Published in final edited form as:

Biomacromolecules. 2011 April 11; 12(4): 1080–1086. doi:10.1021/bm101422j.

Degradation Mechanism and Control of Silk Fibroin

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Abstract

Controlling the degradation process of silk is an important and interesting subject in biomaterials field. In the present study, silk fibroin films with different secondary conformations and nanostructures were used to study the degradation behavior. Silk fibroin films with highest β -sheet content achieved highest degradation rate, different from the previous studies. A new degradation mechanism revealed that degradation behavior of silk fibroin was related to not only crystal content, but also hydrophilic interaction and crystal-noncrystal alternant nanostructures. The hydrophilic blocks of silk were firstly degraded. Then, the hydrophobic crystal blocks which were formerly surrounded and immobilized by hydrophilic blocks, became free particles and moved into solution. Based on the mechanism, which enables the process more controllable and flexible, controlling the degradation behavior of silk fibroin without sacrificing other performances such as mechanical or hydrophilic properties become feasible, and this would greatly expand the applications of silk as a biomedical material.

Keywords

Silk fibroin; Degradation; Biomaterials; Nanostructure

1. Introduction

The degradability is very important for biomaterials used in tissue engineering [1–2]. When damaged or diseased tissues are incapable of self-repair, a substitute biomaterial is often required to aid the healing process. The self-repairing periods of different tissues such as bone, tendons, ligament or vessels are different, meaning that biomaterials used as scaffolds should have corresponding degradation rates to facilitate the formation of new tissues. Therefore, the controlling of the degradation of different scaffolds is still the major goal of tissue engineering research.

Silk fibroin has aroused more and more interests in biomedical field because of its excellent environmental stability, biocompatibility, morphologic flexibility and mechanical properties [3–9]. It has been found that silk fibroin had a promising future in tissue engineering [10–

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11], drug release [12–13] and optical apparatus [14]. In order to satisfy the various requirements of different applications, the researchers still strive to extend the capabilities of silk fibroin from hydrophobic material to hydrophilic material [15–16], from filament to film, sheet, and scaffolds [11, 17–19], and even from soft material to super rigid material [8, 20]. Some of these exciting developments greatly stimulate the studies on the silk forming mechanism and the relation between conformations, microstructures and properties.

However, the degradation is a serious obstacle for the applications of silk-based materials from the beginning. Although the degradability of silk-based materials can be changed sometimes through using different methods or adding different enzymes, the degrading process of silk is still bewildering [21–25]. For example, the β -sheet structure was considered as a critical factor that stabilized silk fibroin in aqueous environments, but silk films, treated by methanol annealing and stretching respectively, showed totally different degradation properties even though the films had similar β -sheet contents. In order to control the degradation behavior, it is necessary to explore potential factors that affect the degradation of silk fibroin.

Different from the general opinion that considers the stability of silk fibroin results from β -sheet formation, water-stable silk films were prepared without the increase of β -sheet in our previous research [26]. Compared with other silk films, the silk films in our research degrade more quickly in PBS and enzyme solutions. The water-stable silk films have special microstructures and flexible properties, making them suitable research objects to explore the relationship between structures and degradation behavior.

In this study, we researched on the degrading process of three different insoluble silk films in enzyme solutions. These silk films had different crystal contents and nanostructures, which is very useful for gaining a clear understanding of the relationship between the structures, processing, and degradability. Furthermore, it will become more predictable to control the degradability of silk-based materials according to the requirements of various tissue regenerations.

2. Materials and methods

2.1 Preparation of silk solutions

B. mori silk-fibroin solutions were prepared according to our previously published procedures [27]. Cocoons were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 , and then rinsed thoroughly with water to extract sericin proteins. The extracted silk was dissolved in 9.3M LiBr solution at 60°C, yielding a 20wt.-% solution. This solution was dialyzed in water using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3500) for 72 h. The final concentration of aqueous silk solution was ~7.5wt%, determined by weighting the remaining solid after drying.

