

Copolymer-Induced Silk-Based Hydrogel with Porous and Nanofibrous Structure

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ABSTRACT: A novel physical blend method was developed to accelerate the self-assembly process of silk fibroin (SF) solution into porous and nanofibrous hydrogel by temperature-sensitive copolymer. Silk-based hydrogel was firstly achieved through blending SF solution with copolymer aqueous solution and then removed the copolymer from blend solution by heat treatment (50°C) after 24 h hydrogelation. Copolymer molecules would interact with SF molecules resulting in reduction of copolymer micelles, which further affect the hydrogelation of SF solutions. Copolymers could be separated from blend solution by heat treatment under an acceptable temperature (50°C), especially the copolymer₂. Fourier transform infrared (FTIR) and X-ray diffraction showed the blending of copolymer significantly accelerated the self-assembly of SF into physically crosslinked β -sheet crystals at room temperature which led to the sol-gel transition. Results from DTA and X-ray diffraction showed that the effect of copolymer on crystalline structure of SF in silk-based hydrogel was very weak. SF molecules transformed from distributed globular nanoparticles to nanofilaments clustered during hydrogelation, resulting in the porous and nanofibrous structure of silk-based hydrogel. Furthermore, silk-based hydrogel was prepared in aqueous solution avoiding organic solvents and harsh processing conditions, suggesting that this silk-based hydrogel could be a potential candidate scaffold for biomedical applications. © 2012 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* 000: 000–000, 2012

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INTRODUCTION

Hydrogels are chemically or physically cross-linked, three-dimensional polymer networks that are durable to swelling in aqueous solutions but do not dissolve in these solutions.^{1–3} Hydrogels have been extensively explored for biomedical and pharmaceutical applications because of their outstanding biocompatibility, hydrophilicity, low adsorption of proteins, and special physicochemical properties.^{4–6} Generally, hydrogels formed from naturally derived polymers have the advantage of typically being biocompatible and biodegradable, whereas hydrogels formed from synthetic polymers showed that gelation and gel properties are more controllable and reproducible through the use of specific molecular weights, block structures, and modes of cross-linking.^{7–9} Among naturally derived biomaterials, silk fibroin (SF) hydrogels have been studied for many biomedical applications, such as bone-filling materials,¹⁰ sustained delivery systems¹¹ and three-dimensional cell culture carriers.¹²

Bombyx mori silk, a self-assembling natural protein of silkworm fibers, has been successfully used in the biomedical field as sutures for several decades.¹³ SF is the predominant component of silk fibers consisting of a heavy (~ 370 kDa) and a light (~ 26 kDa) chain linked by a single disulfide.¹⁴ SF is a hydrophobic structural protein dominated in composition by the amino acids glycine, alanine, and serine, which form antiparallel β -sheets in the spun fibers. In recent years, SF has been processed into various of material formats including films, electrospun fibers, three-dimensional porous scaffolds, microspheres and hydrogels for tissue engineering and cell/drug delivery applications because of its impressive biocompatibility, biodegradability, and mechanical properties.^{15–19} SF aqueous solutions undergo self-assembly into β -sheet structures and form hydrogels, with the rate of hydrogelation dependent on protein concentration, temperature, metal ions, and pH.²⁰

Among synthetic polymers, polylactide (PLA) has been investigated worldwide because of its good degradability and

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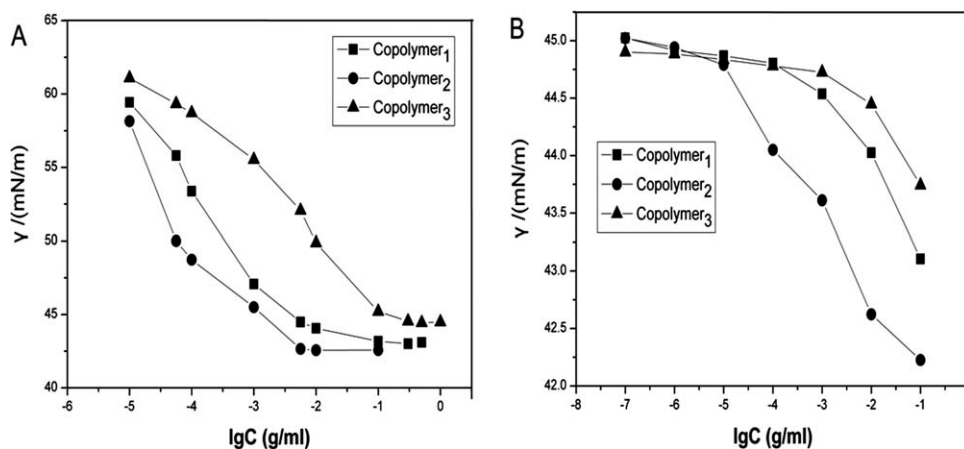


