Investigation on the Structure and Mechanical Properties of Highly Tunable Elastomeric Silk Fibroin Hydrogels Cross-Linked by γ-Ray Radiation

Nier Wu, Huilei Yu, Muyang Sun, Zong Li, Fengyuan Zhao, Yingfang Ao, and Haifeng Chen*

ABSTRACT: Silk fibroin (SF) is a natural polymer with low immunogenicity and good biocompatibility. However, most silk-based hydrogels formed through chemical or physical cross-linking are brittle, the preparation of which also inevitably introduces cytotoxic cross-linking agents. Herein, a simple strategy is presented for synthesizing SF hydrogels with tunable mechanical properties by combining γ-ray radiation with ethanol treatment. Neither toxic initiators nor chemical agents are utilized during the whole preparation procedure. For “soft” hydrogels, the compressive moduli are less than 29 kPa (SF-S hydrogels), while for “tough” hydrogels, the compressive moduli are between 1.21 and 2.41 MPa (SF-D hydrogels). Specifically, γ-ray radiation makes SF form uniform and stable chemical cross-linking sites within and between molecular chains, resulting in “soft” and highly elastic SF hydrogels. The physical cross-linking via ethanol treatment leads to the self-assembly of fibroin chains, transforming those soft hydrogels to tough hydrogels. These double cross-linked SF hydrogels (SF-D hydrogels) exhibit excellent mechanical strength. Effects of various cross-linking conditions on the secondary structure, pore structure, mechanical properties, gelation degree, swelling, and in vitro degradation properties are explored. A series of cell experiments demonstrate that the SF hydrogels with different mechanical strength can stimulate the expression of specific genes of rat bone marrow mesenchymal stem cells (BMSCs) in various differentiation directions. These results also show the application prospects in tissue engineering by customizing hydrogels for the mechanical strength of different tissues.

KEYWORDS: silk fibroin, γ irradiation, double networks, hydrogel, mechanical properties

INTRODUCTION

Hydrogels have high water retention and swelling properties, which are close to living tissues. Besides, hydrogels possess adjustable mechanical properties, which is important for biomaterials to match the resilience and elasticity of the native tissue extracellular matrix (ECM). Recent studies have demonstrated that the mechanical properties of hydrogels would affect the maintenance of cell phenotype and differentiation of stem cells; e.g., neurogenic markers were detected most on the gel near the brain tissue modulus (0.1−1 kPa), and myogenic markers were detected on the gel near the muscle tissue modulus (8−17 kPa). In the case of musculoskeletal tissue engineering, such as meniscus, tendon, and ligament repair, the materials with appropriate stability and load-bearing capacity are also required. At present, natural polymer hydrogels based on alginate, hyaluronic acid, chitosan, and collagen have been widely used in tissue engineering. However, the options of natural polymer hydrogels with a wide range of tunable mechanical properties are rather limited. In comparison, silk fibroin (SF) provides an important material choice for tissue engineering scaffolds and regenerative medical materials due to its excellent biocompatibility and impressive mechanical property. Regenerated Bombyx mori SF is a natural fibrous structural protein with no obvious physiological activity. With its excellent low immunogenicity, good biocompatibility, controllable biodegradability, unique mechanical properties, and easy preparation, SF has been regarded as one of the important biomaterials. Its safety and effectiveness in tissue engineering applications have also been confirmed by a large number of studies, such as SF-based engineering catheter, ligament reconstruction, and so on.

SF-based hydrogels have good swelling ability, pore structure, and slow degradation performance, the properties of which are similar to the extracellular matrix (ECM). For the preparation of physically cross-linked SF hydrogels, a general strategy is to induce the transformation of fibroin chains from the amorphous state to β-sheet secondary structure. This thermodynamic stable structure is regarded as a kind of physical cross-linker. Increasing the concentration...
of SF or adding methanol for dehydration can induce SF molecular chains to self-assemble. Adding external energy is also able to accelerate the gelation of SF by driving the molecular chains’ migration, such as ultrasonication, increasing temperature, and electric field. In addition, SF can also form gels under the situation of low pH or the presence of Ca2+ in the solution. Compared with chemically cross-linked SF hydrogels, the physical cross-linking methods are easy to operate without adding any cross-linking agents. However, the mechanical performances of physical cross-linking SF hydrogels mentioned above are rather limited. Although these random β-sheet crystal structures contribute to the long-term thermodynamic stability, they inevitably brings brittleness, which is not conducive to the maintenance of mechanical strength in vivo.

Chemical cross-linking treatment is considered a feasible way to overcome the brittleness of physically cross-linked silk hydrogels. However, there are few chemical cross-linking methods to choose due to the low utilization efficiency of chemical groups, such as amidogen, carboxyl, or hydroxyl in SF molecular chains. In order to obtain regenerated SF chemically cross-linked hydrogels, chemical cross-linking agents are usually needed, such as genipin and glutaraldehyde. In fact, genipin is a natural cross-linking agent derived from Gardenia jasminoides, which has lower cytotoxicity than glutaraldehyde. However, the cross-linking of genipin mainly depends on lysine and arginine. The account of these amino acids is only about 1.2% of the total amino acid composition in SF, which results in a low gel reaction efficiency. It has been reported that enzymatically cross-linked SF hydrogel can obtain high elasticity hydrogel by utilizing horseradish peroxidase and hydrogen peroxide to cross-link the tyrosine (about 5% in SF molecules), but its loading capacity is too weak. On this basis, Su D et al. introduced additional ethanol treatment to increase the overall mechanical strength and improved the insufficient thermodynamic stability. However, almost all of these chemical cross-linking systems exist with common problems, which are the low reaction efficiency and introduction of bio-unfriendly chemical components.

The biofriendly approaches of preparing tissue engineering scaffolds by energy excitation rather than by introducing extra toxic chemical cross-linkers have attracted more and more attention, such as ultraviolet (UV), electron beam (EB), and γ-ray. In comparison, UV irradiation can hardly reach the deeper part of the materials due to the limitation of light’s penetration depth and will result in an uneven cross-linking network. Besides, the extra photoinitiators are needed to induce the reaction. High-energy EB is an effective energy source for radiation curing. However, for thicker samples, the energy penetration ability is limited as well.

