IJIE Int. J. Indust. Entomol. 45(1) 12-16 (2022)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2022.45.1.12

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Gelation of silk fibroin solution via β-sheet formation promoted by riboflavin-mediated photo-crosslinking

Jaeho Choi[1] and Chang Seok Ki[1,2,*]

[1]*Department of Agriculture, Forestry and Bioresources, Seoul National University, Seoul 08826, Republic of Korea*
[2]*Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Republic of Korea*

Abstract

Di-tyrosine photo-crosslinking of silk fibroin (SF) is recently highlighted as a biocompatible hydrogel fabrication process, because this method does not need potentially harmful chemical species. However, the resulting crosslinking density is often insufficient to obtain a mechanically stiff hydrogel unless additional oxygen is provided during the reaction. In this study, we proposed a combinational crosslinking method to form an SF hydrogel via the di-tyrosine photo-crosslinking with riboflavin (photoinitiator) and physical interaction of SF chains. In the UV light-irradiated SF solution, small particles formed and these particles promoted β-sheet formation of SF molecules, resulting in quick gelation. The di-tyrosine photo-crosslinking produced nuclei that might trigger regular assembly of SF molecules in high temperature condition. Conclusively, this process would contribute to a development of biocompatible hydrogel fabrication for biomedical uses of SF hydrogels.

Int. J. Indust. Entomol. 45(1), 12-16 (2022)

Received : 10 Aug 2022
Revised : 24 Aug 2022
Accepted : 24 Aug 2022

Keywords:
silk fibroin,
photo-crosslinking,
riboflavin,
di-tyrosine,
hydrogel

Introduction

Bombyx mori's silk fibroin (SF) is one of promising materials in biomedical field because of its biocompatibility and tissue regeneration capability. Hence, for decades, diverse SF-based biomaterials have been proposed such as foam-type scaffold, bioprinting ink, and porous fiber-assembly (Gong *et al*., 2020; Park *et al*., 2015; Sun *et al*., 2021). Recently, SF hydrogel received great attention as a biomaterial for not only facile gelation process, but highly tunable physical property (Ki *et al*., 2016). Such a property of SF hydrogel is particularly good for mimicking diverse soft tissues. Hence, SF hydrogel has been extensively explored as a tissue engineering scaffold or three-dimensional cell culture matrix (Ryu *et al*., 2016).

SF hydrogels are readily formed via both physical and chemical processes. For physical process, various physical stimuli (e.g., sonication, shear stress, electric charge, and heat) initiate chain aggregation and β-sheet formation, resulting in physical crosslinks (Ki *et al*., 2016; Kim *et al*., 2018). The physical crosslinking permits a facile fabrication process without harmful reactive reagents. This method is therefore preferred especially in biomedical applications. However, it has some drawbacks such as brittleness, unpredictable gelation time, and incomplete network formation (Kim *et al*., 2018). By contrast, chemical process relies on crosslinking reaction with reactive chemical species. In general, di-aldehyde, di-epoxide, or vinyl

***Corresponding author.**
Chang Seok Ki, PhD
Department of Agriculture, Forestry and Bioresources, Seoul National University, Seoul 08826, Republic of Korea
Tel: +82-2-880-4621 / FAX: +82-2-873-2285
E-mail: ki.cs@snu.ac.kr

reagents are used to form crosslinks between SF chains (Farokhi *et al*., 2021). The chemical crosslinking achieves not only predictable reaction kinetics, but also controllable crosslinking density, resulting in tailored network structure. Despite these advantages, the toxic chemicals possibly remain after chemical gelation. It needs a thorough washing or purifying process for removal of those chemicals.

