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Preparation of cross-linked silk fibroin film by γ-irradiation and their application as supports for human cell culture

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Abstract This study described about preparation of the cross-linked silk fibroin (SF) film by γ-irradiation of the casted SF film, which is fabricated from aqueous solution regenerated via fibers of cocoons and their application as supports for human cell culture. The properties of cross-linked SF film were evaluated by FT-IR spectroscopy, contact angle, solubility to water, thermal analysis, surface area analyzer, and morphology via scanning electron microscopy (SEM). The cross-linked SF films were not dissolved in water and exhibited the rough surface morphology, large surface area, and good thermal properties. The human fibroblast cell (CCD-986sk) and embryo kidney-ft cell were well growed on the surface of cross-linked SF film supports prepared by γ-irradiation. The cross-linked SF film prepared by γ-irradiation can be used as biomaterials for human cell culture.

Key words: cross-linked silk fibroin film, γ-Irradiation, human cell culture, human fibroblast cell, human embryo kidney-ft cell

1. Introduction

Silk fibroin (SF) has been explored as a versatile protein biomaterial for the formation of films, fibers, microspheres, and porous scaffolds for various biomedical applications due to its biocompatibility, slow degradability, and robust mechanical properties.[1-3] Regenerated silk-based materials are normally stabilized by the induction of β-sheet formation through the use of solvents[4] or by physical stretching.[5] The microstructure and porosity of these silk based biomaterials, as well as features involved in cell interactions, can be changed by chemical modification, blending with biocompatible polymers, or by exploiting different preparation processes. The β-sheets serve as physical cross-links in silk-based biomaterials, providing mechanical stability, water insolubility, and relating directly to the rate of degradation via proteolytic action. In general, the SF film casted from aqueous solution regenerated from fibers of cocoons undergoes conformational transitions from random coil to the β-structure. Thus, SF films in the dry state become brittle and unsuitable for practical use. These properties can be improved by blending with other natural or synthetic polymers. Silk blends have been extensively studied with respect to film formation. Blends with polyacrylamide,[6] sodium alginate,[7] cellulose,[8] chitosan,[9] poly(vinyl alcohol),[10] acrylic polymers,[11] poly(ethylene glycol)

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(PEG),[12] poly(β-caprolactone-*co*-D,L-lactide),[13] and S-carboxymethyl keratin[14] have been studied to improve the mechanical or thermal stability or membrane properties of silk films. However, there are no reports that the preparation of the cross-linked SF film using radiation-induced cross-links in order to obtain water-insoluble properties and increase physical properties, to our knowledge.

In the present study, we firstly prepared the cross-linked SF film via γ-irradiation to the casted SF film, which is fabricated from aqueous solution regenerated from fibers of cocoons in order to use as supports of human cell culture. The cross-linked SF film properties were evaluated by FT-IR spectroscopy, contact angle, solubility to water, thermal analysis, surface area analyzer, and morphology via scanning electron microscopy (SEM), respectively. In order to use as supports for human cell culture, the human fibroblast cell (CCD-986sk) and embryo kidney-ft cell were cultured using cross-linked SF film supports, which is prepared by varying experimental condition. From this results, we will discuss the growth effects of the human cell onto croos-linked SF film supports.

2. Experimental Section

2.1. Reagents

Cocoons of *Bombyx mori* silkworm were kindly supplied by Korea Atomic Energy Research Institute (Jeollabuk-do, Korea). LiBr, 2-hydroxyethyl methacrylate (HEMA), and 3-(acryloyloxy)-2-hydroxypropyl methacrylate as cross-linked agents were purchased from Sigma-Aldrich (Seoul, Korea). All other chemicals were used without any purification.

