Novel silk fibroin films prepared by formic acid/hydroxyapatite dissolution method

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ABSTRACT

Bombyx mori silk fibroin from the silkworm was firstly found to be soluble in formic acid/hydroxyapatite system. The rheological behavior of silk fibroin solution was significantly influenced by HAp contents in dissolved solution. At the same time, silk fibroin nanofibers were observed in dissolved solution with 103.6 ± 20.4 nm in diameter. Moreover, the structure behavior of SF films prepared by formic acid/hydroxyapatite dissolution method was examined. The secondary structure of silk fibroin films was attributed to silk II structure ([β-sheet]), indicating that the hydroxyapatite contents in dissolved solution were not significantly affected by the structure of silk fibroin. The X-ray diffraction results exhibited obviously hydroxyapatite crystalline nature existing in silk fibroin films; however, when the hydroxyapatite content was 5.0 wt.% in dissolved solution, some hydroxyapatite crystals were converted to calcium hydrogen phosphate dehydrate in silk fibroin dissolution process. This result was also confirmed by Fourier transform infrared analysis and DSC measurement. In addition, silk fibroin films prepared by this dissolution method had higher breaking strength and extension at break. Based on these analyses, an understanding of novel SF dissolution method may provide an additional tool for designing and synthesizing advanced materials with more complex structures, which should be helpful in different fields, including biomaterial applications.

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1. Introduction

Silk fibroin (SF), a mass-producible natural polymer produced by silkworms, has been used as textile fiber and suture [1]. Recently, many researchers have investigated SF as one of the promising resources of biotechnology and biomedical materials due to its unique properties including outstanding mechanical properties combined with flexibility [2,3], tissue compatibility, biodegradability, and minimal inflammatory reaction [4–6]. The silk fibroin molecules consist of a heavy (H) chain of 390 kDa and a light (L) chain of 25 kDa connected by a disulfide linkage [2,7,8], and their primary sequences of amino acid have been identified recently, consisting primarily of highly repetitive Gly-Ala repeats [8,9]. The crystalline regions of the heavy chain occur primarily in the repeated GAGAG amino acid motifs [10]. These regions assume hydrogen bonded, antiparallel, β-sheet structure in the natural fiber. The hydrogen bonding and the hydrophobic nature of these crystalline regions make dissolution of SF a formidable task [2].

Dissolution of SF is often required when non-textile applications are demanded in the forms of films, porous scaffolds, nanofibers, and hydrogel [11]. In general, SF aqueous solution is obtained by dissolved SF in the concentrated neutral salts, such as lithium bromide and calcium chloride [12]. However, SF aqueous solution obtained by this general dissolved method becomes unstable when stored for a long time. At the same time, regenerated SF materials from aqueous solution show good mechanical properties after post-treated by ethanol [13,14]. These post-treated materials are extremely brittle and unsuitable for practical uses [6,15]. These problems may be solved by improving fiber dissolution methods.

While SF protein can’t directly dissolve in formic acid (FA), the regenerated SF materials are easily soluble in FA. Many research groups have investigated the structural characteristics of protein-based materials prepared from FA [11]. Um et al. reported that the SF/FA solution was remarkably stable and transparent compared to aqueous SF solution system [11,16]. In the acidic solvent system, however, the fibroin chains are gradually degraded with time, possibly due to acid hydrolysis [17]. Despite this disadvantage, the regenerated SF materials dissolved in acidic solvents is much easier to control than concentrated aqueous SF solutions and, therefore, has much better processibility. This may be due to the difference in the size of molecular and its conformations in these solvent systems.