Recently, a non-toxic chemical crosslinking methodology has been proposed using riboflavin (RF). RF is a photosensitive vitamin containing conjugated structure. It absorbs radiations of UV and blue light wavelengths and generates radicals as a non-cleavage type photoinitiator (Cardoso *et al*., 2012; Heo *et al*., 2016; Mu *et al*., 2020). Hence, RF supplies radicals continuously under light irradiation and the radicals produce singlet oxygen or directly oxidize tyrosine residue, resulting in di-tyrosine coupling between SF chains (Raiskup and Spoerl, 2013; Mu *et al*., 2020). In general, the coupling reaction does not produce sufficient chemical crosslinks to form a gel. Fortunately, the di-tyrosine formation is promoted by increasing the concentration of RF or dissolved oxygen concentration, which accelerates the reaction rate and the crosslinking density. Hence, the photo-crosslinking of SF is conducted in the presence of oxygen-releasing agent or under oxygen gas purging. However, the use of additional chemical may cause undesired toxicity of resulting SF hydrogel or need a tedious fabrication process.

The aim of this study was to form a SF hydrogel by combining physical and chemical processes using RF-mediated photo-crosslinking and β-sheet formation of SF chains, excluding any additional harmful compounds. For photo-crosslinking, a diluted SF solution was prepared in the presence of RF. The crosslinking was initiated by shining UV light and modulated by varying the irradiation time. The changes of SF solution were assessed by visible light spectroscopy and dynamic light scattering after photo-crosslinking. To complete the gelation, the SF solution was then incubated at 60 °C, resulting in physical crosslinking. Finally, the presence of β-sheet structure in the hydrogel was confirmed by infrared spectroscopy.

Materials and Methods

Materials

40 g of *Bombyx mori* cocoons were boiled in 1 L of 0.2% (w/v) sodium carbonate solution for 30 min for sericin removal, followed by washing with deionized water and drying. Anhydrous calcium chloride was purchased from Yakuri (Japan). Ethyl alcohol and riboflavin phosphate sodium salt were purchased from and Sigma-Aldrich (St. Louis, MO, USA).

Preparation of silk fibroin solution

The degummed cocoons were dissolved in a ternary solvent of $CaCl_2/H_2O$/EtOH mixture (molar ratio = 1/8/2) at 85 ℃ for 10 min according to an established protocol with some modification (Jo *et al*., 2016). The concentration was 10% (w/v) and the resulting SF solution was transferred into a cellulose acetate dialysis tube (molecular weight cut-off (MWCO): 12–14 kDa). Then, the SF solution was dialyzed against deionized water for 7 days. After dialysis, the concentration of SF solution was 3.65 wt%, which was measured by a moisture analyzer (MB-10, Ohaus, USA). The concentration was adjusted to 3 wt% by diluting with deionized water and stored at 4 ℃ until use.

Hydrogel formation

For preparation of a riboflavin stock solution, riboflavin phosphate sodium salt (RF) was dissolved in saline (0.85% sodium chloride solution) at 10 mM. Then, the 3 wt% SF solution was further diluted with saline and the RF solution was added. Finally, the working concentrations of SF and RF in a precursor solution were 0.6% and 0.2 mM, respectively. The precursor solution was transferred into a transparent polystyrene tube (diameter = 10 mm) and sealed with paraffin film. Then, the tube was shined by UV light (10 mW/cm^2, 365 nm) with a UV light source (Prime-100; Skycares, Korea) for 60 min at room temperature. Subsequently, the solution was incubated at 60 ℃ for 72 h and the formed hydrogel was finally collected.

Photospectroscopy

200 μL of UV light-irradiated SF solution was transferred into a well of transparent 96-well plate and the absorbance at 700 nm was measured by a microplate reader (Synergy HT; BioTek, USA). To obtained the relative absorbance, the absorbance of solution prepared with RF was normalized to the absorbance of unirradiated 0.2 mM RF-contained precursor solution, while no RF-contained solution absorbance was normalized to the absorbance of unirradiated SF solution (no RF).

