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Preparation of 3-D regenerated fibroin scaffolds with freeze drying method and freeze drying/foaming technique

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Abstract Although three-dimensional fibroin scaffolds have been prepared with freeze drying method, the porosity and pore sizes still can not satisfy the requirement of tissue engineering. In this article, fibroin porous scaffold with high porosity and >100 μm diameter interconnected pores was firstly prepared with freeze drying method through adjusting fibroin concentration. The morphology of different scaffolds lyophilized from different fibroin concentration was observed by SEM. A novel freeze drying improved method, freeze drying/foaming technique, was also devised to prepare fibroin scaffolds at different fibroin concentrations. Using the said method, the porosity and pore size of fibroin scaffolds prepared from 12% concentration were $85.8 \pm 4\%$ and 109 ± 20 μm respectively with yield strength up to 450 ± 6 KPa while the porosity and pore size of fibroin scaffolds prepared from 8% concentration were $96.9 \pm 3.6\%$ and 120 ± 30 μm respectively with yield strength up to 30 ± 1 KPa. The freeze drying/foaming technique produced scaffolds with a useful combination of high yield strength, interconnected pores, and pore sizes greater than 100 μm in diameter. Through adjusting fibroin concentration and thawing time, the porosity, pore sizes and mechanical properties could be controlled to satisfy the different requirements of tissue engineering. The results suggested that fibroin scaffolds prepared with the above methods could be formed for utility in biomaterial application.

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Introduction

Tissue engineering has emerged as a potential alternative to tissue or organ transplantation [1, 2]. Porous three-dimensional scaffolds, providing a framework for cells to attach, proliferate and form extracellular matrix, play an important role in manipulating cell functions in this approach [3]. To fulfill these functions, three-dimensional porous matrix should have enough mechanical stability to support cell adhesion and expansion and to degrade at a rate comparable with new tissue growth. It should also have high porosity and >100 μm diameter interconnected pores to provide sufficient opportunity for cell migration and expansion, which is generally considered to be a minimum requirement of tissue engineering [3, 4].

Silks, natural fibers produced by the silkworm, Bombyx mori, have been used traditionally in the form of threads in textiles and sutures for thousands of years [5, 6]. Because of its excellent biological compatibility and mechanical properties, silk fibroins, thread core protein of silk, have also been explored for many other biomedical applications including osteoblast, hepatocyte and fibroblast cell support matrixes and for ligament tissue engineering [7–9]. All these applications needed silk fibroin to form porous three-dimensional scaffolds. So, preparation of fibroin three-dimension scaffold, having high porosity with >100 μm diameter interconnected pores, certainly facilitated the use of silk fibroin in tissue engineering.

Several methods have been developed to fabricate porous biodegradable polymer scaffolds including fiber bonding [10], solvent casting/particulate leaching [4, 11], three-dimensional printing [12], gas forming [13], freeze drying [14], and phase separation [15]. The freeze drying method is one of the most commonly used methods. Recently, freeze-drying method was used to prepare three-dimensional fibroin

scaffolds [16]. However, Liming Zhong *et al*. [14] reported that the porosity of fibroin scaffolds prepared by freeze drying method was below 70% while the pore sizes in fibroin scaffold prepared by Rina Nazarov *et al*. [16] were only about 50 μm with freeze-drying. Both the freeze dried scaffolds were unsuitable for cell migration and expansion.

In this study, fibroin scaffold, having high porosity and >100 μm interconnected pores, was firstly prepared by freeze dying method when fibroin concentration was 6%. More importantly, an improved freeze drying method, named as freeze drying/foaming technique, was devised to prepare fibroin scaffolds suitable for biomedical application. Different fibroin matrixes, having high porosity and >100 μm interconnected pores were prepared at different concentrations.

Experimental

Preparation of regenerated fibroin solution

Bombyx mori silk fibroin was prepared just as described in our earlier procedure [17]. Silk was boiled for 1 h in an aqueous solution of 0.5 wt% Na_2CO_3 and then rinsed thoroughly with water to extract the sericin proteins. The degummed silk was dissolved in $CaCl_2/H_2O/CH_3CH_2OH$ solution (mole ratio, 1/8/2) at 80°C. Then the fibroin solution was filtered and dialyzed against distilled water for 3 days to yield fibroin water solution. The final concentration of the aqueous silk fibroin solution was about 3–4 wt%, which was determined by weighing the remaining solid after drying.