On the contrary, the γ-ray radiation method has specific advantages. The γ-ray can achieve the energy uniform transmission because of the high-intensity penetrating ability. High-energy γ-ray can trigger the production of free radicals between polymer chains, thereby forming a uniform three-dimensional network. The cross-linking and sterilization of the polymer are realized simultaneously without introducing any chemical cross-linkers and initiators. Specifically, γ-ray irradiation promotes the production of hydroxyl radicals in the polymer solution, which attack the polymer chain to generate new free radicals. These free radicals react with each other to form covalent bonds, which causes polymer chains to entangle and finally form hydrogels. Natural polymer hydrogels, such as collagen, chitosan, and alginate, have been prepared by γ-ray radiation. Min Hee Kim et al. successfully prepared SF hydrogel by means of γ-ray radiation, but the mechanical properties are not satisfactory.

Based on the above studies, this research proposed a simple scheme for preparing SF hydrogels with tunable mechanical strength. By introducing ethanol into the gel system, physical networks were constructed on the basis of γ-ray radiation cross-linking, which led to form a “double-network” hydrogel. The mechanical strengths, pore structures, swelling properties, and in vitro degradation time of SF-S hydrogels and SF-D hydrogels were explored in detail. Furthermore, the thermal stability and biocompatibility of SF hydrogels were also evaluated. These tunable protein elastomers may provide new possibilities for meeting the needs of functional biomaterials in regenerative medicine.

### EXPERIMENTAL SECTION

The purchased *Bombyx mori* silk cocoon was from Shandong (China). Sodium carbonate anhydrous (≥99.8%), lithium bromide (99%), and poly(ethylene glycol) (MW: 8000, PEG) were purchased from Aladdin. Anhydrous ethanol (AR) and hexane (AR) were from college of chemistry of Peking University (Beijing, China). Protease XIV from *Streptomyces griseus* (3.5 units/mg solid, powder) and phosphate buffer solution (PBS, 1.0 M, pH 7.4) were purchased from Sigma-Aldrich. Dialysis cellulose membranes (cut off 15 000 Da) were bought from Solarbio Science & Technology Co., Ltd. (Beijing, China). RGD peptides (Gly-Arg-Gly-Asp-Ser-Pro-Cys, GRGDSPC) were supplied by Jier Biochemical Co., Ltd. (Shanghai, China). The water used in all experiments was deionized by a Millipore purification unit (resistivity >18.2 MΩ cm). All the agents were used without any further purification.

**Preparation of SF Solution.** SF solution was prepared according to the established method. Briefly, a *Bombyx mori* cocoon (10 g) was cut into small pieces and boiled for 30 min in 0.02 M sodium carbonate solution to remove the sericin. The degummed cocoon was rinsed twice with deionized water for 10 min and dried overnight in the fume hood. The dried silk fiber was dissolved in 9.3 M lithium bromide solution for 4 h at 60 °C. After that, the yellow viscous SF solution was dialyzed against deionized water for 3 days to remove the salt. The dialyzed solution was centrifuged twice at 9500 rpm for 20 min at 4 °C to remove impurities. The concentration of the final SF solution was 6.9 wt %, which was then concentrated by reverse dialysis against 20% PEG (MW: 8000) aqueous solution at room temperature to reach a higher concentration of about 8.5 wt %.

**Preparation of the SF Hydrogels.** A 5 mL portion of SF solution (8.5 wt %) was put into a 60 mm diameter plastic dish and then encapsulated. The SF-S hydrogels were obtained by γ-ray radiation using a 60Co radiation facility according to the different total doses (45, 75, 105, 135, and 165 kGy) at the same dose rate (196 Gy/min) at room temperature. The γ-ray radiation times were, respectively, 230 min (45 kGy), 383 min (75 kGy), 536 min (105 kGy), 689 min (135 kGy), and 842 min (165 kGy). To further obtain SF-D hydrogels, the SF-S hydrogels were immersed in anhydrous ethanol for 24 h and then repeatedly soaked in deionized water for 30 min 5 times to displace the ethanol in the hydrogel. As a control, the same volume of anhydrous ethanol was directly added to SF solution for 24 h to obtain the hydrogel, namely, SF-E hydrogel.

**Fourier Transform Infrared Spectrum (FTIR).** The samples of SF-S/D hydrogels obtained from different radiation total doses (45, 105, and 165 kGy), SF solution, and SF-E hydrogel were treated with liquid nitrogen, separately, and then freeze-dried for 48 h. The secondary structures of the lyophilized samples were characterized by FTIR (Affinity-1const, SHIMADZU) via the potassium bromide pellet pressing method. The spectra were recorded from 4000 to 400 cm−1, and the resolution was 4 cm−1. The scanning frequency was 5 times. Fourier self-deconvolution (FSD) of the spectra of amide I bands was performed using Origin 9 (OriginLab Corporation, Northampton, MA).
MA). The numbers and positions of peaks were determined in the results of the second derivative spectra and were fixed during the deconvolution process. The proportions of β-sheet, β-turn, and random coil structures were determined by the integral of the Gauss distribution, and the band shape and bandwidth were automatically adjusted by software. The secondary structure content of SF was calculated from the areas of the individual assigned bands and their fraction of the total area in the amide I region.

X-ray Diffraction (XRD). The lyophilized SF hydrogel samples (135 kGy-SF-S/D, SF-E, SF-aq) were examined by XRD (Rigaku, D/max), using Cu Kα radiation (λ = 1.5405 Å) with a Gobel mirror setup at 100 mA and 40 kV. The data were collected from 5° to 70° and were analyzed by Origin 9 software.

CHARACTERISTICS OF PORE MORPHOLOGY AND STRUCTURE

The surface and cross-section pore morphology of lyophilized SF hydrogels was obtained by scanning electron microscopy (SEM, FEI NanoSEM 430) in high vacuum mode and at 15 kV accelerating voltage. For higher resolution in the SEM image, different SF samples (45 kGy-SF-D, 135 kGy-SF-D, 165 kGy-SF-D, and SF-E hydrogel) were gold-coated via a Gatan model 691PIPS instrument (Gatan).