Figure 1. The static surface tension of copolymers in aqueous solution (A) and in 3 wt % SF solution (B) with different weight concentrations.

biocompatibility. But increasing attention is focused on its development of diblock copolymers such as PEG-PLA or PLA-PEG-PLA that have the sol-gel transition near body temperature.^{21,22} Compared with PLA, PLA/PEG copolymers exhibit enhanced hydrophilicity and degradation rate, reduced acidity of degradation products.²³ The copolymer aqueous solution can form micelles with PLA loops in the core and a PEG shell and groups of micelles because of bridging between micelles caused by the PLA blocks with raising temperature. The temperature-induced phase transition is influenced by polymer composition and polymeric molecular structure.^{24–26}

The goal of this study was to develop a new physical blend method to accelerate the self-assembly process of SF solution into porous and nanofibrous hydrogel that is similar to the nanofibrous structure of collagen in extracellular matrix (ECM). At the same time, the blended biomaterial should be easy to remove controllably in the process to get the silk-based hydrogel. Furthermore, the preparation of silk-based hydrogel should be under mild environmental conditions, avoiding organic solvents and harsh processing conditions, facilitated to load drugs or growth factors into process for biomedical applications.

EXPERIMENTAL

Material

L-Lactide from ESUN (China) was recrystallized from ethyl acetate. PEG with molar mass of 400 and 1000 was obtained from MERCK (Germany) and used as received. Stannous octoate from SCR (China) was dried under vacuum before use. Copolymers were synthesized by ring opening polymerization of L-lactide in the presence of dihydroxyl PEG using stannous octoate

as catalyst. Briefly, predetermined amounts of PEG and lactide were introduced into a polymerization tube. The initial molar ratio of ethylene oxide to lactate repeat units (EG/LA) was 3/1 or 1/1. Stannous octoate (0.3 wt %) was then added. After degassing, the tube was sealed under vacuum, and polymerization was allowed to proceed at 130°C. After 24 h, the product was recovered by dissolution in chloroform and precipitation in deionized water. Finally, the product was washed and dried under vacuum.²⁷ Copolymer₁, synthesized by PEG₄₀₀ with EG/LA ratio 1 : 1; Copolymer₂, synthesized by PEG₁₀₀₀ with EG/LA ratio 1 : 1; Copolymer₃, synthesized by PEG₁₀₀₀ with EG/LA ratio 3 : 1.

Raw silk fibers were degummed twice in 0.5% (w/w) NaHCO₃ solution at 100°C for 30 min and then rinsed thoroughly with deionized water to remove glue-like sericin proteins. The extracted silk was then dissolved in 9.3 M LiBr solution at 60°C, followed by dialysis with cellulose tubular membranes (molecular weight cut-off, 8,000–14,000) against distilled water for 3 days. The final concentration of the SF was 6 wt %. Lower concentration silk solutions were prepared by diluting the 6 wt % stock solution with deionized water.

The SF solution was blended with copolymer solution at 5°C using different contents of silk and copolymer by changing the volume ratio. The silk concentrations were kept at 3% whereas the copolymer concentrations were 1, 3, and 9% (SF/copolymer = 3 : 1, 1 : 1, and 1 : 3).

Characterization

The static surface tension of liquids was obtained using Dynamic contact angle meter and tensiometer (DCAT21, data-physics, Germany) at 25°C. Wilhelmy plate method was

Table I. The Concentration of Solutions Before and After Heat Treatment

Before/after heat treatment (%)	SF/copolymer = 3 : 1	SF/copolymer = 1 : 1	SF/copolymer = 1 : 3
SF	3.11/3.07		
SF/copolymer ₁	3.97/3.73	5.98/5.04	11.43/8.65
SF/copolymer ₂	4.05/3.12	6.02/4.35	11.6/6.52
SF/copolymer ₃	4.07/3.84	5.96/5.54	11.72/10.23

Values are average \pm standard deviation of a minimum of $N = 3$ samples for each group ($P < 0.05$).