The relationships between structure and properties are among the most important goals in polymer science. This paper reports a novel dissolution method of Bombyx mori silk fibroin using the formic acid/hydroxyapatite (FA/HAp) system. In this dissolution method, we
test the suitability of FA/HAp solution for dissolving and regenerating SF and examine the structural properties of SF films cast from FA/HAp solutions. Structural analysis of the regenerated SF films may give useful information for preparing SF biomaterials. This study was focused on the structural aspects of the SF both in solution and solid states during the processing for regenerated films. Several analytical techniques, such as rheological studies, Fourier transform infrared analysis (FTIR), X-ray diffraction (XRD), differential scanning calorimetric analysis (DSC), tensile testing, and scanning electron microscopy (SEM) were used to characterize the structure and properties of SF films.

2. Materials & methods

2.1. Materials

*B. mori* silk fiber was purchased from Zhejiang Province, China. All chemical regents (calcium chloride, ammonium dihydrogen phosphate, ammonia solution, formic acid, etc.) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and used without any further purification. HAp nanoparticles were prepared by solution-precipitation method according to our previous published procedures [18].

2.2. Preparation of silk fibroin films

*B. mori* silk fibroins were prepared according to our previous published procedures [18]. Raw *B. mori* silk fibers were boiled in 0.05 wt.% Na₂CO₃ solution for 30 min and then rinsed thoroughly with deionized water to extract the glue-like sericin proteins. Each step was repeated twice, and finally, the degummed SF fiber was dried at room temperature. After drying, the degummed SF was dissolved in FA/HAp solutions at room temperature. In these solutions, HAp contents were 5.0 wt.%, 2.5 wt.%, 2.0 wt.%, 1.5 wt.%, and 1.0 wt.%, respectively. At the same time, the concentration of SF was constant at 5.0 wt.%. After stirring for 3 h, the SF solutions were cast on polystyrene dishes (diameter 90 mm). The solution was slowly dried to obtain the regenerated SF films at room temperature.

2.3. Characterization

The surface morphologies and cross-sections of SF films were platinum-coated and examined by a scanning electron microscope (SEM, Hitachi S4800). Cross-section samples were prepared by freezing fracturing the dried SF films in liquid nitrogen.

The morphology of SF in solution was also observed by SEM. To clearly characterize the dissolved process of silk fiber, silk fiber was dissolved in FA/HAp solution at concentration of 5.0 wt.% at room temperature. In this solution, the concentration of HAp was 2.5 wt.%. After stirring for 3 h, the SF solution was diluted to below 0.001 wt.% by FA solution and dropped on a silicon pellet surface with 1 μl. The SF solution was quickly dried by liquid nitrogen at room temperature.

The secondary structure of SF films prepared by FA/HAp dissolution method was analyzed by FTIR on Nicolet5700 (Thermal Nicolet Company, USA) in absorbance mode. For each measurement, each spectrum was obtained by the performance of 32 scans with the wave number ranging from 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹. At the same time, X-ray diffraction experiments were also measured on X Pert-Pro MPD (PANalytical, Netherlands) in transmittance mode to investigate the crystalline structure of samples. The incident beam wavelength was 0.154 nm. The intensity was finally corrected for changes in the incident beam intensity, sample absorption, and background. The thermal stability of SF films samples was characterized using SDT Q600 (TA Company, USA) under a dry nitrogen gas flow of 100 mL min⁻¹. The samples were heated from 50 to 800 °C, and the heating rate was 10 °C min⁻¹.

Rheological studies were run on a rheometer (AR2000, TA Instruments, and America) with a 40 mm cone plate (Ti, 40/2°). The normal force applied on the sample during lowering of the top plate was limited to 0.1 N. The shear rate was linearly increased from 0.01 to 5000 1/s at 25 °C [19,20].