The porosity of different SF samples (45 kGy-SF-D, 135 kGy-SF-D, and 165 kGy-SF-D hydrogels and SF-E hydrogel) was measured by the liquid displacement method. In brief, the lyophilized hydrogel samples were immersed in a known volume (Vh) of hexane in a 5 mL cylinder for 10 min. The total volume of hexane and sample was recorded as Vt. The hexane-impregnated sample was removed from the cylinder, and the residual volume of hexane was recorded as Vr. The porosity was calculated according to formula 1. All samples in the experiment were carried out four times, and the average value and standard deviation were calculated.

\[
\text{porosity} \% = \frac{V_t - V_r}{V_2 - V_3} \times 100\%
\] (1)

Mechanical Test of the SF Hydrogels. The mechanical properties and loading–unloading stability of different SF hydrogels were tested using an AGS-X precision universal tester (SHIMADZU) with a 50 N force sensor at 25 °C and 32% relative humidity. In the compression tests, SF-S and SF-D hydrogel specimens with a height, length, and width of 5 mm in initial thickness were loaded on a metal plate. The thickness of the specimens was measured by a micrometer caliper. The initial force of 0.1 N was applied to the surface of the sample to ensure that the tester was fully contacted with the sample. The compression speed was set to 3 mm/min. For tensile tests, SF-D hydrogels were cut into rectangle specimens with a height, length, and width of 50, 10, and 2 mm, respectively. Initial stress of the initial length of 5% was applied on the two sides of the sample to extend the hydrogel. The tensile speeds for all of the tensile tests were 10 mm/min. Each sample test was repeated at least four times to ensure reproducibility. The test results were expressed in the form of mean ± standard deviation, which are shown in Table 1.

Rheological Analysis. Rheological measurements were conducted on the different SF-S hydrogels to determine hydrogel elasticity (G′, storage modulus) and viscosity (G″, loss modulus). All rheology tests were carried out on a rheometer (ARES-G2, TA Instruments) using a 35 mm stainless steel conical plate (angle: 0.999 rad) at room temperature. Specifically, the different SF-S hydrogels were cut into 5 mm × 5 mm × 2 mm cubes and carefully transferred to the center of the stainless-steel conical plate. The dynamic time sweep tests were conducted at a frequency of 1 Hz (6.283 rad/s) and 1% strain for 120 s. The test results are shown in Table 2.

In Vitro Degradation Properties. The degradation of SF-S and SF-D hydrogels was evaluated using protease XIV with an activity of 3.5 U/mg. The two types of lyophilized SF hydrogels were cut into small pieces and weighted (Ws). SF samples were immersed in 5 mL of PBS (pH 7.4) containing protease XIV (0.5 U/mL) at 37 °C. The solution was changed daily. As a control, samples were immersed in PBS (pH 7.4) without enzyme. After 1, 3, 7, 14, and 21 days, samples were washed with deionized water three times for 20 min; the detached samples were frozen at −20 °C for 12 h, lyophilized, and weighted (Wd). The degradation rate of samples at different time points was calculated according to the following formula:

\[
\text{Degradation rate} \% = \left(1 - \frac{W_d}{W_s}\right) \times 100\%
\]
The results of gel fraction were displayed as the mean ± standard deviation, as shown in Table 3.

**Table 3. Water content, Gel Fraction, and Equilibrium Swelling Ratio of SF Hydrogels**

<table>
<thead>
<tr>
<th>sample</th>
<th>water content (%)</th>
<th>gel fraction (%)</th>
<th>swelling ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 kGy-SF-S</td>
<td>91.04 ± 3.569</td>
<td>87.97 ± 1.614</td>
<td>427.55 ± 12.7</td>
</tr>
<tr>
<td>75 kGy-SF-S</td>
<td>89.95 ± 2.871</td>
<td>90.28 ± 1.631</td>
<td>393.55 ± 6.6</td>
</tr>
<tr>
<td>105 kGy-SF-S</td>
<td>88.61 ± 2.144</td>
<td>91.49 ± 1.741</td>
<td>360.15 ± 15.3</td>
</tr>
<tr>
<td>135 kGy-SF-S</td>
<td>88.07 ± 2.277</td>
<td>92.96 ± 1.418</td>
<td>332.18 ± 8.6</td>
</tr>
<tr>
<td>165 kGy-SF-S</td>
<td>90.65 ± 1.651</td>
<td>92.79 ± 1.491</td>
<td>273.89 ± 13.2</td>
</tr>
<tr>
<td>45 kGy-SF-D</td>
<td>89.93 ± 4.129</td>
<td>91.02 ± 4.333</td>
<td>118.63 ± 4.8</td>
</tr>
<tr>
<td>75 kGy-SF-D</td>
<td>89.11 ± 1.652</td>
<td>93.07 ± 2.115</td>
<td>112.42 ± 5.4</td>
</tr>
<tr>
<td>105 kGy-SF-D</td>
<td>87.43 ± 5.88</td>
<td>95.25 ± 2.614</td>
<td>106.04 ± 3.8</td>
</tr>
<tr>
<td>135 kGy-SF-D</td>
<td>83.35 ± 4.591</td>
<td>96.31 ± 2.789</td>
<td>102.67 ± 2.3</td>
</tr>
<tr>
<td>165 kGy-SF-D</td>
<td>85.06 ± 5.46</td>
<td>94.65 ± 1.851</td>
<td>101.37 ± 2.7</td>
</tr>
</tbody>
</table>

The gel fraction was determined according to the reported method.41 Specifically, the hydrogel samples were dried in a 45 °C drying oven for 24 h at 37 °C in order to remove the soluble fraction. The hydrogels were then dried again in 45 °C for 24 h (W_d). The gel fraction was calculated according to the following eq 4. Measurements were performed 5 times for each sample, and the results of gel fraction were displayed as the mean ± standard deviation, which are shown in Table 3.

