

Macroporous Silk Fibroin Cryogels

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Supporting Information

ABSTRACT: Silk fibroin cryogels with remarkable properties were obtained from frozen fibroin solutions (4.2–12.6%) at subzero temperatures between -5 and -22 °C. This was achieved by the addition of ethylene glycol diglycidyl ether (EGDE) into the cryogelation system. EGDE triggers the conformational transition of fibroin from random coil to β sheet structure and hence fibroin gelation. One of the unique features of fibroin cryogels is their elasticity that allows them to resist complete compression without any crack development, during which water inside the cryogel is removed. The



compressed cryogel immediately swells during unloading to recover its original shape. The scaffolds obtained by freeze-drying of the cryogels consist of regular, interconnected pores of diameters ranging from 50 to 10 μ m that could be regulated by the synthesis parameters. The mechanical compressive strength and the modulus of the scaffolds increase with decreasing pore diameter, that is, with decreasing gelation temperature or, with increasing fibroin or EGDE concentrations in the feed. The scaffolds produced at 12.6% fibroin exhibit a very high compressive modulus (50 MPa) making them good candidates as bone scaffold materials.

INTRODUCTION

Silk fibroin derived from Bombyx mori is a fibrous protein exhibiting extraordinary material properties such as good biocompatibility, biodegradability, high strength and toughness, and ease of processability.¹⁻³ Silk fibroin has been used for cell culture, wound dressing, drug delivery, enzyme immobilization, and as a scaffold for bone tissue engineering.^{4,5} Silk fibroin has a blocky structure consisting of less ordered hydrophilic and crystallizable hydrophobic blocks.⁶⁻⁹ Hydrophilic blocks provide solubility in water and are responsible for fibroin elasticity and toughness, while hydrophobic blocks form intermolecular β -sheet structures leading to the insolubility and high strength of fibroin. Several techniques have been developed to produce porous fibroin scaffolds such as freezethawing, porogen leaching, gas foaming, electrospinning, and freeze-drying.¹⁰⁻²⁰ The principle of porogen leaching and gas foaming techniques is the use of porogens, such as sodium chloride and ammonium percarbonate acting as a template and gas-forming agent, respectively.^{11,19} After treatment of fibroin/ porogen composites with alcohols to induce β -sheet formation, the porogen is leached out with water to form the pores of the scaffolds. To produce fibroin scaffolds by freeze-drying, aqueous fibroin solutions are mixed with alcohol to obtain a gel precipitate following freezing at a low temperature and finally freeze-drying. It was shown that porogen leaching and gas foaming techniques produce scaffolds having larger pores (100–200 μ m) as compared to the scaffolds formed by freezedrying $(10-50 \ \mu m)$.¹¹ The compressive moduli of the scaffolds vary depending on the preparation conditions between 10 kPa and 3 MPa.

An alternative simple route to produce 3D highly porous fibroin networks is the low-temperature gelation technique, known as cryotropic gelation or cryogelation. Since the pioneering works of Lozinsky and co-workers,²¹⁻²³ a cryogelation technique has been widely used to produce macroporous gels (cryogels) of high toughness and superfast responsivity.²⁴⁻³⁴ By this technique, polymerization and/or cross-linking reactions are conducted in apparently frozen reaction solutions. During freezing of an aqueous polymer solution containing a chemical cross-linker, the polymer chains and the cross-linker molecules expelled from the ice concentrate within the liquid channels between the ice crystals, so that the cross-linking reactions only proceed in these unfrozen domains. After cross-linking and after thawing of ice, a macroporous material is produced whose microstructure is a negative replica of the ice formed. In contrast to the mechanically weak macroporous gels prepared by phase separation technique,³⁵ cryogels are very tough and withstand very large strains without permanent deformation or fracture.

Here, we show, for the first time to our knowledge, that gelation of frozen silk fibroin solutions in the presence of ethylene glycol diglycidyl ether (EGDE) leads to the formation of cryogels with a wide range of tunable properties. EGDE has been widely used for cross-linking of polysaccharides, proteins, and DNA, as well as organic molecules.^{36–39} EGDE contains epoxide groups on both ends that can react with nucleophiles, including amino groups, sulfhydryls, and hydroxyls. Recently,

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set #	$C_{\rm SF}$ w/v %	EGDE mmol/g	$T_{\rm prep}~^{\circ}{ m C}$	TEMED v/v %	$q_{ m w}$	$q_{ m v}$	Р	β -sheets %
1	4.2	20	-18	0 to 0.5	16 ± 2	1.7 ± 0.8	0.92 ± 0.04	31 ± 2
2	4.2	20	-5 to -22	0.25	14 ± 4	1.2 ± 0.1	0.94 ± 0.01	32 ± 2
3	4.2	5 to 30	-18	0.25	15 ± 3	1.2 ± 0.1	0.94 ± 0.01	30 ± 1
4	4.2	20	-18	0.25	16 ± 2	1.6 ± 0.2	0.92 ± 0.04	31 ± 2
	8.4				8 ± 1		0.86 ± 0.02	
	12.6				5.4 ± 0.1		0.77	
${}^{4}q_{w}$ and q_{v} are the weight and volume swelling ratios, respectively, and P is the total porosity of the cryogels estimated using eq 4.								