SF films samples, prepared similar as above, were cut into 50 mm × 5 mm rectangles with thickness of 100–150 μm. The thickness of these samples was measured using a micrometer. Before using an automatic tensile tester (model 3365 electronic strength tester, Instron, Boston, USA) to characterize the mechanical properties of these films, these films were kept for 24 h at standard atmospheric conditions (20 °C and 65% RH). During test process, distance between grips and test speeds were set to 20 mm and 10 mm min⁻¹, respectively. At the same time, the pre-tension was 0.2 N. An average of twenty measurements was reported as the mean ± standard deviation for each sample. The formulas for breaking strength and extension at break were as follows:

\[
\text{Breaking strength (MPa)} = \frac{\text{breaking force(N)}}{\text{(specimen thickness(mm) × specimen width(mm))}};
\]

\[
\text{Extension at break (％)} = \frac{\text{specimen elongation(mm)}}{\text{original length(mm) × 100％}}.
\]

2.4. Statistical analysis

All values were expressed as mean ± standard deviation. Statistical differences were determined by a Mann–Whitney U test (independent t test, SPSS).

3. Results and discussion

3.1. Dissolution behavior of SF in FA/HAp solution

In the previous study of SF dissolution behavior, silk protein can't directly dissolve in FA; however, the regenerated silk fibroin (RSF) materials are easily soluble in FA. Um et al. investigated that the RSF/FA acid solution had low viscosity less than 1 Pa·s [11]. The results showed that RSF/FA solution was relatively stable and transparent, not occurring molecular aggregation or entanglement. Zhu et al. reported that the rheological behavior of RSF/FA solution showed Newtonian fluid and its viscosities were almost independent of shear rate under 20.0 wt.% concentration [21,22]. In this present study, SF fibers were successfully dissolved in FA/HAp solution. To study the SF inter-nanostructure associations present in FA/HAp solutions, viscometry was employed. Fig. 1 showed the rheological behavior of SF in FA/HAp solution.
solutions at HAp content 1.0 wt.% 1.5 wt.% 2.0 wt.% 2.5 wt.% and 5.0 wt.% respectively. Viscosity measurements over a range of shear rates further emphasized the great differences between these solutions (Fig. 1). Over the range of HAp contents tested, the SF dissolved in FA/HAp solution at HAp content 5.0 wt.% exhibited a zero shear viscosity. This solution behaved like Newtonian fluids with significantly lower viscosity [19]. Moreover, the rheological behavior of SF solution dissolved in FA/HAp solution at HAp content 5.0 wt.% was similar to the rheological behavior of SF/FA solution, showing a zero shear viscosity. This result was due to the charge repulsion effect between fibroin molecular chains because most amino acids of SF show positive charges in acid condition [11]. At the same time, SF molecules in FA solution had lower opportunity for the intermolecular association, which was related to the molecular entanglement. Therefore, SF solution exhibited less entangled molecular state [16]. As HAp content is decreasing, the solution exhibited shear-thickening behavior followed by shear-thinning behavior with significantly higher viscosity, which is similar to natural silk dope [23]. At low shear rates, the shear-thickening behavior followed by shear-thinning (<2.0 s⁻¹) might indicate that shear caused a slight extension and interaction of SF chains in solution. At the same time, this instability of the rheology at low shear rates could be followed by a more steady extension leading to the slight shear thickening. At higher shear rates (>10 s⁻¹) the macromolecular chains reached a new equilibrium resulting in an almost constant viscosity [24]. In addition, the viscosity of SF solution was obviously decreased with increasing the content of HAp. This phenomenon was attributed to the size and entangled state of SF nanofibers in the solution.

At the same time, the dissolution process of SF fibers and the nanostructures of SF in FA/HAp solution were assessed by SEM (Fig. 2). Fig. 2 (a–c) showed the dissolution process from SF fibers to SF nanofibers in FA/HAp solution. These nanofibers were observed in FA/HAp solution with 103.6 ± 20.4 nm in diameter (Fig. 2d). The repulsive forces between the negative charges in these nanofibers were suppressed due to the low pH of FA, so that the nanofibers organized in parallel or less entangled, resulting in the increased viscosity and shear-thickening behavior at low shear rates. Moreover, shear-thinning behavior was also observed at high shear rates (≥10 s⁻¹) due to entangled chain networks in solution [11]. At the same time, the viscosity of SF in FA/HAp solution exhibited Newtonian behavior at high shear rates, which is not affected by different HAp contents. This Newtonian behavior might be explained by the strong interaction and aggregation of SF nanofibers in these solutions to form compactly coiled structures [8,24,25].