\[
\text{gel fraction (\%)} = \frac{W_g}{W_d} \times 100\% \\
\text{(4)}
\]

**Swelling Properties.** Generally, the swelling performance of hydrogels was reflected in the swelling ratio. Dried hydrogel samples (W_d) were immersed in PBS (pH 7.4) at 37 °C for different time intervals (1, 2, 4, 8, 12, and 24 h). After each time interval, the surface moisture of the hydrogel was removed, and the weight of the hydrogels (W_s) was measured. The swelling ratio was calculated according to the following eq 5.42 Measurements were carried out 3 times for each sample to ensure the reproducibility of the experiment, and the final results of the swelling ratio were expressed as the mean ± standard deviation, as shown in Table 3.

\[
\text{swelling ratio (\%)} = \frac{W_s - W_d}{W_d} \times 100\% \\
\text{(5)}
\]

**Thermogravimetric Analysis (TGA).** Thermogravimetry could measure the changes of material quality with time and temperature, which was used to evaluate the thermal stability of materials.43 TGA was carried out in a nitrogen atmosphere using a Q500 thermogravimetric analyzer (TA) with a heating rate of 10 °C/min with the detection range 10–400 °C.

**Cell Culture.** Bone marrow mesenchymal stem cells (BMSCs) were isolated from whole bone marrow aspirates of the distal femur of 100 g male Sprague-Dawley rats and were identified according to a previous reported method.44 In short, the washed aspirates were cultured in the complete minimum essential medium α (MEM-α) containing 1% (v/v) penicillin–streptomycin (Gibco) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37 °C with 5% CO₂. After 4 days of incubation, the unattached cells were removed by changing the culture medium, and the adherent cells were cultured to reached confluence, which was defined as passage 0. The medium was changed every 2 days. The passage 3 of BMSCs were used in this study.

**Cytotoxicity Assay.** The cytotoxicity of the SF-S/D hydrogels system was determined by assessing cell proliferation with cell counting kit-8 (CCK-8; Dojindo). The leaching fluids of the hydrogel samples were obtained by immersing the hydrogels in 100 μL of MEM-α at 37 °C for 24 h in a 96-well plate. BMSCs (2 × 10^3 in a 200 μL suspension) were seeded into 96-well plates and incubated in conditioned medium containing the equal volume of leaching fluids and the complete MEM-α containing 10% FBS at 37 °C in a 5% CO₂ humidified incubator for 1, 3, 5, and 7 days. After incubation, cell samples (n = 5) were obtained and then incubated in 100 μL of the complete MEM-α containing 10% CCK-8 solution and 10% FBS at 37 °C in a 5% CO₂ humidified incubator for 4 h. The OD at 450 nm was measured using a microplate reader (Thermo Scientific).45

To further confirm the biocompatibility of BMSCs with the new SF hydrogels, both BMSCs (5 × 10⁵/cm²) seeded on the surfaces of SF-S and SF-D hydrogels modified by RGD peptide46 for 7 days were observed under a confocal microscopy (Leica, Nussloch, Germany) by live/dead staining (Thermo). In brief, the hydrogels were soaked in PBS (pH 7.4) containing 10% FBS at 37 °C and incubated in conditioned medium containing the equal volume of leaching fluids and the complete MEM-α containing 10% FBS at 37 °C in a 5% CO₂ humidified incubator for 4 h. The OD at 450 nm was measured using a microplate reader (Thermo Scientific).45

**Cell Morphology.** The morphology of BMSCs (5 × 10⁵/cm²) seeded on the surfaces of SF-S and SF-D hydrogels modified by RGD peptide46 for 7 days was observed under a confocal microscopy (Leica, Nussloch, Germany) according to a previous reported method.47 In brief, the cell-loaded SF hydrogels were rinsed with PBS (pH 7.4) 2 times and then fixed with 4% paraformaldehyde for 30 min. The cytoskeleton of BMSCs was stained with rhodamine phalloidin (160 nM; Cytoskeleton Inc., Denver, CO) (red fluorescence, dead cells) for 30 min at room temperature. Excitation wavelengths of 488 or 568 nm were used to detect the visualization of calcine AM (green fluorescence, live cells) or ethidium homodimer-1 (red fluorescence, dead cells).

**Real-Time Polymerase Chain Reaction (RT-PCR) Assay.** To evaluate the effects of the SF-S/D hydrogels surface with different mechanical strength on BMSCs, the
expressions of tendon-specific (EGR1, SCX, MKX, and TNMD), osteogen-specific (collagen type I [COL1], osteocalcin [OCN], and runt-related transcription factor 2 [RUNX2]), and chondroid-specific (collagen type II [COL2], aggregan [ACAN], and SOX9) marker genes were analyzed after incubation for 3, 7, and 14 days by RT-PCR using an ABI 7300 RT-PCR system (Applied Biosystems, FosterCity, CA) with SYBR Green PCR Mater mix (Toyobo, Osaka, Japan), as previously reported.48 Briefly, the samples were rinsed three times with PBS (pH7.4) and lysed in TRIzol reagent (Invitrogen, Camarillo, CA). Total RNA was extracted and reverse transcribed to cDNA using the RevertAid First Strand cDNA synthesis kit (K1622, Thermo). The processes of RT-PCR were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 60 s, and 72 °C for 40 s. The 18s RNA was used as the housekeeping gene. The target genes were quantified by normalizing their expression to that of 18s RNA using the ΔΔCt method.49

Statistical Analysis. All results were expressed as mean ± standard deviation. Statistical comparisons between groups were analyzed by using the one-way ANOVA test. A value of p < 0.05 was considered statistically significant.

## RESULTS

Figure 1 shows the preparation process of the SF hydrogels. Compared with other unfriendly cross-linking methods, such as glutaraldehyde50 or toxic hexafluoroisopropanol,56 γ radiation combined with ethanol treatment was a good way to prepare SF hydrogels. The encapsulated SF aqueous solution was irradiated by γ-ray to obtain the SF-S hydrogels, as shown in Figure 1A. Furthermore, the corresponding SF-D hydrogels were obtained after ethanol treatment on the basis of SF-S hydrogels, as shown in Figure 1B. The SF-E hydrogel is shown in Figure 1C.

