphous conformation (random coil) to a more stable structure (β-sheet) [7, 8], and is irreversible under normal physiological conditions.

Hydrogel can be prepared by many difference methods such as freeze-thawing with water miscible organic solvent, salt leaching, gas forming, and freeze-drying. This hydrogel can normally be degraded by enzymatic or oxidative processes. The sol-gel transition of hydrogel depends on the concentration of the protein, temperature, ionic strength and pH [6]. They are capable of absorbing large quantities of water without losing their structural integrity.

The ability of hydrogel to mimic body tissues and respond to external stimuli has made them important and promising forms of biomaterials for various applications including tissue engineering, controlled drug release devices, biosensors, mechanical actuators, biomedical, pharmaceutical etc [9-13].

In this paper, we demonstrated an easy process to fabricate silk fibroin hydrogel by using freezer at -18°C. Effect of Ca^{2+} ions remaining in the silk fibroin solution on formation of hydrogel, morphology, pore sizes, porosity, and swelling behavior of hydrogel was reported.

2. Materials and Methods

2.1. Preparation of silk fibroin solution

Silk cocoons of *B. mori* silkworm were degummed twice by soaking in 0.02 M of aqueous Na_2CO_3 (AR grade, Ajax Finechem Pty Ltd) solution at 85°C for 30 min to remove the sericin protein of the cocoons, and then rinsing in distilled water until pH of waste water was 7 which monitored by pH-indicator strip (Merck KGaA). The fibroin fibers were dried at 37°C for 24 hr. and then dissolved in aqueous $CaCl_2$ (AR grade, Ajax Finechem Pty Ltd) solution at 85°C for 3 hr. The aqueous $CaCl_2$ solution was prepared from dissolved $CaCl_2$ powder in ethanol and distilled water with $CaCl_2$: Ethanol: H_2O ratio of 1:2:8 by molar. The fibroin solution was dialyzed in distilled water using cellulose tubular membrane (Membrane Filtration Productions, Inc., MWCO

3500) at room temperature for different dialysis periods, including 1, 2 and 3 days (denoted as SF1, SF2 and SF3, respectively) to remove Ca^{2+} ion from the solution.

After each dialysis period, the solution was then filtered through the filter paper no.1 to remove any impurity. The final concentration of silk solutions was determined by weight changing measurement after the solution was dried and it was approximately 2.0 wt%.

2.2. Hydrogel formation

SF hydrogel forming was prepared by molding method. The dimension of the plastic tubular mold was 1.2 cm diameter and 2.5 cm long. About 2 ml of SF solution was filled in the mold and it was freezed in a freezer at -18°C for 5 days for the forming of hydrogel.

2.3 Hydrogel characterization

All hydrogel samples were dried at 37°C and then characterized by Field Emission Scanning Electron Microscope (FE-SEM, HITACHI S4700) at 10 kV and 10 nm gold coating and Fourier Transform Infrared Spectrometer (FTIR, Perkin Elmer 2000). Aspect ratio (width/length) of pore size was obtained by using ImageJ software.

2.4 Porosity measurements

The porosity values of the hydrogels were measured by liquid displacement. Briefly, hydrogels were cut into a cylinder shape (diameter 12 mm, thickness 3 mm) pieces, placed in a 10 ml cylinder containing a defined volume of ethanol (V1) and then pressed to force all trapped air out of the hydrogel it had been evacuated. The total volume of ethanol and ethanol-impregnated hydrogel was recorded as V2. The ethanol-impregnated hydrogel was removed from the cylinder and the residual ethanol volume recorded as V3. The porosity of the hydrogel was expressed as:

$$\text{Porosity} = \frac{\text{V1-V3}}{\text{V2-V3}} \times 100$$

2.5 Swelling behavior

2.5.1 Determination of equilibrium swelling in water (ESW)

The swelling studies of the samples were carried out according to the method described by (Yang et al., 2010; Wang et al., 2012) with some modifications. The samples of a cylinder shape (diameter 12 mm, thickness 3 mm) were dried in the oven at 37°C for 24 hr. and weighted (W_0). Then the samples were immersed in distilled water at 37°C for 24 hr. and weighted (W_{24}). The equilibrium swelling in water (ESW) was calculated using the following equation:

$$\%\text{ESW} = \frac{W_{24}-W_0}{W_0} \times 100$$

2.5.2 Sol fraction

The sol fraction remaining after gelation was determined for all hydrogel by modifying the method as described by Behravesh et al. [16]. The samples were prepared and dried completely at 50°C for 3 hr. The total dry weight W_d (sol + gel) was noted down. Next, the dry samples were swollen in distilled water 37°C for 48 hr. After swelling, samples were removed from swelling medium, excess surface water was wiped, and dried as described above. The dry hydrogel weight after redrying (W_{rd}) was recorded. %sol fraction was determined using following equation:

$$\%\text{sol fraction} = \frac{W_d-W_{rd}}{W_d} \times 100$$

3. Results and Discussion

3.1 Hydrogel morphology

Morphology of hydrogels was observed by FE-SEM after dried at 37°C. The FE-SEM cross section images of SF hydrogels are presented in Fig. 1. We observed that the structure of SF hydrogels can be affected by the dialysis time which is directly proportional to concentration of Ca^{2+} ions. At short time of dialysis or high concentration of Ca^{2+} ions, sheet and fibrous structures can be clearly observed in SF hydrogel; for example, dialysis time for 1 day (SF1, Fig. 1A) and 2 days (SF2, Fig. 1B). For longer dialysis time (for 3 days (SF3, Fig. 1C)), the fibrous structure was disappeared. In Fig. 1(A) - 1(C), FESEM images show that fiber structure was decreased when increasing dialysis time of SF solution. Pore size and pore density were also changed as well.