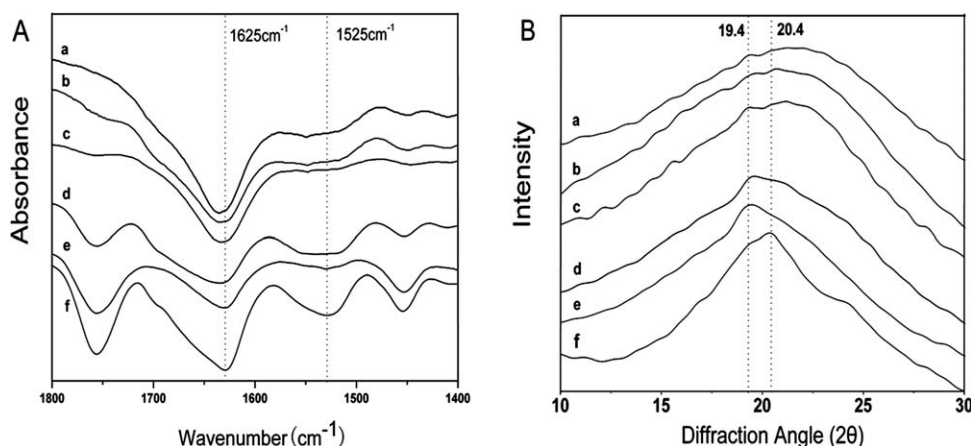


Figure 2. FTIR spectra (A), XRD data (B) of freeze-dried scaffolds derived from SF solution and treated SF solution with different hydrogelation times at room temperature before lyophilized: SF solutions placed in vials after 6 h (a), 12 h (b), and 24 h (c); SF/copolymer solution placed in vials after 6 h (d), 12 h (e) and 24 h (f), and then removed the copolymer by heat treatment.

operated using 50 mm sample pool, surface/interfacial tension measurement range from 1 to 1000 mN/m, accuracy of ± 0.001 mN/m, and the samples' volume was about 10 ml.

Fourier transform infrared (FTIR: Thermo Nicolet corporation, Madison, Wisconsin, U.S.) spectra was obtained using a Magna spectrometer (Nicolet 5700, Thermo Nicolet) in the spectral region of 400–4,000 cm^{-1} , the powdered gels were pressed into potassium bromide (KBr) pellets before data collection.

X-ray diffraction (X'PERT PRO MPD, PANalytical Company, Holland) was operated at 40 kV tube voltage and 40 mA tube current, CuK α radiation was used with diffraction angle $2\theta = 5\text{--}45^\circ$, the scanning rate is $2^\circ/\text{min}$ with powdered gels. Thermogravimetry/differential thermal analysis (TG-DTA, Perkin Elmer) conditions were nitrogen flux at 30 ml/min, heat rate at $10^\circ\text{C}/\text{min}$, temperature range from 50 to 600°C , and the samples' weight was about 5 mg.

The morphology of the structure changes in solutions was observed using atomic force microscope (AFM, Bruker, Germany). The solutions were diluted to below 0.01 wt %, then dripped 3 μL of the diluted solutions on mica surface in air-dry to get single layer.

The surface and cross-section images of the hydrogels were examined by Scanning electron microscope (SEM, Hitach S-480, Japan) at 20°C , 60 RH. Before SEM examination, hydrogels and solutions were directly placed at -20°C for about 24 h to freeze them and then lyophilized for about 72 h.

RESULTS AND DISCUSSION

The Static Surface Tension of Solutions

PLA-PEG-PLA copolymers have both hydrophobic groups (PLA) and hydrophilic groups (PEG) as surfactants, which can

Table II. Characteristic Absorption Peaks of Silk Fibroin

Scaffolds	(a)	(b)	(c)	(d)	(e)	(f)
Amide I (cm^{-1})	1635	1633	1632	1633	1629	1627
Amide II (cm^{-1})	1549	1548	1546	1538	1530	1525