Table 1. Four Sets of Cryogelation Experiments Conducted at Various Reaction Conditions Together with the Properties of the Resulting Cryogels^a

we have shown that the introduction of EGDE cross-links between the fibroin molecules decreases the mobility of the chains, which triggers the conformational transition from random-coil to β -sheet structure and, hence, fibroin gelation.⁴⁰ Gelation reactions conducted at 50 °C showed formation of strong to weak fibroin hydrogels, depending on the pH of the solutions, which was adjusted by the addition of N,N,N',N'-tetramethylethylenediamine (TEMED).⁴⁰

In the present study, we conducted the gelation reactions of fibroin in frozen solutions at subzero temperatures between -5 and -22 °C. In this way, we were able to produce macroporous fibroin cryogels showing 90% porosity and high mechanical stability upon compression. In contrast to weak and brittle fibroin hydrogels, cryogels can be compressed up to about 100% strain without any crack development, during which water inside the cryogel is removed. The compressed cryogel immediately swells during unloading to recover its original shape. The scaffolds obtained by freeze-drying of fibroin cryogels consist of regular, interconnected pores of diameters ranging from 50 to 10 μ m, depending on the synthesis parameters. The scaffolds exhibit a very high compressive modulus up to 50 MPa making them good candidates as bone scaffold materials.

EXPERIMENTAL SECTION

Materials. Ethylene glycol diglycidyl ether (EGDE, Polyscience), 1,4-butanediol diglycidyl ether (BDDE, Aldrich), N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma), poly(ethylene glycol) (10000 g/mol, Fluka), Na2CO3 (Merck), and LiBr (Merck) were used as received. Cocoons of Bombyx mori were kindly provided from Koza Birlik (Bursa, Turkey). Silk fibroin solution was prepared following Kim et al.'s procedure.⁴¹ The cocoons were cut into smaller pieces and placed in 1 L of boiling aqueous solution of 0.02 M Na₂CO₃ for 1 h to remove sericins. Then, silk fibers were rinsed three times in 1 L of distilled water at 70 °C for 20 min each. The extracted silk fibroin was dissolved in a 9.3 M LiBr solution at 60 °C for 90 min, yielding a 20 w/v % solution. This solution was dialyzed using dialysis tubing (10000 MWCO, Snake Skin, Pierce) for 3 days against water that was changed three times a day. The final concentration of the silk fibroin aqueous solution was about 6 w/v %, which was determined by weighing the remaining solid after drying. A 5 w/v % fibroin solution was prepared by diluting this solution with water. Solutions containing 12 and 18 w/v % fibroin were obtained by dialyzing 5% fibroin solution against 1 L of aqueous PEG (10 and 15 w/v %, respectively) using 3500 MWCO dialysis tubing (Snake Skin, Pierce) for 1 day. All fibroin solutions were stored at 4 °C and used within 2 weeks.

Cryogelation Reactions. The reactions were carried out in aqueous solutions at subzero temperatures in the presence of EGDE cross-linker and TEMED catalyst. The cross-linker (EGDE) content of the reaction solution was expressed as mmol/g, which is the mmol epoxy groups added per 1 g of silk fibroin. Four sets of experiments were carried out by changing the gelation temperature (T_{prep}), the amounts of silk fibroin (C_{SF}), EGDE, and TEMED in the feed. Summary of the experimental parameters and the characteristic data of

the resulting cryogels are listed in Table 1. Columns 2–5 of the table contain the synthesis parameters of four sets of cryogels, while columns 6–8 show their weight q_w and volume swelling ratios q_v and the porosities *P*. In the last column, β -sheet contents of the gels estimated from the FTIR spectra are listed. Experiments at various TEMED % (set 1, Table 1) were also carried out using BDDE instead of EGDE as a cross-linker. No differences in the gel properties were observed.

The gels were prepared according to the following scheme: Fibroin solution of known concentration (5, 12, and 18 w/v %) was mixed with aqueous TEMED and finally with EGDE to make the final volume 6 mL. The homogeneous reaction solution was transferred into several plastic syringes of 4 mm internal diameters and then, they were placed in a cryostat at a fixed temperature $T_{\rm prep}$ to conduct the cross-linking reactions for 1 day. For comparison, experiments of set 1 in Table 1 were also carried out at 50 °C to obtain fibroin hydrogels. To study the large strain behavior of cryogel and hydrogel samples, they were also prepared in plastic syringes of 10 mm internal diameters.