Dynamic light scattering

1 mL of UV light-irradiated SF solution was subjected to

dynamic light scattering (DLS) measurement using a Zetasizer (Nano ZS90; Malvern Panalytical, UK) operating at 633 nm and 25 ºC with a 90° detection angle. The particle sizes were presented in Z-average size (nm) with polydispersity index (PDI).

Fourier-transformed infrared spectroscopy

The SF solutions and formed hydrogels were frozen at -80 ºC and lyophilized. The lyophilized samples were analyzed by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) (Nicolet 6700, Thermo Scientific, USA). The measurement was conducted with 32 scans and at 4 cm^{-1} resolution in the range of 1800–1400 cm^{-1}.

Results and Discussion

Particle formation in SF solution under UV light exposure

To initiate di-tyrosine crosslinking, SF solution was exposed to UV light. Fig. 1 shows unirradiated and UV-irradiated SF solutions in the absence/presence of RF. Initially, SF solution was translucent before UV light exposure, regardless of the RF presence. SF molecules form submicro-particles in the SF solution that was obtained from $CaCl_2/H_2O/EtOH$ solvent system. It was caused by structural instability due to partial hydrolysis during the dissolution (Kim *et al*., 2016; Um *et al*., 2003). In case no RF was added, the turbidity of SF solution was not changed for 60-min irradiation. By contrast, the SF containing RF became hazier even after 10-min irradiation, indicating an increase in the number of large size particles. To quantitate the solution turbidity, we measured the relative absorbance of the solution at 700 nm. At this wavelength, both

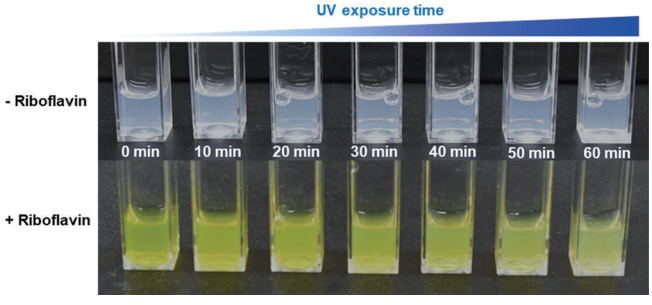

Fig. 1. UV light-irradiated silk fibroin solutions in the absence/presence of riboflavin. SF and riboflavin concentrations were 0.6 wt% and 0.2 mM, respectively. After irradiation, each solution was transferred into a UV disposable polystyrene cell.

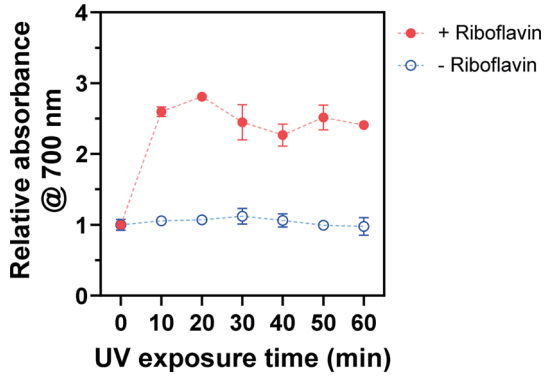

Fig. 2. Relative absorbances of UV light-irradiated silk fibroin solutions in the absence/presence of riboflavin. One-fold is the absorbance of unirradiated SF solution (n = 3, mean ± SD).

SF and RF rarely absorb the radiation. Therefore, it was good for comparing the turbidity increased by light scattering. As shown in Fig. 2, the relative absorbance of the RF-contained SF solution increased significantly after 10-min UV-light exposure, while the relative absorbance of the SF solution containing no RF was not changed under UV light. It was consistent with the solution appearance observation (Fig. 1) and the increased absorbance was not changed even after further UV-light irradiation.