Table 1: The pore size and porosity of SF1, SF2, and SF3

Dialysis time (day)	Width* (μm)	Length* (μm)	Porosity* (%)
1	34.86 ± 20.32	69.33 ± 32.86	69.02 ± 3.47
2	25.68 ± 10.63	55.42 ± 21.23	61.14 ± 4.96
3	17.51 ± 5.47	35.39 ± 11.57	56.76 ± 4.61

* Average value ± standard deviation.

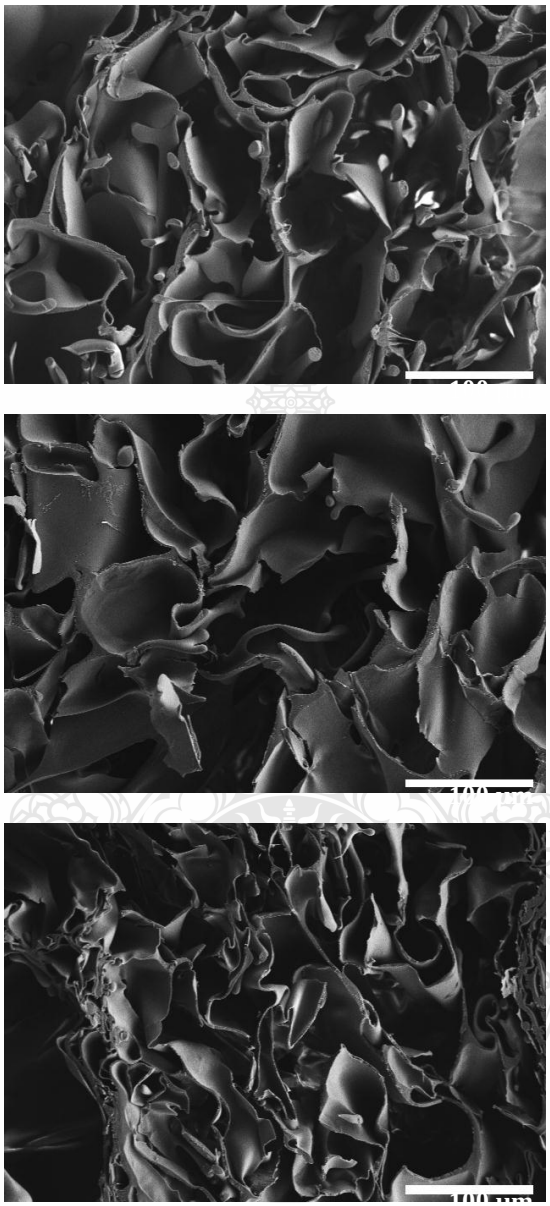

Fig. 1 FE-SEM images for SF hydrogels were varying dialysis time, (A) 1 day, (B) 2 days, and (C) 3 days.

Pore sizes and porosity of SF1, SF2, and SF3 are illustrated by table 1. Its mean pore sizes and porosity decrease with increase dialysis time because the effect of Ca^{2+} ions remaining in the SF solution on pore sizes of hydrogel. Thus, pore size and porosity can be controlled by the dialysis time.

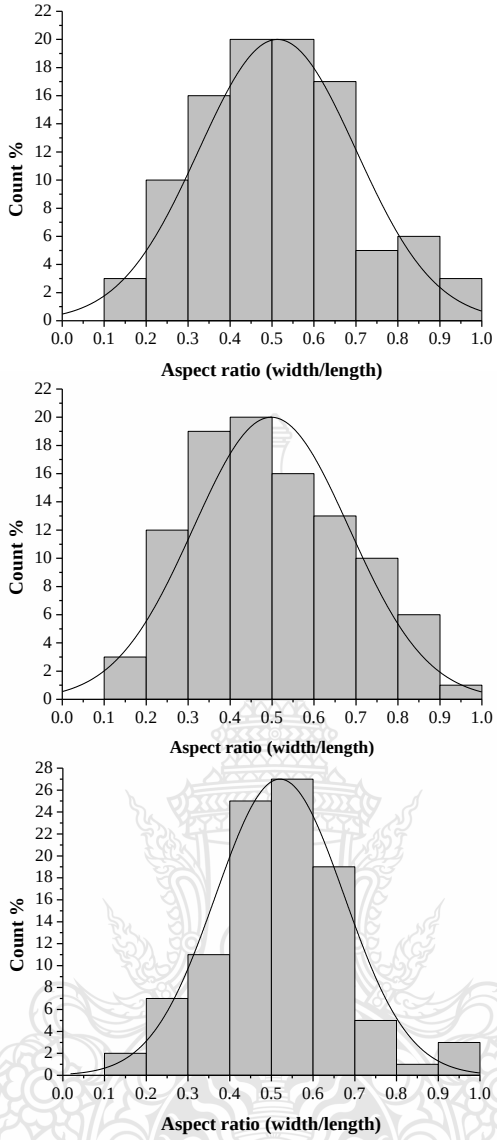

Fig. 2 Aspect ratio of pore size of (A) SF1, (B) SF2, and (C) SF3.