Swelling and Gel Fraction Measurements. Fibroin cryogels were taken out of the syringes and they were cut into specimens of approximately 10 mm in length. Each gel sample was placed in an excess of water at 24 °C and water was replaced every other day over a period of at least 1 month to wash out the soluble fibroin and the unreacted cross-linker. The swelling equilibrium was tested by measuring the diameter of the gel samples by using an image analyzing system consisting of a microscope (XSZ single Zoom microscope), a CDD digital camera (TK 1381 EG), and a PC with the data analyzing system Image-Pro Plus. The swelling equilibrium was also tested by weighing the gel samples. Then, the equilibrium swollen gel samples were immediately frozen at -25 °C for 1 day before being freeze-dried at -40 °C/0.12 mbar for 1 day and -60 °C/0.01 mbar for an additional 1 day. The freeze-drying system was made up of a freezedryer (Christ Alpha 2-4 LD plus) connected to a vacuum pump (Vacuubrand RZ 6). The freeze-drying pressures were adjusted using a valve controller and monitored by an active digital controller. All gels were freeze-dried under the same conditions. The water content of freeze-dried samples determined by Karl Fischer titration (Mettler Toledo DL 38) was $3 \pm 2\%$. The equilibrium weight q_w and volume swelling ratios q_v were calculated using the following equations:

$$q_{\rm w} = (m_{\rm sw}/m_{\rm dry}) \tag{1a}$$

$$q_{\rm v} = (D_{\rm sw}/D_{\rm dry})^3 \tag{1b}$$

where $m_{\rm sw}$ and $m_{\rm dry}$ are the weights of gels after equilibrium swelling, and after drying, respectively, $D_{\rm sw}$ and $D_{\rm dry}$ are the corresponding diameters. The gel fraction, W_{g} defined as the amount of cross-linked (water insoluble) fibroin network obtained from 1 g fibroin, was calculated as

$$W_{\rm g} = \frac{m_{\rm dry}}{m_{\rm o}C_{\rm SF}} \tag{2}$$

where m_0 is the weight of gels after preparation.

Nonfreezable Water Content. For the determination of the nonfreezable water content of the cryogels, DSC measurements were carried out on Perkin-Elmer Diamond DSC. Fibroin cryogels



Figure 1. Gel fraction W_g (A), equilibrium weight swelling ratio q_w (B), and the elastic moduli G_{sw} (C) of swellen fibroin cryogels shown as a function of TEMED % (filled symbols); $T_{\text{prep}} = -18 \,^{\circ}\text{C}$, $C_{\text{SF}} = 4.2 \,\text{wt}$ %, and EGDE = 20 mmol/g. For comparison, the data of fibroin hydrogels formed at 50 $^{\circ}\text{C}$ taken from ref 40 are also shown by the open symbols.

equilibrium swollen in water were placed in an aluminum sample pan of the instrument. The pan with swollen gel was sealed and weighed. Then, it was frozen at -18 °C for 1 day and then heated to 80 °C within the instrument with a scanning rate of 2.5 °C/min. The transition enthalpy ΔH for the melting of frozen water was determined. After the scans, the pans were punctured and dried at 80 °C to constant weight. The total water content of the hydrogel m_w was calculated as $m_w = m_1 - m_2$, where m_1 is the weight of pan with swollen gel and m_2 is the same weight, but after drying. The mass fraction of nonfreezable water in the gel, f_w was calculated as $f_w = 1 - (\Delta H/\Delta H_m)/m_{w_1}$ where ΔH_m is the heat of melting of ice which is 334.45 J/g.

Mechanical Tests. Uniaxial compression tests on cylindrical freeze-dried samples of 4 mm in diameter and 3-4 mm length were performed using a Zwick Roell, 500 N test machine at 24 °C. An initial compressive contact to 0.1 N was applied to ensure a complete contact between the sample and the surface. Load and displacement data were collected during the experiments at a constant crosshead speed of 0.3 mm/min. Compressive stress was presented by its nominal value σ , which is the force per cross-sectional area of the undeformed specimen, while the strain is given by the deformation ratio α (deformed length/initial length). The compression modulus E was calculated from the slope of stress-strain curves between 2 and 4% compressions, while the stress at 3% compression was reported as the compressive stress $\sigma_{\rm comp}$. For reproducibility, at least six samples were measured for each scaffold and the results were averaged. Uniaxial compression measurements were also performed on equilibrium swollen cryogels in water. The stress-strain isotherms were measured by using an apparatus previously described.^{42,43} The elastic modulus G_{sw} was determined from the initial slope of linear dependence

$$\sigma = G_{\rm sw}(\alpha - \alpha^{-2}) \tag{3}$$

At least three gel samples prepared under the same conditions were tested for reproducibility, and the values were averaged to obtain the reported results.

Texture Determination. For the texture determination of freezedried samples, scanning electron microscopy studies were carried out at various magnifications between 20 and 1000 times (Jeol JSM 6335F Field Emission SEM). Prior to the measurements, network samples were sputter-coated with gold for 3 min using Sputter-coater S150 B Edwards instrument.

X-ray Diffraction. X-ray diffraction of freeze-dried fibroin samples was obtained with Ni-filtered Cu K α radiation ($\lambda = 0.15418$ nm) from a Shimadzu XRD-6000 X-ray generator operating at 40 kV and 30 mA. Diffraction intensity was measured in reflection mode at a scanning rate of 0.6° /min for $2\theta = 5-35^{\circ}$.