The formation mechanism of SF-S hydrogel is similar to the gelation mechanism of γ radiation cross-linked collagen hydrogel.51 In brief, H2O in SF solution absorbed an amount of energy from the γ-ray to produce active substances, such as hydroxyl radical (•OH), proton radical (•H), hydrated electrons, and superoxide (•O2−), as shown in Figure 2A1. Among them, hydroxyl radicals were very active, which could attack SF molecular chains to extract hydrogen from polypeptide chains, inducing the formation of SF-derived radicals and H2O, as shown in Figure 2A2. The active hydroxyl radicals could attack many hydrogen atom sites in SF molecular chains (dotted cycles in Figure 2A) to perform the hydrogen extraction reaction. The reaction order was determined by the ease of the reaction.52 These radicals induce SF to covalently cross-linking between the molecular chains, as shown in one of the cross-linking results in Figure 2A3. In addition, the formation of SF-D hydrogel was driven by ethanol under the pre-established γ radiation cross-linking network. A large number of adjacent GAGAGS sequences in
SF were close to each other, forming numerous hydrogen bonds, as shown in Figure 2B.

In order to better understand the structure of SF hydrogels, FTIR was used to study the conformational positions and changes of amide I (C=O stretching vibration, C=O stretching vibration, and bending in the N-H plane, 1700−1600 cm⁻¹), amide II (bending in the N-H plane and C-N stretching vibration, 1600−1500 cm⁻¹), and amide III (mainly C=N stretching vibration and bending in the N-H plane, 1300−1200 cm⁻¹) bands. By fitting the FTIR characteristic peaks of the amide I region of SF, the effects of different cross-linking processes on the relative content of β-sheet, β-turn, and random coil structures in SF could be analyzed. In the transmittance spectra shown in Figure 3A, the positions of the main peaks of SF-S hydrogel samples (45, 105, and 165 kGy) appeared on the 1650 cm⁻¹ (amide I band), 1545 cm⁻¹ (amide II band), and 1245 cm⁻¹ (amide III band) (red area), which had no significant differences with the positions of main peaks of the SF solution, representing the α-helical/random conformation. In the transmittance spectra shown in Figure 3A, the positions of the main peaks of the SF-D hydrogel samples (45, 105, and 165 kGy) appeared on the 1650 cm⁻¹ (amide I band), 1545 cm⁻¹ (amide II band), and 1245 cm⁻¹ (amide III band) (red area), which had no significant differences with the positions of main peaks of the SF solution, representing the α-helical/random conformation. In the transmittance spectra shown in Figure 3A, the positions of the main peaks of the SF-D hydrogel samples (45, 105, and 165 kGy) appeared on the 1650 cm⁻¹ (amide I band), 1545 cm⁻¹ (amide II band), and 1245 cm⁻¹ (amide III band) (red area), which had no significant differences with the positions of main peaks of the SF solution, representing the α-helical/random conformation. In the transmittance spectra shown in Figure 3A, the positions of the main peaks of the SF-D hydrogel samples (45, 105, and 165 kGy) appeared on the 1650 cm⁻¹ (amide I band), 1545 cm⁻¹ (amide II band), and 1245 cm⁻¹ (amide III band) (red area), which had no significant differences with the positions of main peaks of the SF solution, representing the α-helical/random conformation.

The morphologies of the surface and internal pore structure of lyophilized SF-S and SF-D hydrogels were characterized by SEM. The pore structure of the surface (surf) and longitudinal sections (sect) is shown in Figure 3F,G, which had a relatively uniform and interconnected structure. The SEM results of the 45 kGy-SF-S and SF-D hydrogel showed that the ethanol process did not significantly affect the pore structure of the hydrogel, which had been reported similarly. In addition, a larger pore size (>100 μm) could be obtained at a relatively low dose (45 kGy). With the increase of total irradiation dose (45, 105, and 165 kGy), the porosity of lyophilized hydrogel scaffolds gradually decreased, as shown in Figure 3I. It resulted from the increase of chemical cross-linking sites in the SF molecular chains. Compared with SF-S and SF-D hydrogels with a uniform pore distribution, the pore structure of the SF-E hydrogel was rather uneven, as shown in Figure 3H.

The mechanical properties of SF-S and SF-D hydrogels were systematically evaluated in compression and tension tests. Figure 4A shows the representative stress−strain curves of the compressive test for different SF-S hydrogels. When the total radiation dose increased gradually (from 75 to 165 kGy), the failure strength of the SF-S hydrogels increased and then decreased. It was hypothesized that the excessive radiation dose damaged the chemical bonds in the SF-S hydrogels and resulted in the decrease of mechanical strength. 45 kGy-SF-S hydrogels could not be detected due to the weak strength. The different degrees of continuous compressing experiments were carried out to determine the elasticity of the SF-S hydrogel. Figure 4D shows that the loading−unloading curves almost
Figure 3. Secondary structure and morphological structure characterization of SF hydrogels. (A) FTIR spectra of lyophilized SF-S/D hydrogels under the doses of 45, 105, and 165 kGy (FTIR spectra of lyophilized SF aqueous solution and SF-E hydrogel as control). (B) XRD pattern of lyophilized SF-S and SF-D hydrogels (XRD pattern of lyophilized SF aqueous solution and SF-E hydrogel as control). (C) Deconvolution result of the FTIR amide I band of representative lyophilized SF-S hydrogel. (D) Deconvolution result of the FTIR amide I band of representative lyophilized SF-D hydrogel. (E) Structural content fraction of β-sheet, random coil, and β-turn. SEM images of surface pores (F) and longitudinal section pores (G) of lyophilized 45 kGy-SF-S, 45 kGy-SF-D, 105 kGy-SF-D, and 165 kGy-SF-D hydrogels (scale: 100 μm). (H) SEM images of surface pores and longitudinal section pores of lyophilized SF-E hydrogel (scale: 300 μm). (I) Porosity of lyophilized 45 kGy-SF-D, 105 kGy-SF-D, 165 kGy-SF-D, and SF-E hydrogels (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).
overlapped when the compression degree was 80%, which meant that the SF-S hydrogel had good elasticity and automatic recovery ability. In addition, the dynamic oscillatory time sweep test was used to determine the elasticity (storage modulus, $G'$) and viscosity (loss modulus, $G''$) of the SF-S hydrogels. It could be seen from Figure 4G that the storage moduli of the SF-S hydrogels were significantly higher than the loss modulus. Moreover, the elasticity could be enhanced by increasing the total radiation dose (Figure 4I). However, the excessive $\gamma$ radiation dose was not conducive to the SF-S hydrogels. It could be seen from Figure 4G that the storage moduli of the SF-S hydrogels were significantly higher than the loss modulus. Moreover, the elasticity could be enhanced by increasing the total radiation dose (Figure 4I). However, the excessive $\gamma$ radiation dose was not conducive to the SF-S hydrogels.