reduce the surface tension of water and the interfacial tension between oil and water. As seen in Figure 1(A), the surface tension of aqueous solution decreased significantly with increasing the concentration of copolymers, especially copolymer₂, then remained relatively constant at last. The concentration at which surfactants begin to form micelle is known as the critical micelle concentration (CMC). The constant surface tension of aqueous solution means the concentration has reached the CMC of copolymer. The CMC of copolymers also reflected their ability to form micelles in water. Copolymer₂ formed micelles more easily than copolymer₁ and copolymer₃ in water because of its longer hydrophobic PLA chains. Compared with aqueous solution, the surface tension of SF solution also decreased with increasing the concentration of copolymers [Figure 1(B)], but no apparent CMC of copolymers was found in SF solution. These indicated that copolymer molecules would interact with SF molecules resulting in reduction of copolymer micelles. The lower surface tension would affect the hydrogelation of SF solution that occurs because of the formation of inter- and intramolecular interactions among the protein chains, including hydrophobic interactions and hydrogen bonds leading to β -sheet formation, resulting in physical cross-links.²⁰

Heat Treatment

The cloud point of a nonionic surfactant is the temperature where the mixture starts to phase separate and two phases appear, thus becoming cloudy. PLA-PEG-PLA copolymers also have the cloud point, which can undergo a temperature-induced gel-sol transition on heating or cooling of the aqueous solution.²⁸ As is known, PLA-PEG-PLA copolymers form micelles with a core of PLA loops and a shell of PEG. After increasing the temperature, the number of micelles and bridging micelle groups increases, leading to aggregation and sol-gel transition. The aggregation and packing interaction between micelles increases to form a stable gel when the temperature is raised further. If the temperature is raised further, the over-shrunk micelle groups will precipitate in water. Table I showed the concentration of solutions before and after heat treatment. After 24 h hydrogelation at room temperature (25°C), blend solutions

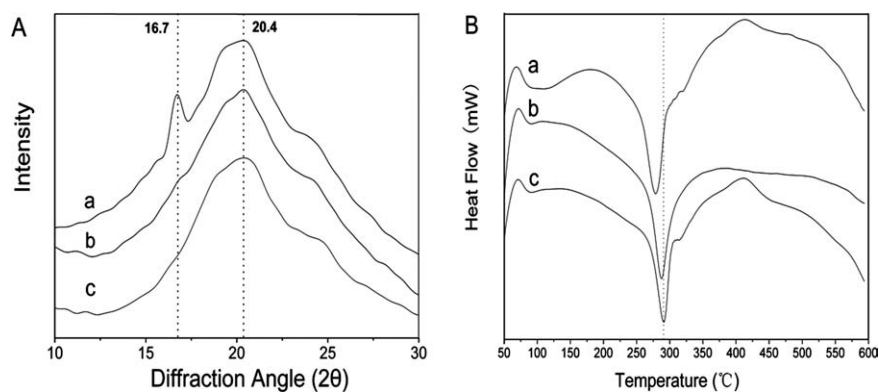


Figure 3. XRD data (A) and DTA curves (B) of freeze-dried hydrogels derived from blend solutions without heat treatment (a), with heat treatment (b), and SF solution (c).

directly placed in vials keeping the temperature at 50°C for 30 min to allow the establishment of equilibrium, and then filtered out the copolymer precipitates at bottom of vials. The concentration of SF solutions had essentially unchanged after heat treatment, whereas all the concentration of blend solutions decreased significantly on the contrary. These results indicated that PLA-PEG-PLA copolymers could be separated from blend solution by heat treatment under an acceptable temperature (50°C), especially the copolymer₂. From the surface tension analysis, we known that copolymer₂ formed micelles more easily because of more hydrophobic. In consideration of the results from the surface tension measurement and heat treatment, we chose the SF/Copolymer₂ solution with the ratio of 3 : 1 in following experiments.

Structural Analysis

Figure 2(A) and Table II show the FTIR spectra of freeze-dried scaffolds derived from SF solution and blend solution with different hydrogelation times. SF solutions placed in vials showed

minor changes during 24 h, which was mainly composed of random and α -helix structures. Treated solutions showed significantly lower peaks at amide I and amide II bands with increasing hydrogelation times, suggesting an increase of β -sheet content.²⁹ Moreover, treated solutions after 24 h hydrogelation showed apparent gel-like behavior, that peaks at amide I and amide II bands also similar to that of SF hydrogel. It illustrated that the blending of copolymer significantly accelerated the self-assembly of SF into physically crosslinked β -sheet crystals. The structural changes of SF during hydrogelation were confirmed by XRD results [Figure 2(B)]. The SF solutions during 24 h hydrogelation all showed an amorphous state, characterized by the presence of a broad peak in the 2θ scattering angle range from 10 to 30°. Compared with SF solutions, treated solutions after 6 h and 12 h hydrogelation mainly showed diffraction peaks at 19.5° (Silk I). When after 24 h hydrogelation, treated solutions showed diffraction peaks at 20.4° (Silk II) and 24.6° (Silk I). The structural transformation from Silk I to Silk II was found during hydrogelation. These results also indicated that