ATR-FTIR Measurements. Spectra of the freeze-dried cryogel samples were collected using a single bounce diamond attenuated total refractance (ATR) module on a Fourier-transform infrared (FTIR) spectrometer (Nicolet Nexus 6700) equipped with a liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector. The resolution of each spectrum was 4 cm⁻¹, and 64 interferograms were coadded in

the range of $500-4000 \text{ cm}^{-1}$. To estimate the conformation of fibroin network chains, the spectra were analyzed using PeakFit software (Version 4.12, SeaSolve Software Inc.). Linear baseline correction was applied to the amide I region ($1580-1720 \text{ cm}^{-1}$) before the band was deconvolved by Gauss Amplitude function. For the curve fitting procedure, the initial band positions at 1620, 1640, 1660, and 1698 cm⁻¹ were fixed, allowing their widths and heights to vary (Figure S1).

RESULTS AND DISCUSSION

Silk fibroin cryogels were prepared by conducting gelation reactions at subzero temperatures in a frozen state. The reactions were carried out at various temperatures, TEMED, EGDE, and fibroin concentrations (Table 1). In the following, the formation and properties of fibroin cryogels are discussed as depending on the synthesis parameters. Then, the porous structure and the mechanical performance of fibroin scaffolds obtained by freeze-drying of the cryogels are discussed and compared with those of freeze-dried hydrogel samples.

Swelling, Elasticity, and Network Structure of Fibroin Cryogels. The reactions were first carried out at a fibroin concentration of 4.2 w/v % in the presence of 20 mmol epoxide/g fibroin, while the amount of TEMED was varied. These synthesis conditions are the same as we reported before for the preparation of fibroin hydrogels,40 except that the gelation temperature $T_{\rm prep}$ was decreased from 50 to -18 °C. Results from the swelling and elasticity tests are shown in Figure 1. The gel fraction W_{g} , the equilibrium weight swelling ratio q_w and the elastic modulus G_{sw} of water-swollen cryogels are plotted against the TEMED content. For comparison, the data for fibroin hydrogels formed at $T_{\text{prep}} = 50 \text{ }^{\circ}\text{C}$ are also shown in the figure by the open symbols.⁴⁰ Gelation at $-18 \text{ }^{\circ}\text{C}$ results in cryogels with a gel fraction $W_{\rm g} \cong 1$ over the whole range of TEMED investigated. Both the swelling ratio q_w and the modulus G_{sw} of the cryogels do not change much with the TEMED content and they remain at 16 ± 2 and 53 ± 19 kPa, respectively. This is in contrast to the fibroin hydrogels where $G_{\rm sw}$ decreases while $q_{\rm w}$ increases with a rising amount of TEMED and no gel forms at or above 0.24% TEMED (for details see ref 40).

Figure 1C also shows that, at low TEMED contents, the modulus G_{sw} of fibroin hydrogels approaches to that of the cryogels, indicating similar elastic behavior of both types of gels at low compression ratios (<10%). However, as the gel samples are further compressed, significant differences were observed. Fibroin hydrogel formed at 50 °C fractured under low deformation, indicating that the mechanical stress applied is localized without effective dissipation (Figure 2A). However,



Figure 2. (A, B) Photographs of fibroin hydrogels (A) and cryogels (B) formed at 50 and -18 °C, respectively, during the compression tests; $C_{SF} = 4.2\%$, EGDE = 20 mmol/g, TEMED = 0.10%. (C) Stress-strain curves of these gel samples as the dependence of the nominal stress σ on the degree of compression.



Figure 3. (A) Typical ATR-FTIR spectra of EGDE (dashed red curve), freeze-dried silk fibroin before (thin curve) and after cryogelation (thick curve). TEMED = 0.07%. (B, C) Amide I region of FTIR spectra (B) and X-ray diffraction (C) of freeze-dried cryogels. Data obtained by freeze-drying of silk fibroin solutions before gelation are also shown by the dotted curves. TEMED % is indicated in C, while in B, 0.07 (solid curve), 0.10 (long dash), 0.13 (short dash), 0.17 (short–long), 0.25 (dash-dot-dot), 0.33 (short–long–short), and 0.50% TEMED (dash-dot). $T_{\text{prep}} = -18 \text{ °C}$; $C_{\text{SF}} = 4.2$ wt %; and EGDE = 20 mmol/g.

fibroin cryogel remained mechanically stable up to complete compression (Figure 2B). Important point is that, as the cryogel is squeezed under the piston or via manual hand compression, the gel releases all its water so that it can completely be compressed. Although no energy dissipation mechanism was introduced in the cryogels, release of water from the pores under stress seems to prevent crack formation at large deformation ratios. After the release of the load, the gel sample immediately recovers its original shape by absorbing the released water, as shown Figure 2B and in the movie attached as the Supporting Information. Figure 2C shows typical stress—strain curves of swollen gel samples under compression. Hydrogels rupture at about 10% compression and at a compressive nominal stress of 15 kPa while the cryogels sustain 99.8% compression at 640 kPa stress. Successive compression tests conducted on the same gel sample between 0 and 99.8% strain showed reversibility of the stress strain curves of the cryogels revealing that no crack occurs during the experiments. The results indicate that fibroin cryogels prepared as beads or high flow-path monolith columns can be used in separation processes in which the separated compounds such as particulate matter or proteins deposited in the internal pore surface can easily be recovered by compression of the gels under a piston.