3.2. Structure characteristic

The crystal structure of SF films prepared by FA/HAp dissolution method containing different HAp contents was analyzed by FTIR and XRD. FTIR spectrum was a good tool for structural investigations because of the knowledge of the vibration origins of the amide bonds and others. FTIR spectra of SF films prepared by FA/HAp dissolution method were shown in Fig. 3. Our observations demonstrated that both phosphate and hydrogen phosphate groups were obviously presented in SF films prepared at HAp content 5.0 wt.% in dissolved solution (Fig. 3a). The weak absorption at 1722 cm⁻¹ is due to hydrogen phosphate groups [26], while P=O associated stretching vibrations were observed at two different wavenumbers (1135 and 1067 cm⁻¹). Peak intensity of hydrogen bonded hydrogen phosphate ions at 1384 cm⁻¹ [27]. Likewise, the P–O–P asymmetric stretching vibrations were observed at 1003, 898, and 793 cm⁻¹. However, the strong absorptions at 575 cm⁻¹ and 528 cm⁻¹ are due to acid phosphates (H–O–P=O) bond vibrations [28]. These absorptions are attributed to the characteristic peaks of calcium hydrogen phosphate dehydrate (CHPD) [26,29]. At the same time, the characteristic peaks were coincid-ent with several references reporting crystallization characterization of CHPD crystals using FTIR spectroscopy [28,30]. From these results, it is thus clear that the HAp crystalline was decomposed under current condition. However, following the content of HAp decreased in dissolution

Fig. 2: SEM images of SF fiber dissolution process in FA/HAp solution: (a) SF fiber; (b) the dissolved process of SF fiber; (c) SF nanofibril bundle; (d) the alignment of SF nanofibrils.
method, the characteristic peaks of CHPD did not obviously appear. In addition, Fig. 3 also revealed the secondary structure of SF prepared by FA/HAp dissolution method. Um et al. investigated that the β-sheet crystallization of SF molecules occurred through the elimination of formic acid upon drying [11,16]. Wang et al. showed that SF in aqueous solution was mainly in random coil conformation [31]. However, when the SF films were prepared by FA/HAp dissolution method (Fig. 3a), the peak at 1627 cm$^{-1}$ (amide I) was attributed to silk II structure (β-sheet). The structure of SF films was similar to the film prepared by regenerated SF dissolving in FA. Furthermore, the HAp content was decreasing, the peak of amide I was also located in 1627 cm$^{-1}$, indicating that HAp contents in dissolved solution were not significantly affected the secondary structure of SF. The silk II structure of SF films may be closely related to the fact that FA has two opposite functions of dissolution and crystallization simultaneously [16].

Fig. 4 was the XRD results of SF films prepared by FA/HAp dissolution method containing different HAp contents. At HAp content 5.0 wt.%, the SF samples was characterized by diffraction peaks at 2θ values of 9.8° and 20.1°, attributing to the silk II structure (β-sheet). Um et al. discussed that amorphous regenerated SF was crystallized to β-sheet structure by dissolving and casting in formic acid [11]. Fig. 4a also confirmed that the HAp crystalline nature remained as evidenced from the existence of 20 peaks at approximately 26.4°, 31.4°, 38.1°, 44.6°, 46.7°, and 49.6° appearing, corresponding to the diffraction planes (002), (211), (310), (222), (213), and (004) of the HAp crystallites, respectively. At the same time, the minor peaks observed at 20 values 11.8° and 21.2° could be indexed for (020) and (121) planes of CHPD. When the HAp content decreased to 1.5 wt.%, Fig. 4 (b–d) also obviously confirmed that the HAp crystalline nature existed in SF films. At the same time, the diffraction peaks at 2θ values of 9.8° and 20.1° exhibited that the structure of SF in the films was predominantly silk II structure. In addition, the HAp content decreased to 1.0 wt.%, the secondary structure of SF was also silk II structure (Fig. 4e). The XRD data of the sample did not obviously exhibit the existence of the HAp crystal (Fig. 4e). From XRD results, it clearly demonstrated the presence of HAp crystal existing in the SF films prepared by FA/HAp dissolution method.