2.2. Preparation of SF film by γ-irradiation

Fig. 1 shows the preparation procedure of the cross-linked SF film including three processes such as degumming process, dissolving process, and manufacturing process with γ-irradiation. According to reference,[15] SF solution was prepared. Cocoons were boiled for 30 min in aqueous solution of 0.02 M Na_2CO_3 and then rinsed through with distilled water to extract the sericin protein. After drying the extracted SF was dissolved in 9.3 M LiBr solution at 60 for 4 hrs, yielding 20% (w/v) solutions. This solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (Pierce, molecular

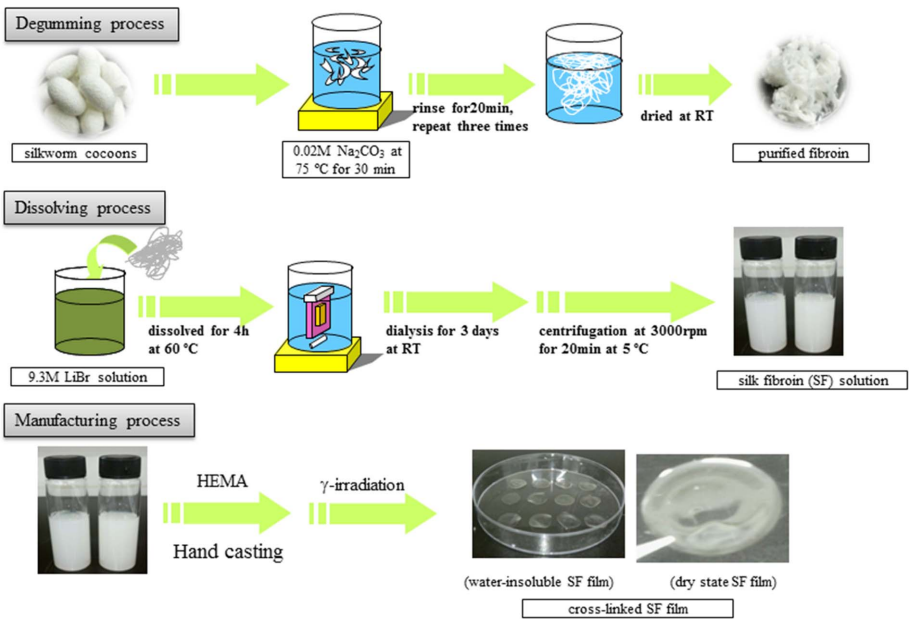

Fig. 1. Schematic preparation procedure of the cross-linked SF film from B*ombyx mori* silk cocoon.

Table 1. Preparation condition and water solubility, contactangle (°), and pore volume (cc/g) of the cross-linked SF film using γ-irradiation

Total irradiation dose	HEMA (0.00 wt.%)	HEMA (1.00 wt.%)	HEMA (3.00 wt.%)	HEMA (5.00 wt.%)
0 kGy	No. 1 S, 58.72°, 0.00128 cc/g	No. 2 S, 54.63°, 0.00138 cc/g	No. 3 S, 47.53°, 0.00134 cc/g	No. 4 S, 42.49°, 0.00129 cc/g
10 kGy	No. 5 S, 59.28°, 0.00138 cc/g	No. 6 S, 57.52°, 0.00136 cc/g	No. 7 I, 55.36°, 0.00129 cc/g	No. 6 I, 51.48°, 0.00128 cc/g
30 kGy	No. 9 S, 54.28°, 0.00135 cc/g	No. 10 S, 51.63°, 0.00129 cc/g	No. 11 I, 46.22°, 0.00131 cc/g	No. 12 I, 42.73°, 0.00136 cc/g
50 kGy	No. 13 S, 57.36°, 0.00130 cc/g	No. 14 S, 55.72°, 0.00139 cc/g	No. 15 I, 53.42°, 0.00138 cc/g	No. 16 I, 52.42°, 0.00136 cc/g

Water-soluble film and water-insoluble film was denoted as "S" and "T", respectively.

weight cut-off 35,000) for 3 days to remove salt. The solution was optically clear after dialysis and was centrifuged (at 3,000 rpm for 20 min) to remove the small amount of the silk aggregate that formed during the process. The final concentration of aqueous silk solution was ~7.5 wt.% determined by weighing the remaining solid after drying.