Cryogelation reactions conducted in the absence of EGDE did not lead to gel formation after one day of reaction time, indicating that the cryo-concentration alone does not induce fibroin gelation. This suggests that, in accord with our previous report,⁴⁰ even in the frozen state, fibroin gelation is mediated by the presence of diepoxide cross-linker. The reaction between EGDE and fibroin was assessed by the ATR-FTIR spectra of freeze-dried fibroin samples. Figure 3A presents the spectra of the samples before and after cryogelation together with the spectrum of EGDE cross-linker. In addition to a shift of the Amide I absorption band to lower wavenumbers, new bands at 1040–1100 cm⁻¹ appear upon gelation, which were assigned to the ether stretching bands of EGDE cross-linkages. The ether stretching bands were observed in the spectra of all cryogel samples listed in Table 1.

Figure 3B is a zoom-in of the Amide I band region of the spectra presenting the carbonyl stretching vibration of amide groups on silk fibroin. The spectrum of fibroin before gelation (dotted curve) is characterized by a peak at 1640 cm^{-1} indicating the presence of primarily random coil and/or α helix conformations.^{44,45} After cryogelation, all samples display a main peak at 1620 cm⁻¹ which was assigned to β -sheet conformation.⁴⁵ In addition to the main peak, shoulders at 1660 and 1698 cm⁻¹ are seen in the figure, which can be assigned to α -helix and β -turn conformations, respectively. This indicates the occurrence of a conformational transition from random coil to β -sheet structure in frozen fibroin solutions. A further evidence for the β -sheet formation comes from the Xray profiles of freeze-dried cryogels (Figure 3C). Silk fibroin before gelation (dotted curve in the figure) exhibits a broad peak at around 22°, indicating an amorphous structure.⁴¹ After gelation, all the cryogels show a distinct peak at 20.9° and two minor peaks at 9.8 and 24.5°. These are the characteristic peaks of the β -sheet crystalline structure of silk fibroin corresponding to β -crystalline spacing distances of 4.3, 9.0, and 3.6 Å, respectively.41,46

To estimate the conformation of the fibroin network chains, peak separation of Amide I band was carried out after baseline correction by selecting a Gaussian model for curve fitting, as described previously.⁴⁰ The peak positions were fixed at 1620, 1640, 1660, and 1698 cm⁻¹, representing β -sheet, random coil, α -helix, and β -turn conformations, respectively (Figure S1). The results of β -sheet contents show that fibroin chains before gelation have 12 \pm 2% β -sheet structures, while their contribution increases to $33 \pm 2\%$, independent of the amount of TEMED. In the case of fibroin hydrogels formed at 50 °C, the contribution of β -sheets was 55% between 0.07 to 0.17% TEMED, while this percentage decreased as the TEMED content at gelation is further increased.⁴⁰ The decrease in the β sheet content of fibroin hydrogels with rising TEMED content is due to the simultaneous increase of pH of the gelation solution.⁴⁰ The osmotic pressure of the fibroin counterions in basic solutions causes chain expansion and thus inhibits the formation of β -sheets. This also leads to a decrease in the gel fraction and in the modulus of fibroin hydrogels above 0.17% TEMED corresponding to pH = 9.7 (Figure 1). In contrast, present results show that, if the reactions are conducted in a frozen state at -18 °C, the amount of TEMED or pH of the solution prior to freezing does not affect the gel properties. A similar finding was observed by Lozinsky in cryogelation of ovalbumin solutions in the presence of urea at concentrations above 0.5 $\mathrm{M.}^{26}$

We attribute the occurrence of the gelation reactions and the conformational transition of fibroin in frozen solutions to the cryo-concentration that effectively increases the reaction mixture concentration. When the reaction solution containing fibroin, EGDE and TEMED was cooled to a temperature below the nominal freezing temperature, the majority of water formed ice crystals whereas bound water and soluble substances accumulated in the unfrozen domains as a result of the freezing point depression. This phenomenon referred as cryoconcentration leads to a high concentration of the reactants in the unfrozen domains accelerating the reactions. To estimate the amount of unfrozen water in the reaction system, we conducted DSC measurements on equilibrium swollen fibroin gels frozen at -18 °C. Prior to freezing, fibroin concentration of the gel samples was 6.4 \pm 0.8 wt %. After freezing at -18 °C, the mass percent of nonfreezable water in gels was found to be $12 \pm 3\%$, indicating that the concentration of fibroin in the unfrozen domains becomes 37 ± 6 wt %. These values are in agreement with previous reports.^{31,47} When swollen polyelectrolyte hydrogel samples are frozen, they contain about 6% of water, which are bound to the polymer chains and remain unfrozen, even at -24 °C.³¹ It was also shown that, starting from an initial 6% monomer solution of dimethylacrylamide, the concentration of solute in the unfrozen domains becomes 33 and 46% at $T_{\text{prep}} = -10$ and -20 °C, respectively.⁴