2.2 Film formation

1.5 ml of silk solution was cast on polystyrene Petri dishes (diameter 30 mm). A lid with nine holes was placed over the dish to control the drying rate. The area of each hole was 3.13 mm², and the drying time was 3 days when the hood airflow maintained at 0.20 m s⁻¹. Once dried, the insoluble silk films with high silk I structure were directly prepared without any further treatment. The water-insoluble silk fibroin films prepared by slow drying were termed SD-SF. After the soluble silk fibroin films were formed by drying silk fibroin solutions in dishes without lids, insoluble silk fibroin films were also prepared by the water annealing treatment. The soluble films were placed in a water-filled desiccator with a 25 in. Hg vacuum for 4h to produce water-insoluble films which were termed WA-SF. The other water-insoluble silk fibroin films were prepared by adding glycerol as additive. The silk

fibroin solution (1.5 ml) was mixed with glycerol at weight ratios of 20% (w/w), and then poured into a Petri dish (diameter 30 mm) and dried at room temperature in a flow hood overnight. Once the films were prepared, they were stretched to 250% of their original length through using an Instron 3366 testing frame (Instron, Norwood, MA, USA), resulting in the formation of silk II structure. Then the treated films were incubated in distilled water for 24h at room temperature to remove glycerol [28]. After dried in the air overnight, the silk fibroin films prepared by stretching were termed ST-SF.

2.3 Silk degradation

Silk fibroin films were incubated at 37°C in 40ml PBS solutions which contained 0.23 U/ml protease XIV at pH 7.4. Each solution contained an approximately equivalent (40 ± 5 mg) amount of silk films. Solutions were replenished with enzyme and collected daily. At appointed time points, groups of samples were rinsed in distilled water and prepared for mass balance.

2.4 Differential Scanning Calorimetry (DSC)

Samples of about 5mg were encapsulated in Al pans and heated in a TA Instruments Q100 DSC (TA Instruments, New Castle, DE) under a dry nitrogen gas flow of 50 mL/min. Standard mode DSC measurements were performed at a heating rate of 2K/min. Temperature-modulated differential scanning calorimetry (TMDSC) measurements were also performed through using a TA Instruments Q100, equipped with a refrigerated cooling system. The samples were heated at 2K/min with a modulation period of 60s and a temperature amplitude of 0.318 K.

2.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed with a Bruker Equinox 55/S FTIR spectrometer, equipped with a deuterated triglycine sulfate detector, a multiple-reflection, and a horizontal MIRacle ATR attachment (using a Ge crystal, from Pike Tech.). For each measurement, 32 scans were coded with resolution 4 cm^{-1} , with the wave number ranging from 400 to 4000 cm^{-1} . Fourier self-deconvolution (FSD) of the infrared spectra covering the amide I region ($1595\text{--}1705 \text{ cm}^{-1}$) was performed by Opus 5.0 software. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. FSD spectra were curve-fitted to measure the relative areas of the amide I region components [29].

2.6 Scanning electron microscopy (SEM)

The surface morphologies of different silk films were imaged with a Zeiss Supra 55 VP SEM (Oberkochen, Germany). Then these films were fractured in liquid nitrogen and sputtered with platinum. The cross-section images were also investigated with a Zeiss Supra 55 VP SEM.

2.7 Atomic force microscopy (AFM)

The morphology of degraded silk fibroin in enzyme solution was observed by AFM (Veeco, Nanoscope III) in air. A $225 \text{ }\mu\text{m}$ long silicon cantilever with a spring constant of 3 N/m was used in tapping mode.

3. Results and discussion

3.1 Structure of silk fibroin films

Changes in the structure of silk films prepared with various methods were determined by FTIR. The infrared spectral region within $1700\text{--}1500 \text{ cm}^{-1}$ was assigned to absorption by