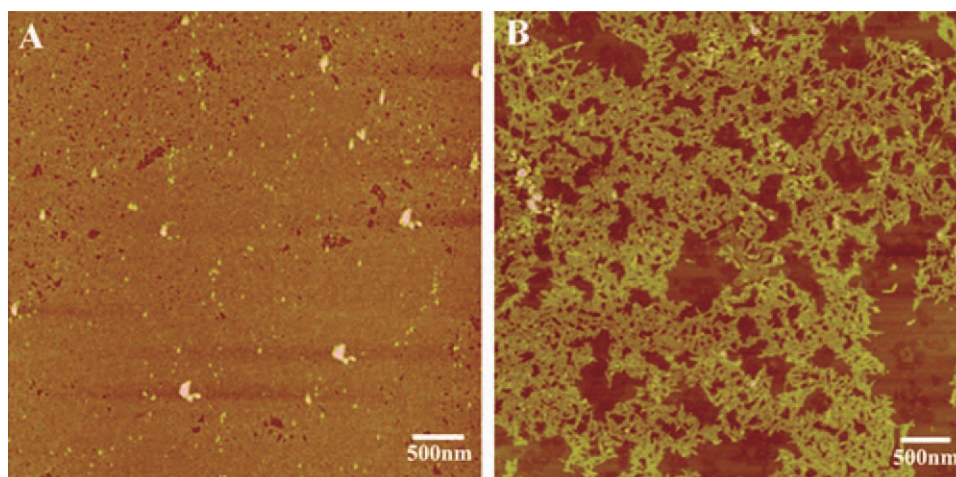


Figure 4. AFM images of the structure changes in solutions after overnight hydrogelation: Pure SF solution (A) and blend solution with heat treatment (B) diluted to below 0.01 wt % to get single layer on mica surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