In the absence of cryo-concentration, although fibroin hydrogels could be obtained at 50 °C after a reaction time of $1 \text{ day},^{40}$ no gel formed by lowering the temperature to 25 or 0 °C due to the slowing down of the reactions. However, a further decrease of the temperature below the nominal freezing point of fibroin solution leads to the formation of gels with a gel fraction around unity (Table 1). This suggests that the decrease in the reaction rates at subzero temperatures is compensated by cryo-concentration of the reactants in the unfrozen reaction zones leading to the formation of fibroin cryogels. Moreover, relatively low β -sheet content of the cryogels as compared to hydrogels (33 vs 55%) can be explained on the basis of the backbone mobility of the chains. Due to the cryo-concentration, fibroin in the nonfrozen domains of the reaction system is in a highly concentrated solution, which restricts alignment of the crystallizable protein segments and so, the extent of the conformation transition. This explanation is also supported by the fact the degree of denaturation of proteins in frozen solutions is smaller as compared to that in unfrozen solutions.²⁶

Gelation reactions were also conducted at various temperatures $T_{\rm prep}$, EGDE and fibroin concentrations. Within the ranges of the synthesis parameters listed in Table 1, cryogels with complete gel conversion were obtained. All the cryogel samples were very tough and could be compressed up to about 100% strain without any crack development, during which water inside the cryogel was removed. Upon unloading, the compressed cryogel immediately swelled to recover its original shape. The swelling ratios and the conformational contents of the cryogels showed no significant changes, except that the swelling ratio q_w decreased with increasing fibroin concentration $C_{\rm SF}$ (Table 1). However, as will be shown in the next section, both the morphologies and the mechanical properties of freeze-dried cryogels significantly depend on the synthesis parameters.

Morphology and Mechanical Properties of Fibroin Scaffolds. Macroporous fibroin scaffolds were obtained by



Figure 4. Images of the cryogel (up) and hydrogel samples (down) formed at -18 and 50 °C, respectively, in swollen and dry states. $C_{SF} = 4.2$ wt %; EGDE = 20 mmol/g; and TEMED = 0.10%. The scaling bars of SEM images (far right) are 1 mm, while the bar of the inset is 20 μ m.



Figure 5. SEM images of fibroin scaffolds formed at -18 °C. TEMED = 0.10 (A), 0.33 (B), and 0.50% (C); $C_{SF} = 4.2$ wt %; EGDE = 20 mmol/g; and scaling bars = 10 μ m.



Figure 6. SEM images of cryogel networks formed at various temperatures, EGDE, and fibroin concentrations; see Table 1 for the synthesis parameters. Scaling bars = $100 \ \mu m$ (first and second rows) and $10 \ \mu m$ (bottom row).

freeze-drying of the cryogels and hydrogels under identical conditions. Typical images of equilibrium swollen and freezedried cryogels and hydrogels formed at $T_{\rm prep} = -18$ and 50 °C, respectively, are shown in Figure 4. The hydrogel samples were transparent (>0.10% TEMED) or translucent, indicating the existence of scattering centers for light, while all the cryogels had an opaque white color. After freeze-drying, scaffolds derived from the cryogels retained their original shape while a lateral distortion in the cylindrical shape of the hydrogel scaffolds was observed. SEM images of these samples after freeze-drying (far right in Figure 4) also show that the cylindrical shape of the hydrogel scaffold is partially destroyed due to the weak network structure. In contrast, cryogel scaffolds were mechanically stable and consisted of regular pores of sizes

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 $10^1 \,\mu$ m, which are typical for macroporous networks created by the cryogelation technique.

Different morphologies of the hydrogel and cryogel networks are attributed to the different modes of the porosity formation. The pores in the hydrogels appear after the gel formation during the freeze-drying procedure while those in the cryogels form before the onset of gelation. The frozen state of the cryogelation system produces macropores that are templated from the spaces occupied by the ice crystals. The pores are separated from each other by well-defined thick fibroin network due to the cryo-concentration during gelation which produces a high concentration of fibroin network building the pore walls. Figure 5 showing SEM images of the cryogel scaffolds formed at $T_{\rm prep}$ = -18 °C and in the presence of 20 mmol epoxide/g fibroin at a larger magnification indicate that they consist of interconnected pores. By measuring random samples of at least six pores from the images, the average pore size was calculated as $33 \pm 8 \,\mu\text{m}$, independent of the amount of TEMED at the gel preparation.