the peptide backbones of amide I ($1700\text{--}1600\text{ cm}^{-1}$) and amide II ($1600\text{--}1500\text{ cm}^{-1}$), which were usually used for the analysis of different secondary structures of silk fibroin. The peaks at $1610\text{--}1630\text{ cm}^{-1}$, $1695\text{--}1700\text{ cm}^{-1}$ and $1510\text{--}1520\text{ cm}^{-1}$ were characteristics of silk II secondary structure, while the absorptions at $1648\text{--}1654\text{ cm}^{-1}$ and $1535\text{--}1542\text{ cm}^{-1}$ were indicative of silk I conformation [29, 30]. As shown in Fig 1A, the amide I band for SD-SF showed one strong peak at 1651 cm^{-1} , corresponding to silk I structures. In the stretched films (ST-SF), the amide I band showed one strong peak at 1624 cm^{-1} , with a shoulder at 1651 cm^{-1} , while in the water annealed samples (WA-SF), one peak at 1651 cm^{-1} appeared, with a shoulder at 1624 cm^{-1} . The same trend in structural change was also found in the amide II region. From the SD-SF to the WA-SF and the ST-SF samples, the peak at 1539 cm^{-1} (silk I) decreased, while the peak at 1515 cm^{-1} (silk II) increased. The results indicated that silk films with different crystal structures were achieved by changing the processing.

Structural changes in the silk films after different processes were confirmed by DSC (Fig 1B). In our previous studies, it was found that silk I crystal degraded at around 250°C and silk II degraded at around 260°C [26]. The SD-SF samples showed a main degradation peak at 251°C and a minor degradation peak at 258°C , indicating the formation of the stable silk I structure. After silk fibroin films were prepared by water annealing process (WA-SF), the strength of degradation peak at around 250°C decreased and two minor peaks at around 260°C appeared, implying the decrease of silk I crystal and the increase of silk II structure. Only one degradation peak at 261°C was found for the ST-SF samples, meaning that they were mainly composed of silk II structure. The DSC results are consistent with the FTIR results, confirming that the ST-SF samples had the highest silk II structure and thermal stability, while the SD-SF samples had the lowest silk II structure.

In our previous studies we reported the observation of a lower $T_g(1)$ which provided information about the removal of bound water, indicating an interaction between silk and bound water and further hydrophilic interaction in silk fibroin [26]. As shown in Fig 1C, the ST-SF samples had no $T_g(1)$, meaning that little water formed strong interactions with the silk fibroin protein. $T_g(1)$ was found for both the WA-SF and the SD-SF samples, with a higher heat capacity at $T_g(1)$ being achieved for SD-SF samples. The results indicated that stronger hydrophilic interaction formed in SD-SF films.

The nanostructure of samples was investigated by SEM. The special nanostructure of the SD-SF samples was studied in our previous studies [26]. Although the surface was relatively flat, the inside of silk fibroin films formed special core-layer globules with about $200\text{--}1000\text{ nm}$ in diameter, in which the core of globules was composed of nano-filaments containing high crystal content and surrounded by random nano-filaments (Fig 2a–b). Likewise, the core-layer structure composed of nanofilaments was also formed inside the WA-SF films (Fig 2c–d), which was confirmed by the following SEM, FTIR and DSC results of the degraded WA-SF samples (Fig 4–6). The nanostructure of the ST-SF samples was different from the SD-SF and the WA-SF samples (Fig 2e–f). Their main structure consists of nanoparticles and short nanofilaments rather than core-layer globules structure. Some short nanofilaments with a size of several ten nanometers were surrounded by nanoparticles with below 20 nm in diameter. The results indicated that nanostructures, as well as secondary conformations, could be controlled by adjusting the preparation conditions, which would be useful for the control of the degradation behavior.

3.2 Degradation of silk fibroin films

In the previous research the degradation of methanol-annealed and water-annealed silk films was studied [21]. The results indicated that there was no weight change in water-annealed or methanol-annealed silk films after 2 weeks in PBS solution, serving as controls. After

incubation in protease XIV solution (5.6Uml⁻¹) at 37°C for 24h, the weight loss of the water-annealed and methanol-annealed silk films was about 20%. This study also revealed that degradation time could be controlled through the content of silk II structure. As silk II structure increased, the degradation time also increased. In the present studies, silk films with much higher degradation rate were achieved in different ways (Fig 3). More interestingly, these silk fibroin films showed reversed trend between degradation rate and silk II content. The ST-SF samples containing high silk II content degraded most rapidly, with a weight loss of about 80% after 24h in protease XIV solution (2.3 U ml⁻¹), while the WA-SF and SD-SF samples, having low silk II content, degraded slowly, losing only about 40% of their original weight after 24h. Although the WA-SF samples had higher silk II content than the SD-SF samples, the two kinds of films had similar degradation rate, which was totally different from the previous studies [21]. The results implied that besides silk II content, there were other undetected but critical factors in controlling the degradation of silk fibroin.