![Figure 4](https://dx.doi.org/10.1021/acsabm.9b01062)
hydrogels. Besides, the elasticity modulus of SF-S hydrogel was similar to the natural soft tissue modulus, such as brain tissue \((0.1−1 \text{ kPa})\) and muscle tissue \((8−17 \text{ kPa})\) moduli.\(^4\) Therefore, SF-S hydrogels are expected to be promising candidates for soft tissue engineering. Surprisingly, the mechanical properties of SF-D hydrogel had greater advantages. The compression (Figure 4B) and tensile (Figure 4C) fracture strengths could be observed from the representative stress–strain curves of SF-D hydrogels. The highest fracture strengths reached \(1.37 ± 0.09\) and \(0.44 ± 0.024\) MPa (135 kGy-SF-D hydrogel, water content \(83.35 ± 4.59\) wt %). Correspondingly, the results of the compression modulus (Figure 4H) and tensile modulus (Figure 4J) of SF-D hydrogels showed that the highest values were up to \(2.41 ± 0.232\) and \(6.69 ± 1.015\) MPa (135 kGy-SF-D hydrogel), which were tens to hundreds of times higher than that of conventional hydrogels \((0.01 ± 0.1\) MPa).\(^3\) These elastic modulus ranges already contained the natural cartilage tissue modulus \((0.45−0.8\) MPa) and natural meniscus tissue modulus \((0.09−0.23\) MPa),\(^5\) and thus, the SF-D hydrogel could be considered as a load-bearing tissue engineering scaffold material. In addition, the loading–unloading tests at a fracture strain of 20%, 40%, and 60% were performed to evaluate the self-recovery ability of SF-D hydrogels (representative 135 kGy-SF-D hydrogel), as shown in Figure 4E,F. The SF-D hydrogel recovered rapidly and had a small hysteresis loop after the first stretching cycle, indicating that the SF-D hydrogel had no obvious plastic deformation and had good fatigue resistance during the 60% strain of the stretching process. However, the SF-D hydrogel showed a significant hysteresis loop under the compressing process, which indicated that there was greater energy dissipation in the loading–unloading cycle. This might be due to the loss of moisture under the initial compression loading.\(^6\) Additionally, for the different strain degrees (Figure 4E,F, top left results), the representative loading–unloading cycles showed that the SF-D hydrogels almost had no plastic deformation after the first cycle. The results of uninterrupted cyclic loading were able to truly reflect the comprehensive mechanical properties of the SF hydrogels and got closer to the actual applications of scaffolds in vivo. Besides, the SF-D hydrogel could withstand various forms of deformations, including large bending and twisting, as shown in Figure 4K.

Swelling capacity is one of the most important properties of hydrogels. In order to determine the swelling properties of SF-S and SF-D hydrogels, the dried SF hydrogel samples were immersed in PBS at 37 °C for different times until swelling equilibrium. Equation 5 was used to calculate the swelling ratio of SF hydrogels at different points in time. Figure 5A shows the swelling results of SF-S hydrogels obtained by different \(\gamma\) radiation cross-linking doses. Obviously, the swelling ratio of all SF hydrogels increased significantly within 1 h and reached swelling equilibrium until 12 h. With the increase of \(\gamma\) radiation cross-linking dose, the equilibrium swelling ratio of SF-S hydrogels gradually decreased from \(427.55 ± 12.7\) % to \(273.89 ± 13.2\) %, which might be due to the increase of gel fraction. The swelling results of SF-D hydrogels (Figure 5D) showed a similar trend as well. Compared with highly hydrophilic SF-S hydrogels, the equilibrium swelling ratios of SF-D hydrogels were obviously lower. The chemical–physical double networks made the intermolecular entanglement closer, weakening the gel swelling property.

The biodegradation behavior of materials is an important index in tissue engineering and regenerative medicine applications. Figure 5B shows that the 45 kGy-SF-S hydrogel almost completely degraded within 1 week under the action of protease XIV, and the biodegradability was not significantly improved by increasing the cross-linking dose. After ethanol...
Figure 6. Biocompatibility evaluation of SF-S and SF-D hydrogels in vitro and specific gene expression of BMSCs in different differentiation directions. The cell viability and morphology of BMSCs cultured on the surfaces of SF-S and SF-D hydrogels for 7 days were observed via confocal fluorescence microscopy results of live/dead (A, i) and Phalloidin/Hoechst (A, ii) assay. (B) The cytotoxicity of SF-S and SF-D hydrogel systems was tested by CCK-8 assay, and the OD value at each point was normalized against the average of the first day in each group. There was no statistical difference among different groups at the same time point. Specific gene expression results of BMSCs cultured on the surfaces of SF-S and SF-D hydrogels with different mechanical strength for 3, 7, and 14 days: tendon-specific gene expression of EGR1, SCX, MKX, and TNMD (C–F), osteogen-specific gene expression of COL1, OCN, and RUNX2 (G–I), and chondroid-specific gene expression of COL2, ACAN, and SOX9 (J–L) (*p < 0.05, **p < 0.01, ***p < 0.001).
treatment, the biodegradabilities of 45 kGy-SF-D and 105 kGy-SF-D scaffolds were greatly enhanced (Figure 5E). At the same cross-linking dose, the significant difference in the enzymatic degradation ratio of SF-S and SF-D scaffolds is mainly due to the SF molecular crystallinity. Generally, the degradation initially took place in the amorphous region in SF, such as random coil or α-helix regions, while a large number of closely arranged β-sheet domains in SF-D scaffolds could effectively resist enzymatic hydrolysis.60 In summary, the degradation properties of the new SF hydrogels can be adjusted by the total γ radiation dose and the crystallinity of the protein in order to meet the requirement of complex applications.

