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Novel two-step method to form silk fibroin fibrous hydrogel

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ABSTRACT

Hydrogels prepared by silk fibroin solution have been studied. However, mimicking the nanofibrous structures of extracellular matrix for fabricating biomaterials remains a challenge. Here, a novel two-step method was applied to prepare fibrous hydrogels using regenerated silk fibroin solution containing nanofibrils in a range of tens to hundreds of nanometers. When the gelation process of silk solution occurred, it showed a top-down type gel within 30 min. After gelation, silk fibroin fibrous hydrogels exhibited nanofiber network morphology with β-sheet structure. Moreover, the compressive stress and modulus of fibrous hydrogels were 31.9 ± 2.6 and 2.8 ± 0.8 kPa, respectively, which was formed using 2.0 wt.% concentration solutions. In addition, fibrous hydrogels supported BMSCs attachment and proliferation over 12 days. This study provides important insight in the in vitro processing of silk fibroin into useful new materials.

1. Introduction

Hydrogels are insoluble three-dimensional polymer chain networks that swell in aqueous solutions and that can hold or entrap liquid components [1,2,3]. Due to their high water contents, hydrogels possess excellent biocompatibility [4,5,6]. The amount of water in the equilibrium swollen state is a balance between the osmotic force and the entropic retroactive force in the hydrogel networks [7]. The network structures of hydrogels are cross-linked by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces [7,8,9]. The cross-link types of hydrogels are often classified to chemical and physical [10,11]. These chemical cross-links can be formed by disulfide formation, polymerization, or other chemical reactions [12]. In contrast, physically cross-linked hydrogels avoid these chemical reactions. Ionic interactions, chain entanglements, association bonds including hydrogen bonds or strong van der Waals interactions between chains, or even topological cross-linking, etc. can be used to form the cross-linker regions in these hydrogels [13]. Therefore, physically cross-linked hydrogels are attractively researched, because toxic reactive molecules are evitable in the cross-linking process [12].

Silk fibroin (SF) from *Bombyx mori* (*B. mori*) was investigated by researchers as one of the promising resources of biotechnology and biomedical materials [14,15]. One important material option for biomaterials is the formation of hydrogels. SF aqueous solutions form hydrogels directly through self-assembly, with the rate of sol–gel transition dependent on protein concentration, temperature, metal ions, and pH [16,17,18]. The mechanism of gelation is the self-assembly of protein chains into physically crosslinked β-sheet crystals [19,20].

Dissolution is a necessary and key step to form hydrogels with different features, such as different materials shapes and pore size, controllable degradation rates, and rich β-sheet [21,22]. In previous studies, degummed silk fibers have been successfully dissolved in several solvents, such as inorganic salts (LiBr solution and $CaCl_2$/ethanol/H_2O solution), environmental friendly N-methyl morpholine N-oxide (NMMO), acid solution (phosphoric acid/formic acid mixture), and ionic liquids [23,24,25]. These dissolved methods share a common feature of silk dissolution at the molecular level [26]. SF aqueous solution prepared above dissolution methods needs much more time for gelation without external intervention at room temperature, showing changes from clear to turbid solutions. The gelation is irreversible, since the hydrogen bonds are too strong to be broken at mild conditions [18]. After lyophilization, silk hydrogels mainly exhibit leaflike and/or porous structure. However, in order to prepare flexible fibrous hydrogels, a two-step method was introduced that dissolves silk at room temperature. The solution obtained from the two-step method is easily processed into fibrous hydrogels.

In this present study, we developed a facile and effective method to prepare silk fibrous hydrogels, and also investigate SF sol–gel transition processes in detail. The effects of physical parameters such as fibroin concentration, temperature, and metal ions on gelation and structure were determined. Furthermore, cell proliferation and viability of bone marrow mesenchymal stem cells (BMSCs) on the silk fibrous hydrogels were characterized.

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2. Experimental

2.1. Preparation of SF aqueous solution

B. mori SF aqueous solution was prepared by a two-step method according to the following procedures. Silk fibers were boiled in 0.05 wt.% Na_2CO_3 solution for 30 min and then rinsed thoroughly with deionized water to extract the glue-like sericin proteins. Each step was repeated twice. Degummed silk was then dissolved in formic acid/$CaCl_2$ (FA/$CaCl_2$) solvent at room temperature. This solution (SF–FA–$CaCl_2$) was used for characterization by SEM/AFM and for film formation (Fig. S1 & Fig. S2). The formed films were redissolved in 9.3 M LiBr solution at room temperature for 4 h, yielding a 10% (w/v) solution. This solution was dialyzed against distilled water using Slide-A-Lyzer dialysis cassettes (Sigma, USA, molecular weight cut-off 3500) for 72 h to remove salt ions. The final concentration of aqueous silk solution was ~1.0 wt.%, determined by weighing the remaining solid after drying. This preparation process was a two-step dissolution method, and its obtained aqueous solution was named SF-F solution, storing at 5 °C for hydrogel formation.

In addition, SF-L solution was prepared according to the published procedures [27,28]. Degummed silk was dissolved in 9.3 M LiBr solution at 70 °C for 4 h, yielding a 10 g dL^{-1} solution. After dialyzing, the aqueous silk solution was obtained with ~1.0 wt.% concentration. This solution was used to form hydrogel as control.