To prepare crossed-linked SF film, the cross-linked agents such as 2-hydroxyethyl methacrylate (HEMA) was added in SF solution as ratio of 1.00 wt.% (w/w, cross-linked agent/silk fibroin), 3.00 wt.%, and 5.00 wt.%, respectively. After then the solution was cast on polystyrene Petri dishes. The SF film with cross-linked agents generally dried within 12 hrs, which was formed water-soluble films at room temperature. In order to prepare water-insoluble film, the SF film was irradiated γ-ray from a Co-60 source at 1.0×10^4 Gy/h at atmospheric pressure and ambient temperature as wet state. *Table* 1 exhibits the cross-linked SF film prepared by varying experimental condition such as total irradiation dose and HEMA concentration as cross-linked agents.

2.3. Cell culture

Human fibroblast cell (CCD-986sk) and human embryonic kidney (HEK) 293T cell were washed 1 time with 2 mL 1X DPBS. The cell were added in 1 mL of 37 °C, 0.25% Trypsin-EDTA evenly coat flask surface containing the cell and Trypsinize for 2 min. The cell were cultured in 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% heat inactivated fetal bovine serum (FBS), 1% penicillin streptomycin. The cell number was determined using a hemacytometer. Cell grown in 37.5 cm^2 cell culture flasks were trypsinized, counted and plated at a density of 450,000 cell/cm^2. The cross-linked SF membrane was put onto the plate. The cell placed in the place flasks, and the cells were allowed to settle/adhere at 37 °C in an atmosphere of 5% CO^2.

2.4. Instrumentation

DSC (TA2910, Instrument , USA), Contact Angle (Phoenix 300, Surface electro optics, USA), FT-IR (Nicolet is 10, Thermo fisher scientific, USA), FE-SEM (S-4800, Hitachi science system, Japan).

3. Results and Discussion

Fig. 2 displays FT-IR spectra of the cross-linked SF film with HEMA prepared by γ-irradiation. The pure silk fibroin film (No. 1 in *Table* 1) has absorption bands at 1630 cm^{-1} (amide I), 1530 cm^{-1} (amide II) and 1265 cm^{-1} (amide III), which indicates both β-sheet and random conformation exist in the fibroin film.[16, 17] When fibroin was blended with HEMA, the absorptions bands was not shifted, this indicated that there is no change of the conformation by adding HEMA as cross-linked agents.

On the other hand, the characteristic peaks at 1725

cm^{-1} (>C=O stretching), 1483 cm^{-1} (CH_2 deformation), and 750 cm^{-1} (>C-O out of plane bending) were obtained from cross-linked SF film prepared by γ-irradiation. However, the characteristic amide bond peaks were not changed after γ-irradiation. As results, we could obtain the cross-linked SF film with no change β-sheet structure by γ-irradiation.

Fig. 3 shows the DSC curves for the cross-linked SF film prepared by varying total γ-irradiation dose. The SF film samples, which are prepared without HEMA as cross-linked agents, shows an endothermic peak at around 100 °C, a non-isothermal crystallization peaks at around 213 °C and degradation peak at 257 °C regardless of total irradiation dose. The endothermic peaks at around 100 °C were due to the evaporation of bound water and indicated SF film interacted with water. By adding HEMA as cross-linked agent, the crystallization peaks around was disappeared in 0 kGy total γ-irradiation dose because of the formation of β-sheet. However, there are no changes of structure of SF film when γ-ray irradiated to SF film.

Fig. 4 displays the contact angle image of the cross-linked SF film prepared by γ-irradiation. The contact angle (°) of the sampes was slightly decreased with increasing HEMA as cross-linked agents becasue of β-sheet structure. However, we could not find the regular change of the sample contact angle after γ-irradiation. The detailed contact angle for samples prepared by γ-irradiation was also listed in *Table* 1. As results, the β-sheet structure of the crosslinked SF film was not changed after γ-irradiation.