Particle size change via photo-crosslinking

Fig. 3 presents particle size distributions in SF solution before/after 60-min UV-light exposure. When the SF solution was irradiated in the presence of RF, an obvious increase in large particle fraction was observed (Fig. 3B and 3D). In contrast, the no RF-contained SF solution did not show any change in particle size distribution after UV-light exposure (Fig. 3A and 3C). This indicated that the photo-crosslinking that was initiated by RF induced the aggregation of SF molecules although the photo-crosslinking was not sufficient to form a stiff hydrogel. To analyze the photo-crosslinking kinetics, we compared the SF particle Z-average sizes and PDI, which were measured over time evolution by dynamic light scattering. As expected, the no RF-contained SF solution did not show any changes in both Z-average size and PDI for 60 min (Fig. 4A). For RF-contained SF solution, the only 10-min UV-light irradiation increased not only Z-average size, but PDI (Fig. 4B). This supported the large particle formation, resulting in a wide size distribution. However, the longer light exposure did not contribute to the greater difference in both Z-average size and PDI, which means the particle formation process was completed almost within 10 min.

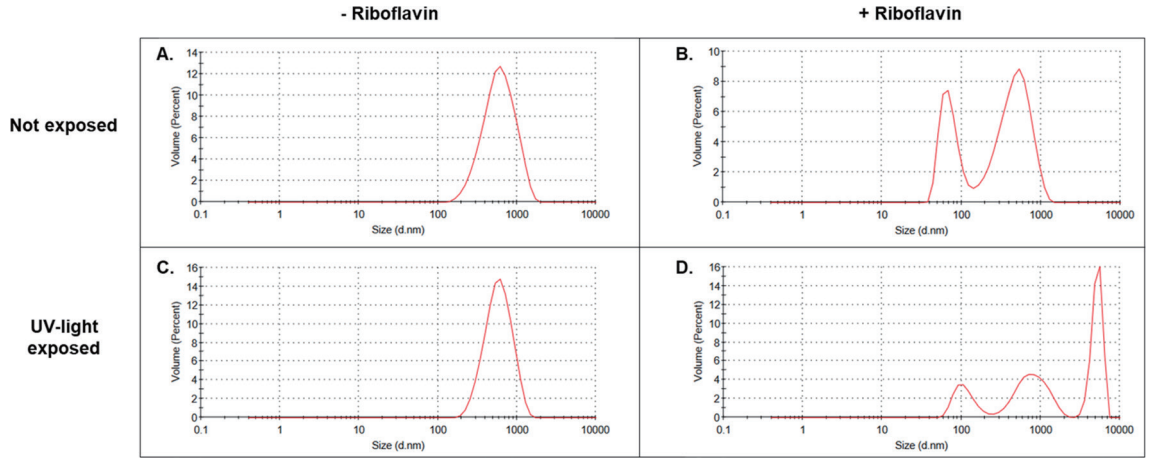

Fig. 3. Particle size distributions of UV light-irradiated silk fibroin solutions in the absence/presence of riboflavin in volume percent. (A) Unirradiated solution containing no riboflavin. (B) Unirradiated solution containing 0.2 mM riboflavin. (C) 60-min UV-light irradiated solution containing no riboflavin. (D) 60-min UV-light irradiated solution containing 0.2 mM riboflavin.

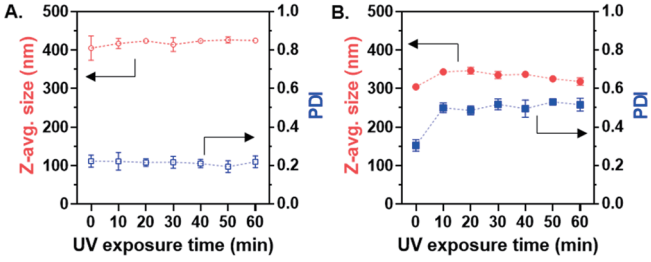

Fig. 4. Zeta-average (Z-avg.) sizes and polydispersity indices (PDI) of UV light-irradiated silk fibroin solutions in the absence/presence of riboflavin. (A) No riboflavin and (B) 0.2 mM riboflavin (n = 5, mean ± SD)