Three-dimensional scaffold fabrication by freeze drying

The fibroin solutions with 4 wt%, 6 wt%, 8 wt% and 12 wt% concentrations were obtained through concentrating the above fibroin solution at 50–55°C with stirring. These solutions were put into the glass disks and then frozen in refrigerator at −20°C for 12 h. The ice/silk composites were then lyophilized leaving a porous matrix. After the drying porous matrixes were obtained, they were immersed in methanol for about 1 h to induce crystallization and insolubility in water. The insoluble fibroin three-dimensional scaffolds were then prepared after methanol evaporated at room temperature.

Three-dimensional scaffold fabrication by freeze drying/foaming technique

The fibroin solutions with 8 wt% and 12 wt% concentrations were obtained through concentrating the above fibroin solution at 50–55°C with stirring. These solutions were put into the glass disks and then frozen in refrigerator at −20°C for 12 h. Unlike freeze drying method, the ice/silk composites were firstly placed in atmosphere at 20°C for different minutes to make them partly thaw and then lyophilized leaving a porous matrix. After experiments, it was found that the appropriate thawing times were 3 min and 8 min respectively for 8 wt% and 12 wt% fibroin concentrations. The said porous matrixes were immersed in methanol for about 1 h to induce crystallization and insolubility in water. The insoluble fibroin three-dimensional scaffolds were then prepared after methanol evaporated at room temperature.

SEM

The surface and cross-section morphologies of fibroin scaffolds and pore distributions, sizes, and interconnectivity were observed with JEOL JSM-6460LV SEM. Segments of the outer surface and inner area of the scaffold were prepared by fracture of scaffolds in liquid nitrogen. The specimens were sputter coated with gold. The pore sizes were determined by measuring random samples of 10 pores from the SEM images.

Density and porosity

The density and porosity of the scaffolds were measured by liquid displacement. Just as described by Rina Nazarov [16], hexane was used as the displacement liquid and the procedure was as follows. A sample of weight W was immersed in known volume (V_1) of hexane in a graduated cylinder. The sample was left in the hexane covered for 5 min. The total volume of the hexane and the hexane-impregnated scaffold was V_2. The hexane-impregnated scaffold was then removed from the cylinder and the residual hexane volume was recorded as V_3. The density (d) of the scaffold is expressed as

$$d = W/(V_2 - V_3)$$

and the porosity of the scaffold (ε) was obtained by

$$\varepsilon = (V_1 - V_3)/(V_2 - V_3)$$

ATR-FTIR

The infrared spectra of silk fibroin structures were measured with an ATR-FTIR (NICOLET 560, American) spectrophotometer. Each spectrum for samples was acquired by accumulation of 256 scans with a resolution of 4 cm^{-1}.

Mechanical properties

The compression modulus of the scaffold was evaluated at room temperature on an Instron-6022 instrument. The cross-head speed was set at 2 mm/min. Cylinder-shaped samples were 9 mm in diameter and 10–20 mm in height. Since

the scaffolds were ductile and spongelike in behavior, the cells of the scaffold no longer maintained their shape when the maximum compression was reached. So the yield strength rather than the compress strength was used to express the mechanical property in this study.

Results

Morphology of scaffold prepared with freeze drying method

The different scaffolds were obtained by adjusting fibroin concentration before freezing. Figure 1 shows SEM images of freeze-dried scaffolds dried from 12% fibroin solution. The general size of pores observed was 81 ± 63 μm. The pore structure was not highly interconnected; both open and closed pore structures were observed. Most fibroin was aggregated to sheet like morphology which could also be apparently seen on the surface of scaffold. A skin layer formed on the surface. Under the skin layer, some parallel flake-like layers were also observed.

The morphologies of the scaffold dried from 8% fibroin solution were shown in Fig. 2 The general size of pores observed was 88 ± 80 μm. In the cross section of fibroin scaffold, there were two different structures, an interconnected pores structure with diameters in the range of 90 ± 75 μm and a more condensed and compact pores structure in which 10–20 μm disconnected small pores distributed in big pores with diameters of 85 ± 51 μm. A flake-like layer formed on the surface of the scaffold, which had many pores with a size about 50 μm. The above results indicate that the pore size distribution of fibroin scaffolds dried from 8% and 12% fibroin solution has so large scope that it is difficult to be statistically justifiable. The results also indicated the scaffold prepared from 8% and 12% fibroin solution apparently has great difficulty to be used in tissue engineering.