3.3 Structural changes in the degradation process

In order to reveal the undetected factors, the structural changes in the degradation process were further investigated. As shown in Fig 4, for the degraded WA-SF and ST-SF samples, non-crystal and silk I structures degraded more quickly than silk II structure, similar to previous studies [21, 26]. The silk II structures increased quickly within 2h and then kept stable in the degradation process. The results indicated that non-crystal structure and unstable crystal (silk I) were firstly degraded in protease XIV solution, resulting in the increase of crystal structure content (silk II) in the degraded samples. On the other hand, the crystal structures were mainly composed of silk I rather than silk II structure in the SD-SF films. The silk I structure in the SD-SF films increased continuously in the degradation process, showing higher stability than that in the WA-SF and ST-SF films. The results confirmed that besides silk II structure content, other factors also have great influence on the degradation of silk fibroin. The DSC results further confirmed the degradation behavior of silk fibroin (Fig 5). After incubated in protease XIV solution for 12h and 24h, the T_g (1) disappeared in the degraded WA-SF and SD-SF samples respectively, confirming the degradation of the hydrophilic non-crystal structure. The present structural results indicated that the non-crystal structure was degraded firstly among all the samples, which was consistent with other previous studies. But the ambiguous problem was that silk fibroin films containing high silk II structures degraded quickly rather than that containing low crystal structures. More interestingly, the content of silk II structure kept stable after the WA-SF and SD-SF samples were incubated in protease XIV solution for 2h, implying that the crystal structures and non-crystal structures seemed to degrade at a similar rate after 2h.

The morphological changes in the degradation process were shown in Fig 6. The WA-SF and ST-SF films degraded from surface while the SD-SF degraded more randomly. The changes of nanostructure in the degradation process revealed the latent factors which could control degradation behavior of silk fibroin. Our previous studies have revealed the formation of the SD-SF films with special core-layer globule structure in which the crystal core was surrounded by non-crystal nano-fibrils [26]. From the SEM images (Fig 2), we found that similar core-layer globule structure with higher crystal content and weaker water-silk fibroin interaction was also formed in the WA-SF samples. Interestingly, similar degradation processes were found in the WA-SF and SD-SF samples (Fig 6a–b). The non-crystal structures degraded firstly in the WA-SF and SD-SF samples. Following the degradation of the non-crystal hydrophilic structures, the filaments surrounding the core ruptured, resulting in the formation of free core. These results clarified the degradation behavior which hid the ambiguous degradation rate of the WA-SF and SD-SF samples. As reported in many other studies [22–25, 31–35], the non-crystal hydrophilic blocks degraded

firstly. After the degradation of hydrophilic blocks, the hydrophobic crystal blocks became free particles, and then moved rather than degraded into solution (Fig 7). Although the WA-SF samples had higher β -sheet content than the SD-SF samples, the hydrophilic blocks degraded easily because of the weak hydrophilic interaction, and then the crystal blocks moved to the solution, resulting in the similar degradation rate of the two samples. The degradation behavior of the ST-SF samples further confirmed the degradation mechanism. With the highest β -sheet content in our samples, the ST-SF samples degraded more quickly than the WA-SF and SD-SF samples, because the ST-SF films were composed of nanoparticles and short nanofilaments with little hydrophilic interaction. Fig 8 showed the morphology of the degraded materials in protease XIV solution. Particles with size of several hundred nanometers appeared in the degraded solutions of the SD-SF and WA-SF samples while only particles about the size of several ten nanometers and short nanofilaments were found in the degraded solutions of the ST-SF samples, validating that crystal-rich parts with different nanostructures in these samples were firstly “dissolved” in the protease solution rather than degraded.

In the present studies, more complicated degradation behavior was found, which related to not only β -sheet content, but also hydrophilic interaction as well as special crystal-noncrystal alternant nanostructures. In previous studies, crystal content was regarded as pivotal and even the only factor in controlling the degradation properties. Since the crystal content also had great influence on the mechanical and hydrophilic properties, the superiority of silk fibroin over other natural biomaterials often has to be sacrificed to satisfy the requirements for degradation rate. Based on our present degradation mechanism, although the degradation behavior of hydrophobic blocks in the enzyme solutions needs further investigations and more controllable and flexible processes, controlling the degradation behavior of silk fibroin without the sacrifices of other performances such as its mechanical or hydrophilic properties become feasible, which would further expand the applications of silk as a biomedical material.