3.3. Thermal analysis

Fig. 5 illustrated thermal behavior of SF films prepared by FA/HAp dissolution method. From DSC results, a weight loss about 2.5 wt.% around 83 °C was detected in SF films, which was ascribed to the evaporation of water (Fig. 5a). As the temperature was increased further, the weight residue started to decrease sharply at 181 °C, which indicated the loss of lattice water of CHPD and HAp crystals. The endothermic peak in DSC around 137 °C indicated the removal of water during this temperature range. In the region (154–223 °C), two molecules of CaHPO$_4$ combined and resulted in the elimination of a water molecule leading to the formation of calcium pyrophosphate and nearly the sample was stable. The following chemical reactions were expected to occur during the dehydration and decomposition stages [27]. When the temperature was increased to 309 °C, the endothermic peak appeared, due to the thermal degradation of SF [32].

$$2\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \rightarrow 2\text{CaHPO}_4 + 4\text{H}_2\text{O}$$

$$2\text{CaHPO}_4 \rightarrow \text{Ca}_3\text{P}_2\text{O}_7 + \text{H}_2\text{O}$$

When the HAp content decreased to 2.0 wt.% in dissolved solution (Fig. 5c), the similar water content of SF films obtained about 4.0 wt.% was also observed around 93 °C. Moreover, similar endothermic peaks at 180 °C with the associated shoulders (144 °C), indicate the stepwise removal of water from crystals during this temperature range. As the temperature was increased further, similar thermal degradation behavior with a dramatic decrease in weight residue was observed at around 309 °C (Fig. 5b–c). Decreasing the HAp content in dissolved solution, the thermal behavior was similar to the SF films obtained under containing 5.0 wt.% HAp content in dissolved solution (Fig. 5d). At the same time, the content of HAp decreased to 1.0 wt.%, the endothermic peak of moisture evaporation ranging from 80–100 °C disappeared. However, the thermal degradation of SF also appeared at 304 °C. According to the XRD and FTIR results, the crystal structure of SF was mainly silk II structure in this dissolution method (Figs. 3 and 4). This demonstrated that the crystal structure of SF was not influenced.
under FA/HAp dissolution method containing different contents of HAp crystals.

3.4. Mechanical properties

The results of the mechanical tests were shown in Fig. 6. At dry state, the breaking strength and extension at break were 6.06 ± 1.34 MPa and 1.40 ± 0.08%, respectively, for SF film obtained under the HAp content 5.0 wt.% in FA/HAp dissolved solution (Fig. 6A-a). From Fig. 6A, the breaking strength of SF films at dry state was increased with the HAp content decreasing in dissolved solution. The breaking strength of SF films was 22.50 ± 7.34 MPa, 23.19 ± 6.80 MPa, and 29.19 ± 8.89 MPa, respectively, when the SF films were prepared by FA/HAp solution containing 2.5 wt.%, 2.0 wt.%, and 1.5 wt.% HAp contents (Fig. 6A-b, c, d). At the same time, the SF films obtained with HAp content 1.0 wt.% in dissolved solution showed higher breaking strength and extension at break for 28.99 ± 9.57 MPa and 3.9 ± 0.98% (Fig. 6A-e), obviously higher than the SF films prepared by FA solution. Rajkhowa et al. showed the breaking strength and extension at break of SF/FA films were 23.6 ± 7.7 MPa and 1.4 ± 0.1% at dry state [33]. The results exhibited that the mechanical properties of films were decreased with increasing the addition of HAp in dissolution process. This phenomenon was attributed to the entangled degree of SF fibers existing in the formation of SF film and its silk II structure.