2.2. Preparation of SF fibrous hydrogels

SF fibrous hydrogels were prepared according to the following procedures. A total of 1 mL of SF solution was placed in 2.5 mL flat-bottomed vials (diameter 10 mm). The vials were sealed and kept at 37 °C. After gelation, the sample had developed an opaque white color and did not fall from an inverted vial within 30 s. And then, hydrogels were frozen at −20 °C and freeze-dried for characterization.

2.3. Morphology analysis of hydrogels

The morphology of SF in acid solution and aqueous solution was observed by AFM (Veeco, CA) in air. A 225 μm long silicon cantilever with a spring constant of 3 N m^{-1} was used in tapping mode. For clear observation, solutions (SF–FA–$CaCl_2$ solution and SF aqueous solution) were diluted to 1.0×10^6 to get dispersed silk fibrils on silica plates. Once diluted, 1 μL diluted SF solution was quickly dropped onto fleshly silica surface and dried under nitrogen gas. The morphology of degummed silk, regenerated SF solution, and SF hydrogels were observed by using SEM (Hitachi S4800, Japan). Samples were sputter coated with gold prior to imaging. For SF solution observation, diluted solution was dropped 1 μL on silica surface and dried under nitrogen gas. In addition, based on SEM images the average diameter of fibrous hydrogel was determined by measuring 50 randomly selected nanofibers using Image J 1.44p software [29].

2.4. Structural analysis of hydrogels

FTIR spectra were obtained using Nicolet5700 spectrometer (Thermo Nicolet Company, USA) in the wavenumber region of 400–4000 cm^{-1}. SF hydrogels were pressed into potassium bromide (KBr) pellets prior to data collection. The structure of various hydrogels was also examined by XRD on X Pert-Pro MPD diffractometer (PANalytical, Netherlands) with CuKα radiation, operating at 40 kV and 40 mA. Diffraction intensity was measured in reflection mode at a scanning rate of 1.0° min^{-1} for 5–45°. Fourier self-deconvolution of the infrared spectra for amide I region (1600–1700 cm^{-1}) was performed by Opus 5.0 software to identify silk secondary structures. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. Fourier self-deconvolution spectra were curve-fitted to measure the relative areas of the amide I region components [30].

2.5. Circular dichroism (CD)

CD spectra were used to determine the nanostructural transition of silk solution. Spectra were recorded with a 0.1 mm path length, quartz sample cell at concentrations of 1.0 mg mL^{-1} on a Jasco-815 CD spectrophotometer (Jasco Co., Japan) equipped with a peltier temperature controller [31]. All samples were scanned ranging from 250 to 190 nm at a scanning speed of 100 nm min^{-1} at 25 °C. CD results represented the average of three measurements and were smoothed.

2.6. Porosity

The porosity of SF hydrogels was measured by liquid displacement [32]. Hexane was used as the displacement liquid since it was a nonsolvent for SF and was able to easily permeate through SF hydrogels without swelling or shrinking the matrix. SF hydrogels scaffolds (dry weight, W) were immersed in a known volume (V_1) of hexane in a graduated cylinder for approximately 5 min. The total volume of hexane and the hexane-impregnated scaffold was recorded as V_2. The hexane-impregnated scaffold was then removed from the cylinder and the residual hexane volume was recorded as V_3. The total volume of the hydrogel scaffold was

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3,$$

V_2-V_1 was the volume of the SF hydrogel scaffold and V_1-V_3 was the volume of hexane within the scaffold. The porosity of the hydrogel scaffold (E) was obtained by

$$E(\%) = (V_1 - V_3)/(V_2 - V_3) \times 100.$$

2.7. Mechanical properties

The compression properties of hydrogel specimens ($d = 16$ mm, $h = 15$ mm) in dry condition before sterilization by gamma rays were measured with a cross head speed of 2 mm min^{-1} at standard conditions (20 °C, 65% RH) using an Instron 3365 electronic strength tester (Boston, USA). All samples were measured in triplicates.

2.8. In vitro enzymatic degradation

SF hydrogels were incubated at 37 °C in 50 mL of phosphate-buffered saline (PBS, Hyclone) containing protease XIV (5 U mL^{-1}, Sigma-Aldrich). Each solution contains an approximately equivalent mass (50 ± 5 mg) of hydrogel samples. Solutions were replenished with enzyme and samples were collected daily. At designated time points the samples were rinsed with distilled water and prepared for mass balance assessment [33].

2.9. Cell culture

BMSCs were used to evaluate the cytocompatibility of SF fibrous hydrogels. Samples of BMSCs were obtained from the Second Affiliated Hospital of Soochow University (Suzhou, China). Firstly, for in vitro testing, two male Sprague-Dawley (SD) rats, two months old, were purchased and housed at the center for experimental animals, Suzhou University. All animal related experimental protocols have been approved by the institutional animal care and use committee of Suzhou University. Unless otherwise stated. Secondly, BMSCs were isolated by rapid plastic adhesion of rats bone marrow-derived cells. Cells were cultured in Dulbecco's modified eagle medium/F-12 (DMEM/F-12, Hyclone) supplemented with 10% fetal bovine serum (FBS) (Invitrogen,