4. Conclusion

The degradation mechanism of silk fibroin was studied based on the films with different secondary conformations and nanostructures. Besides β -sheet content that had confirmed by other groups, the degradation of silk fibroin was also related to hydrophilic interaction as well as special nanostructures. In the degradation process, the hydrophilic blocks were firstly degraded, making stable blocks with high content of crystal structures move to protease solutions. Based on the new degradation mechanism, the preparation of silk fibroin materials with more controllable degradation properties become feasible, facilitating the applications of silk in tissue engineering and drug release systems.

Acknowledgments

The authors thank the NIH (EB002520) and National Natural Science Foundation of China (30970714) for support of this research.

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Figure 1.

(A) FTIR spectra of silk fibroin films prepared with different processes; (B) DSC data from silk fibroin films prepared with different processes and (C) TMDSC data from silk fibroin films prepared with different processes: (a) silk fibroin film derived from slow drying process, SD-SF, (b) silk fibroin film derived from water-annealing process, WA-SF and (c) silk fibroin film derived from stretching process, ST-SF.

Figure 2.

SEM images of silk fibroin films prepared with slow drying process (a–c), water annealing treatment (d–f) and stretching treatment (g–h). (a) surface image of film prepared with slow drying, (b) cross-section image of film prepared with slow drying, low magnification, (c) cross-section image of film prepared with slow drying, high magnification, (d) surface image of film prepared with water annealing, (e) cross-section image of film prepared with water annealing, low magnification, (f) cross-section image of film prepared with water annealing, high magnification, (g) surface image of film prepared with stretching, and (h) cross section image of film prepared with stretching. The circles show the hydrophobic core of the globules and the arrows point to the disjunct nanoparticles.

Figure 3. Enzymatic degradation of water-insoluble silk fibroin films prepared by slow drying process (SD-SF), water annealing treatment (WA-SF) and stretching treatment (ST-SF), respectively. The silk fibroin films were cultivated in protease XIV solution. N=5, bars represent standard deviation.

Figure 4.

(A) FTIR spectra of degraded silk fibroin films prepared by slow drying process, SD-SF; (B) FTIR spectra of degraded silk fibroin films prepared by water annealing treatment, WA-SF; (C) FTIR spectra of degraded silk fibroin films prepared by stretching treatment, ST-SF; and (D) crystal content changes with degradation time. The crystal content was calculated from the deconvoluted amide I band using the method of Fourier self-deconvolution. $n=3$, bars represent standard deviation. The crystal structure was silk II in ST-SF and WA-SF samples while changed to silk I in SD-SF. All samples were cultivated in protease XIV solution.

Figure 5. TMDSC data from degraded silk fibroin films that were cultivated in protease XIV solution for 0h, 12h and 24h. (a) SD-SF, silk fibroin films prepared by slow drying process, and (b) WA-SF, silk fibroin films prepared by water annealing treatment.

Figure 6.

SEM images of different degraded silk fibroin films that was cultivated in protease XIV solution for 12 h. (a), films were prepared with slow drying process, SD-SF; (b), films were prepared with water annealing treatment, WA-SF; and (c), films were prepared with stretching treatment, ST-SF. (Left, low magnification; Right, high magnification of the degraded area of the films)

Figure 7. Degradation mechanism of silk fibroin. The Non-crystal or unstable crystal structures were firstly degraded in enzyme solutions, resulting in the formation of free crystal structure. Then the crystal structure was dissolved in enzyme solutions.

Figure 8.

AFM images of degraded silk fibroin particles that dissolved in protease XIV solution when the samples were cultivated in protease solution for 12h: (a) protease XIV solution without degraded silk fibroin particles; (b) films were prepared with slow drying process, SD-SF; (c) films were prepared with water annealing treatment, WA-SF and (d) films were prepared with stretching treatment, ST-SF.