The pore size of the cryogel scaffold could be regulated depending on the cryogelation conditions. Decreasing the gelation temperature $T_{\rm prep}$, increasing EGDE or fibroin concentrations decreased the average diameter of the pores (Figure 6). For example, at $T_{\text{prep}} = -18$ °C, the average pore diameter decreased from 55 ± 10 to $23 \pm 7 \mu m$, while the poresize distribution became narrower as the amount of EGDE is increased from 10 to 30 mmol/g. This could be due to the increased rigidity of the fibroin gel in the unfrozen domains as the amount of EGDE is increased. Moreover, because lower gelation temperature means faster freezing of the reaction solution, decreasing pore size with decreasing T_{prep} is consistent with the fact that a larger number of small ice crystals form as the freezing rate is increased. Additionally, because water in large voids is preferentially frozen relative to that in small capillaries due to a smaller freezing-point depression, it is thought that, at $T_{\text{prep}} = -5$ °C, only water in large voids freezes during gelation, leading to large pores.^{48,49} A similar effect of temperature on the pore size of fibroin scaffolds was observed by Nazarov et al.¹¹ By decreasing the freezing temperature of aqueous 6% fibroin solutions from -20 to -80 °C, the pore diameter of freeze-dried scaffolds decreased from about 50 to 15 μ m. Increasing fibroin concentration C_{SF} led to scaffolds with thicker pore walls but a smaller pore size (Figure 6). For instance, at $T_{\text{prep}} = -18$ °C, the pore diameter decreased from 33 ± 10 to $10 \pm 3 \,\mu\text{m}$ as C_{SF} increased from 4.2 to 12.6%. The inverse relation between C_{SF} and the pore size is possibly related to the higher amount of unfrozen microdomains during gelation as the amount of fibroin is increased.

We estimated the total volume of the pores in the cryogel scaffolds from their weight q_w and volume swelling ratios q_v . Because the weight swelling ratio includes water locating in both pores and in the fibroin region of the gel, while, assuming isotropic swelling, the volume swelling only includes water in the fibroin gel, the larger the difference between q_w and q_v , the larger the amount of water in the pores, that is, the larger the volume of pores in the scaffolds. From the weight and volume swelling ratios of the cryogels (Table 1), the porosity *P* (volume of pores/volume of scaffold) was estimated using the following equation:³⁵

$$P = 1 - q_{\rm v} [1 + (q_{\rm w} - 1)\rho]^{-1}$$
(4)

where ρ is the density of fibroin (1.35 g/mL). *P* of all cryogel scaffolds formed at $C_{\rm SF}$ = 4.2% is 93 ± 1%, while it slightly decreases with increasing fibroin concentration (Table 1).

Mechanical properties of the scaffolds were investigated by uniaxial compression tests. Figure 7A shows typical stress-



Figure 7. Stress-strain curves of fibroin scaffolds as the dependence of the nominal stress σ on the degree of compression. (A) Freeze-dried cryogel and hydrogel samples formed at $T_{\rm prep} = -18$ and 50 °C, respectively; $C_{\rm SF} = 4.2\%$; EGDE = 20 mmol/g; and TEMED = 0.07%. (B) Freeze-dried cryogels formed at various $C_{\rm SF}$ indicated; $T_{\rm prep} = -18$ °C; EGDE = 20 mmol/g; and TEMED = 0.25%.

strain curves of cryogel and hydrogel scaffolds formed at T_{prep} = -18 and 50 °C, respectively. Three different regimes can be observed in the stress-strain curve of the cryogel scaffold. First, the curve is quite linear indicating that the porous structure remains mechanically stable in this range of strain. This linear elastic regime is followed by a near-plateau regime, indicating that the network easily deforms due to the collapse of its pores under the pressure. The critical stress corresponding to the plateau regime, denoted by $\sigma_{\rm p}$, is thus a measure of the mechanical stability of the porous structure. Finally, the steep increase of the curve in the third regime corresponds to the compression of the nearly nonporous fibroin network. Although freeze-dried hydrogels are also porous, no distinct plateau was observed in stress-strain curves, which is attributed to the weak network structure and irregularity of the porous structure.

Inspection of stress-strain curves of cryogel scaffolds revealed that the critical stress $\sigma_{\rm p}$ increases with decreasing size of the pores, that is, with decreasing $T_{\rm prep}$ down to -18 °C or with increasing EGDE or fibroin contents $C_{\rm SF}$ (Figure S2). The most striking increase in $\sigma_{\rm p}$ was observed when the fibroin concentration was increased, as seen in Figure 7B. $\sigma_{\rm p}$ increases from 0.6 to 4.4 MPa as $C_{\rm SF}$ is increased from 4.2 to 12.6%. The results thus suggest increasing mechanical stability of the porous structure of fibroin scaffolds with decreasing size of the pores. Indeed, the compressive modulus and the compressive stress measurements of the scaffolds support this finding.