In order to evaluate the thermostability, the weight loss of freeze-dried SF hydrogel samples was measured by TGA. The thermogravimetric curves of SF-S and SF-D hydrogel samples are shown in Figure 5C. Two different samples all underwent three main decomposition stages in TGA curves. The first stage showed slight weight loss (<15%) within 110 °C; the thermal decompositions were kept in the second stage with a slower rate, and the third one contained an abrupt SF decomposition. At 287.74 °C, the weight loss rate of SF-S and SF-D samples reached the maximum, and the weight residual rates were 68.64% and 70.35%, respectively, which was mainly caused by the cleavage of peptide bonds and side groups. In addition, the different radiation dose had no significant effects on the thermostability of the SF-S samples (Figure 5F).

In order to evaluate the potential cytotoxicity of the SF hydrogel system, the cell proliferation of BMSCs cocultured, respectively, with the leaching fluids of SF-S and SF-D hydrogels for 1, 3, 5, and 7 days was determined by standard CCK-8 assay (Figure 6B). The absorbance values of two experiment groups were not significantly different with the control group, indicating that the new SF hydrogel system had no obvious cytotoxicity. The cell viability and morphology of BMSCs cultured on the SF-S and SF-D hydrogels modified with RGD peptide are displayed in Figure 6A. The live/dead results (Figure 5Ai) indicated that most of the BMSCs on SF-S and SF-D hydrogels showed green fluorescence (living cells), and almost no red fluorescence (dead cells) appeared. Moreover, the cell adhesion number on the different SF hydrogels could be also evaluated by live/dead assay. Obviously, the quantity of BMSCs on the surface of “tough” SF-D hydrogels was more than that of “soft” SF-S hydrogels, which was consistent with previous studies.61 Similarly, the cell morphology demonstrated by Phalloidin/Hoechst assay (Figure 5Aii) showed that the cytoskeleton of BMSCs on the SF-D hydrogel surface had good spreading, while the cell spreading area was small and irregular on the SF-S hydrogel. It was well-known that the surface stiffness of the hydrogel would affect the specific differentiation of MSCs.4 Here, the expressions of tendon-specific (EGR1, SCX, MKX, and TNMD), osteogen-specific (COL1, OCN, and RUNX2), and chondrogen-specific (COL2, ACAN, and SOX9) genes were detected by RT-PCR. The expression of tendon-specific genes increased with incubation time, and the expression values of tendon-specific genes and osteogenic-specific genes in the “tough” SF-D hydrogel group (1.5–2 MPa) were significantly higher than that in the “soft” SF-S hydrogel group (20–25 kPa). However, on the contrary, the expression values of cartilage-related specific genes in the “soft” SF-S hydrogel group were significantly higher than that in the “tough” SF-D hydrogel group (Figure 6J–L).

**DISCUSSION**

The development of tissue engineering and regenerative medicine depends on the preparation of good biocompatibility, biodegradability, and cheaper biomaterials, which is usually up to the continuous optimization of preparation methods. Herein, γ radiation can initiate the polymerization of active radicals at room temperature, which make the SF solution system ionized after absorbing high-energy γ-ray radiation. The generated ions and excited molecules, due to chemical instability, react easily and then convert into large free radicals and neutral molecules, triggering a series of chemical changes to form the gel network. Because of the instability of free radicals, they usually exist in a stable form at the end of the reaction, such as H2O2, which can avoid the potential cytotoxicity. The cell results (Figure 6) can support this perspective. In addition, the γ radiation cross-linking method greatly increases the cross-linking efficiency and breaks through the limitations of some chemical cross-linking methods,62 such as peroxidase and genipin. They usually depend on a few active amino acid residues in SF, such as amidogen groups on arginine or lysine residues and phenolic hydroxyl groups on tyrosine residues. Compared with the enzyme cross-linked SF hydrogel,25 SF-S hydrogel has a more stable property at 37 °C, which does not transform from gel to solution, as shown in Figure 5A. At the same time, this method avoids the risk of cytotoxicity in the early or later chemical cross-linking process and omits the steps of removing cytotoxic initiators from hydrogel network.25 Of course, the effects of conventional factors, such as the concentration of SF solution and reaction temperature, also exist in the γ radiation cross-linking method. However, these may not be the most crucial factors that affect the final results of γ radiation cross-linking. As we all know, the γ radiation process is usually accompanied by the polymerization and degradation of polymers, which is usually associated with the total radiation dose. The different natural polymers, such as collagen, gelatin, sercin, hyaluronic acid, and alginate, have a relatively appropriate radiation dose of cross-linking. Therefore, it is particularly significant to explore the effects of different radiation doses on the properties of SF hydrogels. In the previous reported study of the γ radiation cross-linking SF hydrogel,56 the researchers set the range of reaction dose at 15–90 kGy and thought that the maximum bearable reaction dose of 7.9 wt % SF solution was about 60 kGy, which was different with our results. In our study, 8.5 wt % SF solution could withstand a dose far greater than 90 kGy, even up to 165 kGy, and achieve the best cross-linking effect at about 135 kGy shown in the results of gel fraction (Table 3) and the mechanical properties (Figure 4A,G,I). However, as the radiation dose increased, the internal molecular chains of SF were destroyed, which may affect the mechanical properties (Figure 4), swelling properties (Figure 5A), and in vitro degradation properties (Figure 5B) of the SF hydrogels. In terms of the secondary structure of SF-S hydrogels (Figure 3A,B), interestingly, the random coil structure was not induced to the β-sheet structure in advance as the increasing of radiation dose. The different properties of SF-S hydrogels may be related to the tighter connectivity within SF molecular chains, which was not relative to the β-sheet. The results of the pore size and porosity of the lyophilized SF hydrogels (Figure 3F,G,I) could also indirectly prove that viewpoint.