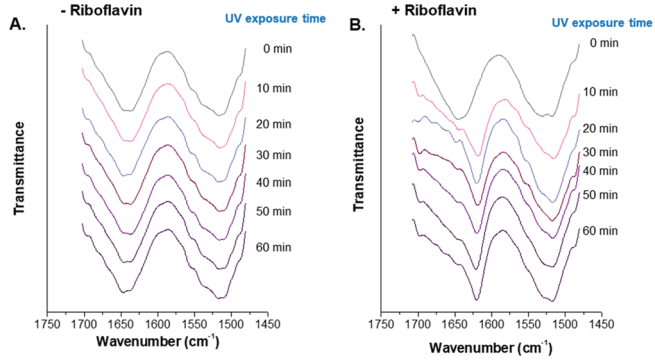

Fig. 5. FT-IR spectra of lyophilized SF solutions after UV irradiation in the absence/presence riboflavin. (A) No riboflavin and (B) 0.2 mM riboflavin.

For the lower Z-average size of RF-contained solution compared with that of no RF-contained solution, we reasoned that some part of particles participated in the large particle formation, while the other remained small in size. It might cause a relative lower Z-average size in spite of the wide size distribution (i.e., higher PDI).

Hydrogel formation and secondary structure of photo-crosslinked SF solutions

To form a hydrogel, the SF solutions were subsequently incubated at 60 °C for 72 h, and the gelation was confirmed by a tilting method (Hao *et al.*, 2013). As a result, the only UV-light irradiated SF solutions in the presence of RF turned into hydrogels. Fig. 5 shows amide I and II bands of FT-IR spectra of the freeze-dried SF solutions and hydrogels. In Fig. 5A, the amide I and II peaks of no RF-contained SF samples appeared at ~1650 and 1515 cm^{-1}, respectively, regardless of UV exposure time. These peak positions are attributed to random coil conformation of SF chains, which means that no conformational transition occurred during the high-temperature incubation. For RF-contained SF samples, the UV-light irradiation caused a significant change in peak position. The amide I peaks of the UV-light irradiated samples shifted to ~1620 cm^{-1}, while the peak position of the unirradiated sample was close to 1650 cm^{-1} (Fig. 5B). Such a peak shift indicates an obvious conformational change to β-sheet from random coil and this was caused after photo-crosslinking, not by RF itself. The β-sheet formation in solution phase induced self-assembly of SF molecules, ultimately resulting in crystalline structure. It is known to be accelerated by diverse stimuli (e.g., alcohol treatment, shear stress, ultrasonication, electric charge) (Ma *et al.*, 2011; Ki *et al.*, 2009; Kim *et al.*, 2016, Leisk *et al.*, 2010). In any case, the

first step of the structural transition was initiated from nucleation of SF molecules. The regular structure propagates and forms a network physically. In this context, we speculated that the particles generated by the di-tyrosine photo-crosslinking acted as a nucleating agent and the nucleation contributed to accelerate the structural transition of SF molecules to β-sheet in a high temperature condition.

Conclusion

In this work, an SF hydrogel was formed via RF-mediated photo-crosslinking and physical interaction of SF chains at a high temperature. Although the photo-crosslinking was insufficient to create hydrogel network in the absence of additional oxygen suppliers, the formed particles in the solution promoted the β-sheet formation during the high temperature incubation, resulting in a significant reduction in gelation time. In conclusion, we demonstrated that SF hydrogel formation via photo-crosslinking and physical SF crosslinks without use of any harmful compounds. Hence, we expect this method will be used to enhance biosafety and biocompatibility of silk hydrogel-based biomaterials.

Acknowledgement

This research was supported by the INNOPOLIS foundation funded by the Ministry of Science and ICT (2021-IT-RD-0168).

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