The cryogel scaffolds formed at $T_{\text{prep}} = -18$ °C and $C_{\text{SF}} = 4.2\%$ exhibit a compressive modulus E of 8 ± 1 MPa over the whole range of TEMED with a compressive nominal stress σ_{comp} of 0.22 ± 0.04 MPa (Figure 8A). These values are about 1 order of magnitude higher than those of the hydrogel scaffolds formed below 0.25% TEMED ($E = 1.0 \pm 0.3$ MPa, $\sigma_{\text{comp}} = 0.030 \pm 0.004$ MPa). The variations of the modulus and strength of the cryogel scaffolds depending on the synthesis conditions follow the same trend as the plateau stress σ_{p} . Both E and σ_{comp} increase with decreasing T_{prep} or with increasing EGDE or fibroin concentrations (Figure S3). For example, the



Figure 8. (A) The compressive modulus *E* and compressive stress σ_{comp} of fibroin scaffolds shown as a function of TEMED %. Freeze-dried cryogels (red circle) and hydrogels (O) formed at $T_{prep} = -18$ and 50 °C, respectively; $C_{SF} = 4.2\%$ and EGDE = 20 mmol/g. (B) *E* and σ_{comp} of cryogel scaffolds shown as a function of fibroin concentration C_{SF} in the feed; $T_{prep} = -18$ °C; EGDE = 20 mmol/g; and TEMED = 0.25%.

modulus E increased from 2 to 15 MPa as $T_{\rm prep}$ is decreased from -5 to -18 °C; a further decrease in \hat{T}_{prep} to -22 °C decreased again the modulus to 11 MPa. We have to mention that the plateau stress also decreased in the range of T_{prep} between -18 and -22 °C (Figures S2 and S3). This may be attributed to the glass transition of fibroin (below -20 °C) slowing down the reactions.^{11,14} Drying conditions of the cryogels slightly affected the strength of the resulting scaffolds. Drying at 24 °C under vacuum instead of freeze-drying decreased the size of the pores and increased the modulus Efrom 8 to 10 MPa (Figure S4). The most significant variations in the modulus and strength of the cryogel networks were observed by changing fibroin concentration C_{SF} at the gel preparation. As illustrated in Figure 8B, E increases from 7 to 48 MPa, while $\sigma_{\rm comp}$ increases from 0.2 to 1 MPa as $C_{\rm SF}$ is increased from 4.2 to 12.6%. Comparison of the SEM images with the modulus data clearly show that a decrease in the average pore diameter increases the mechanical stability of fibroin scaffolds formed by cryogelation.

Previous work shows that fibroin scaffolds formed by freezedrying of fibroin solutions pretreated with alcohols exhibit compression moduli of 10 to 100 kPa, while salt leaching method generates scaffolds with a modulus of 0.5 MPa.¹¹ The largest compressive modulus and compressive strength reported so far for fibroin scaffolds are 3 MPa and 60 kPa, respectively, which were produced by gas foaming technique.¹¹ Cryogel scaffolds prepared in this study by the cryogelation technique and using 12.6% fibroin in the feed exhibit 16-fold larger modulus and strength (48 \pm 10 MPa and 970 \pm 50 kPa, respectively). The extraordinary strength of the cryogel scaffolds originates from the high fibroin concentration of the pore walls; gelation in frozen solutions confines fibroin in a small region of the reaction volume forming the pore walls of the final material. This also provides a high degree of toughness to cryogels in their swollen states.

CONCLUSIONS

Silk fibroin cryogels could be obtained from frozen fibroin solutions (4.2–12.6 w/v %) at subzero temperatures between -5 and -22 °C using EGDE as a cross-linker. EGDE triggers the conformational transition of fibroin from random coil to β -sheet structure and hence fibroin gelation. One of the unique features of fibroin cryogels is their elasticity that allows them to resist complete compression without any crack development,

during which water inside the cryogel is removed. The compressed cryogel immediately swells during unloading to recover its original shape. The reversibility of the compression cycles between 0 and 99.8% compressions suggests that fibroin cryogels can be used in separation processes. The scaffolds obtained by freeze-drying of the cryogels consist of regular, interconnected pores of diameters ranging from 50 to 10 μ m, depending on the synthesis parameters. The mechanical compressive strength and the modulus of the scaffolds increase with decreasing pore diameter, that is, with a decrease in the gelation temperature or with an increase of fibroin or EGDE concentrations at gelation. The scaffolds produced at 12.6% fibroin exhibit a very high compressive modulus (50 MPa), making them good candidates as bone scaffold materials.

ASSOCIATED CONTENT

Supporting Information

Figure S1, ATR-FTIR spectra of fibroin scaffolds together with the curve fitting results; Figure S2, stress—strain curves of the scaffolds formed at various $T_{\rm prep}$ and EGDE contents; Figure S3, *E* and $\sigma_{\rm comp}$ of the scaffolds formed at various $T_{\rm prep}$ and EGDE contents; Figure S4, SEM images and stress—strain curves of the scaffolds depending on the drying conditions; and the movie showing the compression of the cryogel and hydrogel samples prepared at $T_{\rm prep} = -18$ and 50 °C, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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