Fig. 2 shows the distribution in aspect ratio of pore sizes of SF1, SF2, and SF3. Aspect ratio indicates the shape of pore size. Obviously, most of the pores (85%) found in SF1, SF2, and SF3 hydrogels are ellipse shape with the aspect ratio of 0.3-0.7.

3.2 Hydrogel formation

Fig. 3 shows the FTIR spectra of natural silk (red) threads and the fibroin hydrogel after 3 days of dialysis (black). FTIR analysis is generally used for determining the conformation and crystallization behavior of silk protein. The peak positions of amide I, II, and III are referred to C=O, N-H, and C-N stretching modes, respectively [1, 15-16]. From the data, both natural silk and the fibroin hydrogel (SF3) show the similar pattern of FTIR spectra. FTIR spectra of natural silk refer to the random coil structure [17, 19], but FTIR spectra of SP3 hydrogel in amide I, II, and III positions are at 1639, 1524, and 1235 cm^{-1}, respectively, which is referred to the β-sheet structures [17-20]. FTIR spectra of SF1 and SF2 are the same pattern, but not show in Fig. 3, which mean SF1, SF2 and SF3 are same structure, β-sheet structure. Thus, it can be concluded that the protein structures of natural silk and fibroin hydrogel are very similar.

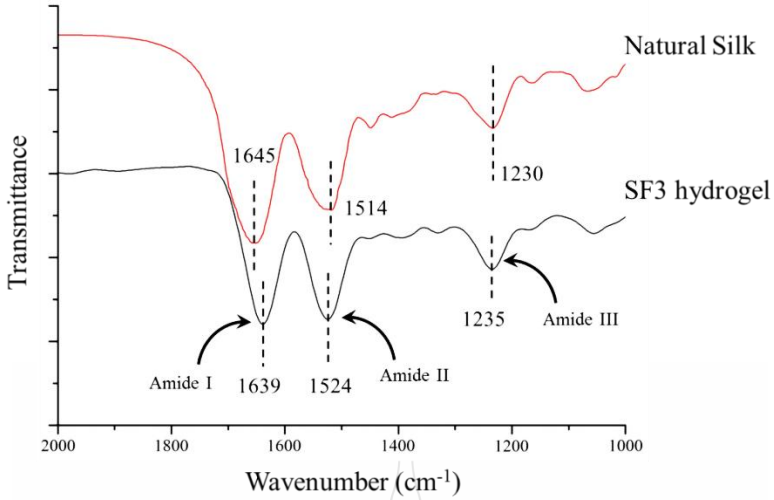

Fig. 3 FTIR spectra of natural silk (red) threads and the fibroin hydrogel (SF3) (black).

3.3 Swelling behavior

The swelling behaviors of the samples were investigated. Table 2 shows the equilibrium swelling in water (ESW) of the samples, which indicated the water retention ability of the samples. With the increase of dialysis time, ESW of the hydrogels decreased significantly and the maximum value was reached by SF1 hydrogel. Table 2 also shows the %sol fraction for different dialysis time. The results indicated that the sol fraction decreased with an increase dialysis time with maximum sol fraction (6.65%) remaining in SF1 and minimum (0.54%) in SF3 hydrogels. These results support the fact that with more tightly crosslinked matrix, expansion in water is less compared to loosely crossliked matrix. At lower dialysis time (1 day) the network is loose and has a high hydrodynamic free volume to accommodate more of the solvent molecules, thereby increasing matrix swelling. Intense crosslinking hinders mobility and relaxation of the polymer chains, which in turn impedes the mobility of water, hence lowering the equilibrium swelling in water and porosity.

Table 2: The equilibrium swelling in water and sol fraction (%) of SF1, SF2, and SF3

Dialysis time (day)	Equilibrium swelling in water* (%)	Sol fraction* (%)
1	929.09 ± 194.77	6.65 ± 0.36
2	780.09 ± 78.56	3.43 ± 0.41
3	626.67 ± 76.20	0.54 ± 0.22

* Average value ± standard deviation.

4. Conclusion

In this work, we demonstrated the easily process to fabricate hydrogel from silk fibroin protein. The process is much easier than conventional methods. Pore sizes of hydrogel can be controlled easily by dialysis time. FTIR spectra show that fibroin hydrogel has similar protein structure to that of natural silk, including random coil and *β*-sheet structures. The controllability of pore size and sol fraction of the hydrogel may broaden its application in bioengineering, especially cell scaffold.

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