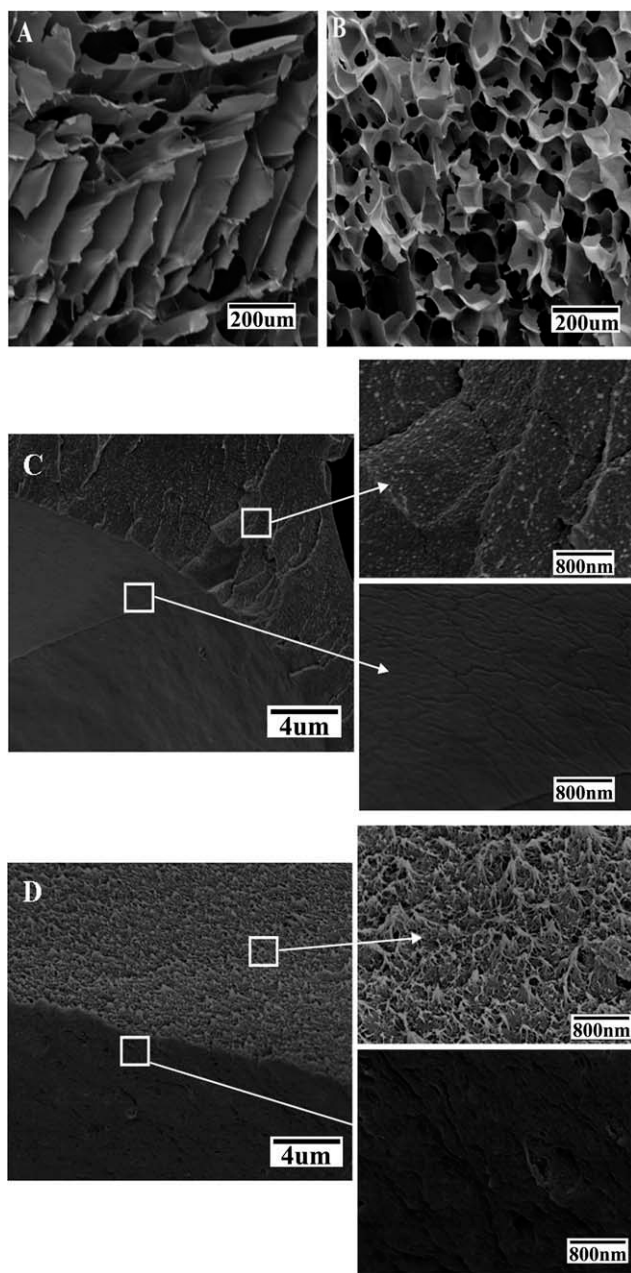


Figure 5. SEM images of SF hydrogel (A) and silk-based hydrogel (B) after lyophilized; surface and cross-section images of macropore wall of SF hydrogel (C) and silk-based hydrogel (D).

the blending of copolymer accelerated the structural transformation of SF, which led to the sol-gel transition.

X-ray diffraction was performed to study the crystalline structure changes of hydrogels [Figure 3(A)]. SF hydrogel showed diffraction peak at 19.5° (Silk I), 20.4° (Silk I), and 24.6° (Silk II), whereas SF/copolymer hydrogel showed a different diffraction peaks at 16.7° (copolymer), indicating that the copolymer and SF were only blended but phase separation. With heat treatment, the diffraction peak at 2θ values of 16.7° was disappeared, suggesting that almost all of copolymer had been removed. This also indicated that the silk-based hydrogel was

mainly composed of Silk I and Silk II structure, especially the Silk I structure, as SF hydrogel.³⁰ The DTA results confirmed that the effect of copolymer on crystalline structure of SF had been weakened [Figure 3(B)]. SF/copolymer hydrogel had the degradation peak at 278°C , whereas SF hydrogel had the degradation peak at 290°C , demonstrating that the copolymer had affected the crystal structure of SF. With heat treatment, the degradation peaks of silk-based hydrogel increased to 287°C , illustrating that the effect on crystalline structure of SF had been weakened. These results also confirmed that most of copolymer had been removed from blend solution by heat treatment.

Morphology

The structural changes in solution during hydrogelation were observed by AFM (Figure 4). SF solution showed many globular nanoparticles distributed uniformly on mica surface after overnight hydrogelation. When blend solution removed the copolymer by heat treatment, it showed many nanofilaments clustered single layer on mica surface. These different changes in solution would affect the hydrogelation and the final structure of hydrogel.

Morphological features of hydrogels were observed by SEM after lyophilized. The hydrogel from pure SF solution formed leaf-like lamellar structures rather than porous structures [Figure 5(A)], whereas silk-based hydrogel showed porous structures [Figure 5(B)], indicating that blending copolymer lower the lamellar formation of SF during hydrogelation. In particular, besides the improvement of porous structure, the blending of copolymer also induced the formation of nanofilaments. As seen in Figure 5(C), no specific nanostructure was found in the macropore walls of SF hydrogel. But the nanofibrous structures with diameters of 20–100 nm were formed in the macropore walls of the silk-based hydrogel [Figure 5(D)], and part of nanofilaments inside the walls appeared on the surface. From the structural analysis, we know that the copolymer would accelerated the secondary structure transition of SF from non-crystalline structures (random coil, α -helix) to crystal structures (Silk I and Silk II). Consistent with AFM results, the early formation of nanofilaments in solution correlated with the nanofibrous structures in the hydrogel are likely influenced by silk secondary structural changes.³¹ The copolymer could induce the self-assembly of SF to nanofilament, which would further restrain the formation of lamellar structures in the hydrogelation.³²

CONCLUSION

A novel physical blend method was developed to accelerate the self-assembly process of SF solution into porous and nanofibrous hydrogel by temperature-sensitive copolymer. Silk-based hydrogel was first achieved through blending SF solution with temperature-sensitive copolymer aqueous solution, and then removed the copolymer from blend solutions by heat treatment (50°C) after 24 h hydrogelation. The copolymer could be removed easily in the hydrogelation and its effect on crystalline structure of SF in silk-based hydrogel was very weak. In particular, the self-assembly of SF solution into porous and

nanofibrous hydrogel induced by copolymer provided a new strategy to mimic the nanofibrous structure of collagen in extracellular matrix (ECM). Furthermore, silk-based hydrogel was prepared in aqueous solution avoiding organic solvents or harsh processing conditions, facilitated to load drugs or growth factors into process for biomedical applications.

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