The SEM of pure fibroin film (No. 1 of *Fig.* 5) shows a smooth surface structure typical of homogeneous film with densely packed fibroin chains. In No. 3 sample, the surfaces were also smooth; the spot in the SF film surface was obserbed due to HEMA aggregation onto SF film surface. After γ-irradiation (No. 5, 6, 7, 8, 9, 13, 16), the typical of homogineous film with rough surface structure with low density fibroin chains was observed. This results

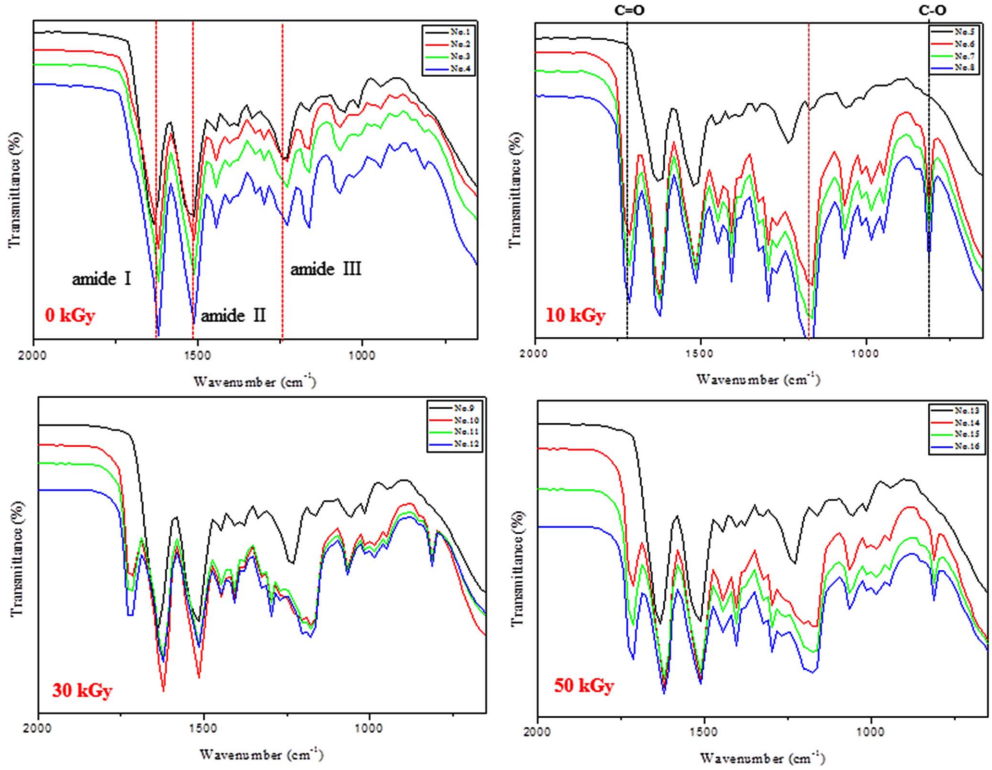

Fig. 2. FT-IR spectra of the cross-linked SF film prepared by γ-irradiation as function of total irradiation dose.