However, when the samples were in wet state condition, the SF films exhibited the higher extension at break better than dry state. Under the HAp content 5.0 wt.%, the breaking strength and extension at break was 1.15 ± 0.21 MPa and 30.54 ± 3.27%, respectively (Fig. 6B-a). When the HAp content decreased, the breaking strength of SF film samples enhanced, simultaneously, the extension at break of the samples was also increased. The breaking strength of SF films at wet state was 2.32 ± 0.70 MPa, 3.81 ± 0.25 MPa, and 4.43 ± 0.21 MPa, respectively, for SF film samples prepared by FA/HAp solution containing 2.5 wt.%, 2.0 wt.%, and 1.5 wt.% HAp contents (Fig. 6B-b, c, d). At the same time, the extension at break of samples was 53.27 ± 8.45%, 54.74 ± 3.18%, and 67.54 ± 3.27%, respectively. As the HAp content decreased to 1.0 wt.%, the breaking strength of SF films achieved 8.16 ± 1.46 MPa, homologous, the extension at break was 74.12 ± 19.82% higher than SF films prepared by other conditions (Fig. 6B-e). These results may explain the pronounced effect of water in silk films. Water can easily alter the regenerated SF structure leading to large changes in the mechanical properties of silk films [33].

3.5. Morphology

The surface and cross-sectional SEM images of the SF films prepared by FA/HAp dissolution method were shown in Fig. 7. Fig. 2 depicted that this dissolution method could keep the dissolution process from SF fiber to SF nanofibers. The surface image of SF films showed many microfibers appearing, indicating that SF nanofibers self-assembled in drying process (Fig. 7f). At the same time, these nanofibers existing in SF films can enhance the breaking strength of SF films, which was confirmed by the mechanical tests of SF films (Fig. 6). The cross-sectional images of the SF films showed that unconspicuous nanofibers existed in SF films (Fig. 7a). This may be attributed to much more HAp co-existed induced. As the HAp content decreases in FA/HAp solution, obviously nanofibers were existing in the SF films (Fig. 7b-e). Through this dissolution method, many nanofibers were seen from the cross-section of the SF films. These nanofibers may contribute to the improved elasticity and mechanical strength of the regenerated SF films despite their somewhat brittle nature.

4. Conclusions

SF fibers were successfully dissolved in FA/HAp solution. The rheological measurement showed that the rheological behavior of SF was significantly influenced by the HAp content in dissolved solution. At the same time, SF nanofibrils were observed in FA/HAp solution with 103.6 ± 20.4 nm in diameter. The structure behavior of SF films prepared by FA/HAp dissolution method was also examined. The XRD results exhibited that obviously the HAp crystalline nature existed in SF films; however, when the HAp content was 5.0 wt.% in FA/HAp solution, some HAp crystal was converted to CHPD crystals in dissolution process. This result was also confirmed by FTIR and DSC measurements. In addition, the SF film prepared by FA/HAp dissolution method had higher breaking strength and extension at break. At wet state, the extension at break of samples reached up to 74.12 ± 19.82%, homologous, its breaking strength achieved 8.16 ± 1.46 MPa. Therefore, an understanding of the novel SF dissolution method in FA/HAp provided an additional tool for designing and synthesizing advanced materials with more complex structures, which should be helpful in different fields, including biomaterials applications.

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Fig. 7. The cross-section morphology of SF films prepared by FA/HAp solution containing (a) 5.0 wt.%, (b) 2.5 wt.%, (c) 2.0 wt.%, (d) 1.5 wt.%, and (e) 1.0 wt.%, respectively, and (f) the longitudinal-section of SF films.

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