Compared with SF-S hydrogels, SF-D hydrogels have more excellent mechanical properties, which is closely related to
their internal $\beta$-sheet domains. Many studies have shown that the formation of small and uniformly distributed $\beta$-sheet domains during the conformational transitions is an effective way to enhance the mechanical properties of SF hydrogel. Achieving this goal may lie on the first cross-linking network. Herein, the size of $\beta$-sheet domains is limited by the covalently cross-linked sites of the previous $\gamma$ radiation network, which makes an important contribution to the outstanding mechanical properties of SF-D hydrogels. XRD results (Figure 3B) can also prove this perspective. Compared with SF-E samples, SF-D samples show a broader peak at about 20.5°, which indicates that the lower crystallinity of $\beta$-sheet domains exists in the SF samples.26 In addition, a large number of secondary bonds, such as intermolecular van der Waals forces, also provide mechanical assistance for the whole gel system. When the SF-D hydrogel is stretched or compressed, the hydrogen bonds inside of the hydrogel can be used as a kind of reversibly sacrificial bond to dissipate energy. The small $\beta$-sheet serving as a stress transfer center is able to effectively absorb energy and undergo deformation. Moreover, the porous structures of the SF hydrogels (Figure 3F,G) can transfer mechanical stress, and the relatively uniform pore distribution makes the stress distribution more uniform. Besides, the smaller pore size and greater pore number also play a crucial role in preventing crack growth.19

It is surprising that the mechanical properties of SF-D hydrogels happen to be contained in the “empty soft materials space” region of the elastic modulus/strain Ashby plot summarized by Miserez et al.64 The research shows that the mechanical properties of the commonly used natural polymer hydrogels and other “soft” biomaterials are mostly divided into two extremes. They either have higher elastic modulus with lower strains, such as the harder native or cross-linked collagen (elastic modulus $>300$ MPa, fracture strain $<20\%$), or have strong ductility with limited elastic modulus, such as alginate (elastic modulus $<0.1$ MPa, fracture strain value $>40\%$), elastin (elastic modulus $<1$ MPa, fracture strain value $>70\%$), and double-network hydrogels (elastic modulus $<0.3$ MPa, fracture strain $>100\%$). Few “soft” biomaterials (mostly hydrogels) with both good biocompatibility and comprehensive mechanical strength are covered in the space of “empty soft materials”. As far as the single component SF hydrogels are concerned, the cross-linking methods of SF hydrogels covered in that space are limited,25,36 and many problems still exist, such as the complex preparation process, the many influential factors, and the introduction of cytotoxic substances. However, SF-D hydrogels avoid the above-mentioned problems while filling a part of the “empty soft materials” space, thus expanding the selection scope of biomaterials for tissue engineering applications.

It is well-known that MSCs have the ability to differentiate into osteoblast, chondrocyte, adipocyte, and tendon/ligament like cells, and thus, they are always considered as a “natural tissue repair system”.13 In tissue engineering, MSCs need the assistance of appropriate scaffold materials to build bone, cartilage, or other tissues. However, the microenvironment of scaffolds generally has a great influence on the differentiation of BMSCs, especially the surface hardness of the scaffolds. The RT-PCR results (Figure 6C–L) show that BMSCs had a tendency to differentiate into bone or tendons on the “tough” SF-D hydrogel substrate on day 14, while on the “soft” SF-S hydrogel substrate, the chondroid-specific gene expression of BMSCs was more significant. Similar views had been reported.4 Butcher et al.66 believed that all kinds of cells are exposed to isometric force or tension, including those in traditionally mechanically stable tissues, such as breast or brain. These stresses are caused by the interactions of cell–cell or cell–extracellular matrix and affect the cell functions. All of these views emphasize the critical association between tissue phenotype and matrix rigidity. Therefore, optimizing the mechanical strength of biocompatible natural polymer hydrogel substrates is specifically critical for MSCs’ culture and regenerative medicine.

## CONCLUSION

In summary, we have proposed a simple and clean method to prepare a kind of elastic SF hydrogel with various mechanical strengths suitable for diverse tissue engineering applications. This kind of new gel system is easy to make and has high reaction efficiency without any extra toxic cross-linking agents. The $\gamma$ radiation cross-linking technology combined with ethanol can trigger and regulate the properties of SF hydrogels by inducing conformational transition. The first network of the $\gamma$ radiation cross-linking provides fixed sites for the subsequent physical cross-linking and also lays a significant foundation for the uniform distribution of $\beta$-sheet domains in the second step. Thus, the new SF hydrogels have excellent mechanical properties and tunable Young’s modulus, which can achieve the mechanical distribution of $<$29 kPa or 1.21–2.41 MPa (even higher). Furthermore, these elastic SF hydrogels have a relatively uniform pore structure, tunable porosity, degradation time, good biocompatibility, and adjustable mechanical strength, which contributes a wider choice scope of biomaterials to the complex tissue engineering applications.

## AUTHOR INFORMATION

### Corresponding Author

Haifeng Chen — Peking University, Beijing, China; Email: haifeng.chen@pku.edu.cn

### Other Authors

Nier Wu — Peking University, Beijing, China; orcid.org/0000-0001-5304-5673

Huilei Yu — Peking University Third Hospital, Beijing, China

Muyang Sun — Peking University Third Hospital, Beijing, China

Zong Li — Peking University Third Hospital, Beijing, China

Fengyuan Zhao — Peking University Third Hospital, Beijing, China

Yingfang Ao — Peking University Third Hospital, Beijing, China; orcid.org/0000-0002-8909-2022

### Complete contact information is available at:

https://pubs.acs.org/10.1021/acsabm.9b01062

### Notes

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