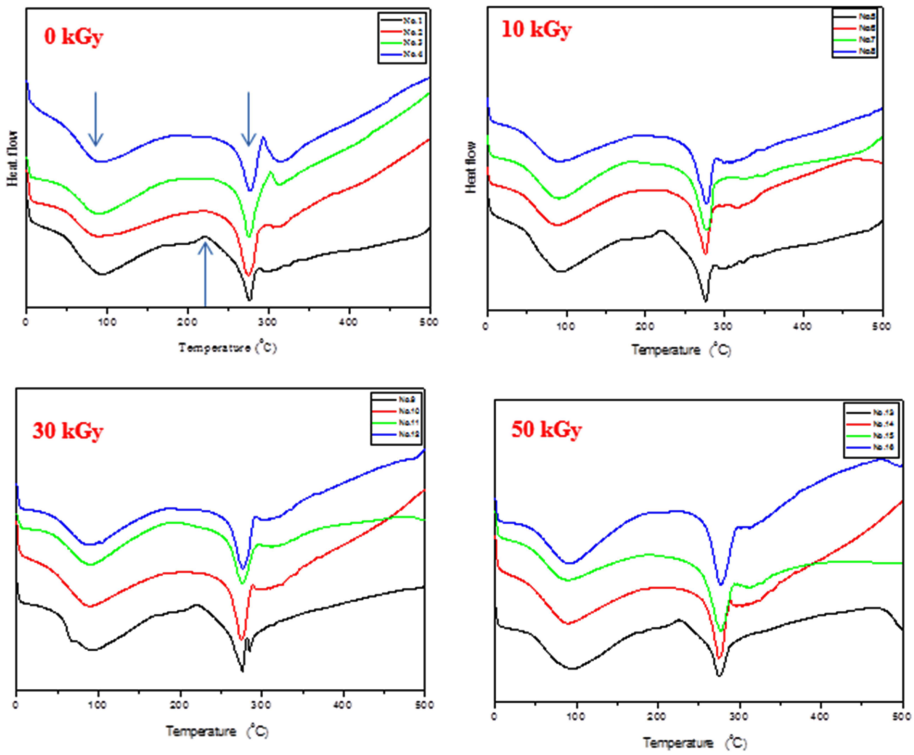

Fig. 3. DSC curves for the cross-linked SF film prepared by γ-irradiation as function of different total irradiation dose.

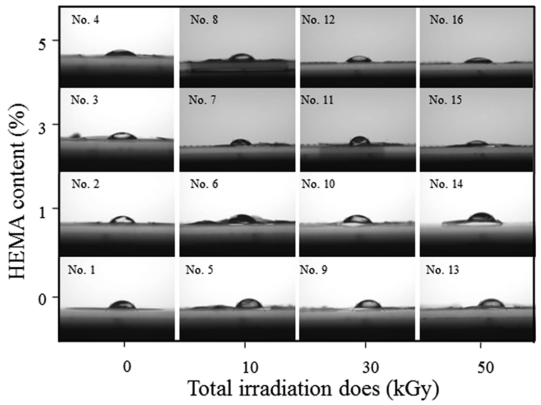

Fig. 4. Contact angel image of the cross-linked SF film prepared by γ-irradiation.

were expected the large surface area and pore volum. However, the pore volum of the prepared samples were in the range of 1.28×10^{-3}~1.39×10^{-3} cc/g as shown in *Table* 1.

The properties of water solubility, contact angle, and pore volum for the prepared cross-linked SF film using γ-irradiation are summarized in *Table* 1. The prepared samples (No. 1, 2, 3, 4, 5, 6, 9, 10, 13, 14) were dissolved to water, while the prepared cross-linked samples (No.7, 8, 11, 12, 16) have water-insoluble properties. These samples with water-insoluble properties could be used for application cell culture biomaterials.

Fig. 6 shows the SEM images (upper) of the the cross-linked sample surface of after cell culture [human fibroblast cell (CCD-986sk)] and OM images (down) of the cross-linked SF film surface after cell culture (human embryo kidney-ft cell). The cell culture was performed using only water-insoluble SF film (No. 7, 8, 11, 12, 15, 16 in *Table* 1). As shown in *Fig.* 6, human fibroblast cell (CCD-986sk) and embryo kidney-ft cell were well growed on the surface of cross-linked SF film supports prepared by γ-irradiation.

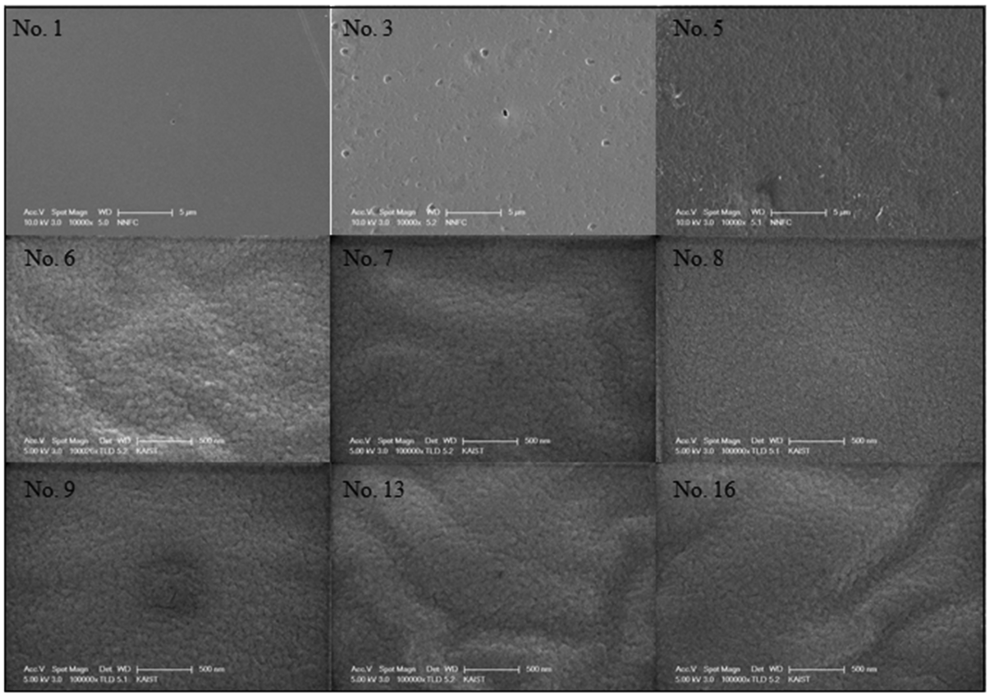

Fig. 5. SEM images of the cross-linked SF film from silkworm cocoons (see, condition in Table 1).

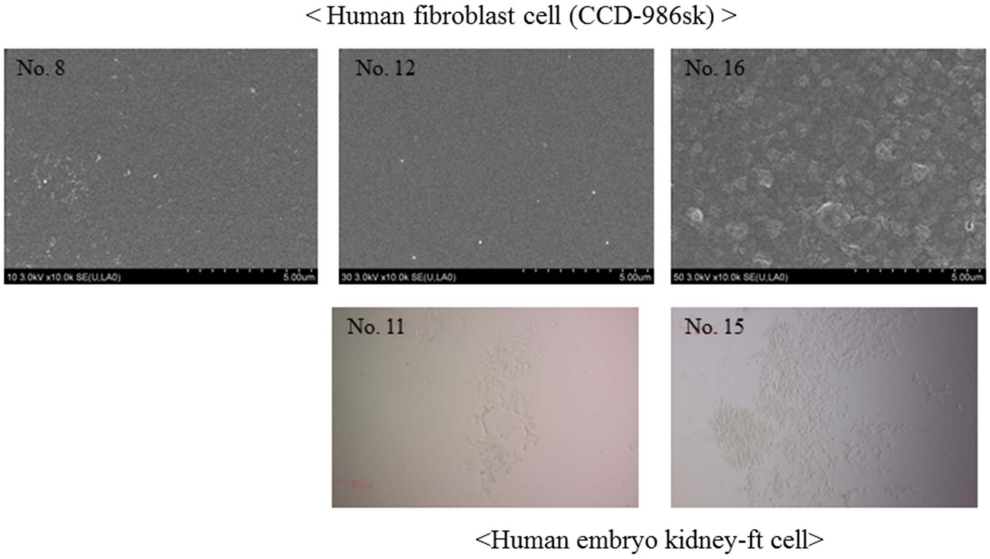

Fig. 6. SEM image (upper) of the surface of the cross-linked SF film after cell culture [human fibroblast cell (CCD-986sk)] and OM images (down) of the surface of the cross-linked SF film after cell culture [human embryo kidney-ft cell].

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