When fibroin concentration decreased to 6%, the suitable three-dimensional porous scaffold for tissue engineering was succeed to prepare using drying method. As shown in Fig. 3, the scaffold had highly interconnected and porous structures. The general size of pores observed was appropriate for cell migration and expansion, with diameter of 151 ± 40 μm. In the preparation process, only the upper surface formed a layer that can be automatically separated from scaffold after the scaffold was lyophilized. Both of upper and bottom surface morphologies were porous structure with >100 micron diameter pores.

Figure 4 shows the morphology of scaffold prepared from 4% fibroin solution. When fibroin concentration decreased to 4%, the fibroin scaffold prepared with freeze drying method

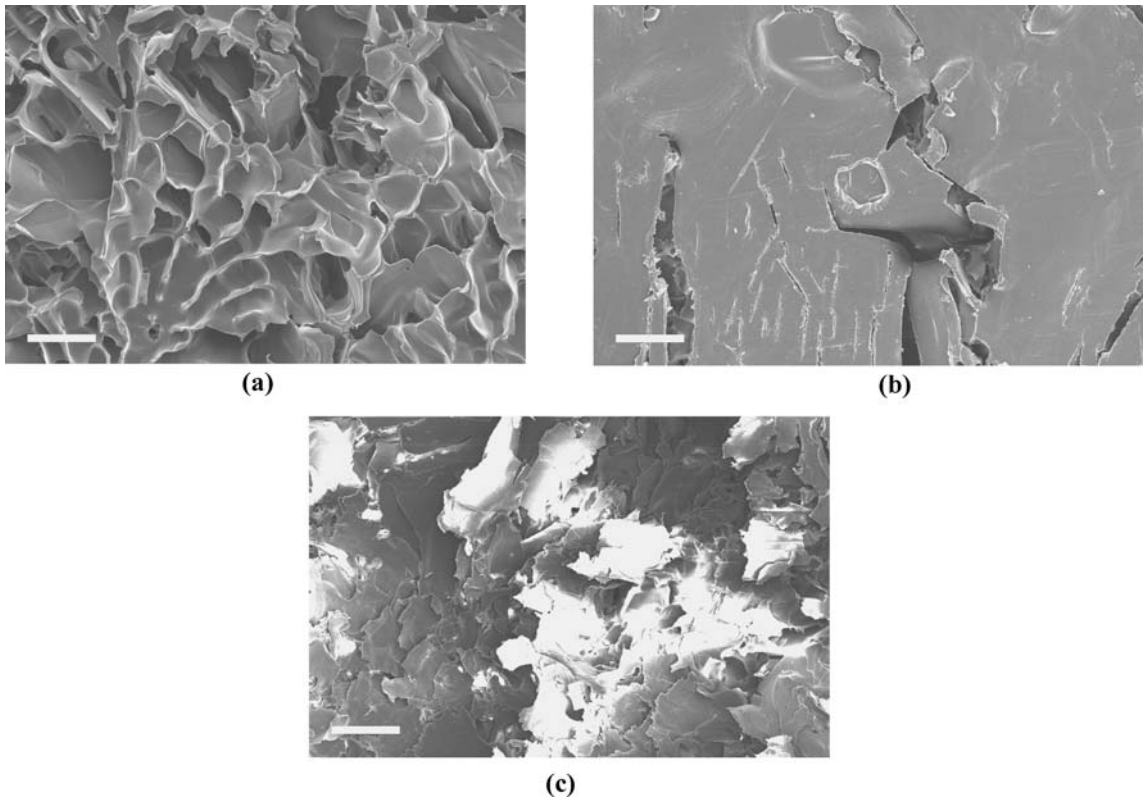

Fig. 1 SEM images of the cross section and surface structure of silk scaffold from 12% solution: (a) the cross section morphology (scale bar, 100 μm) and (b), (c) the surface morphology (scale bar, 100 μm).

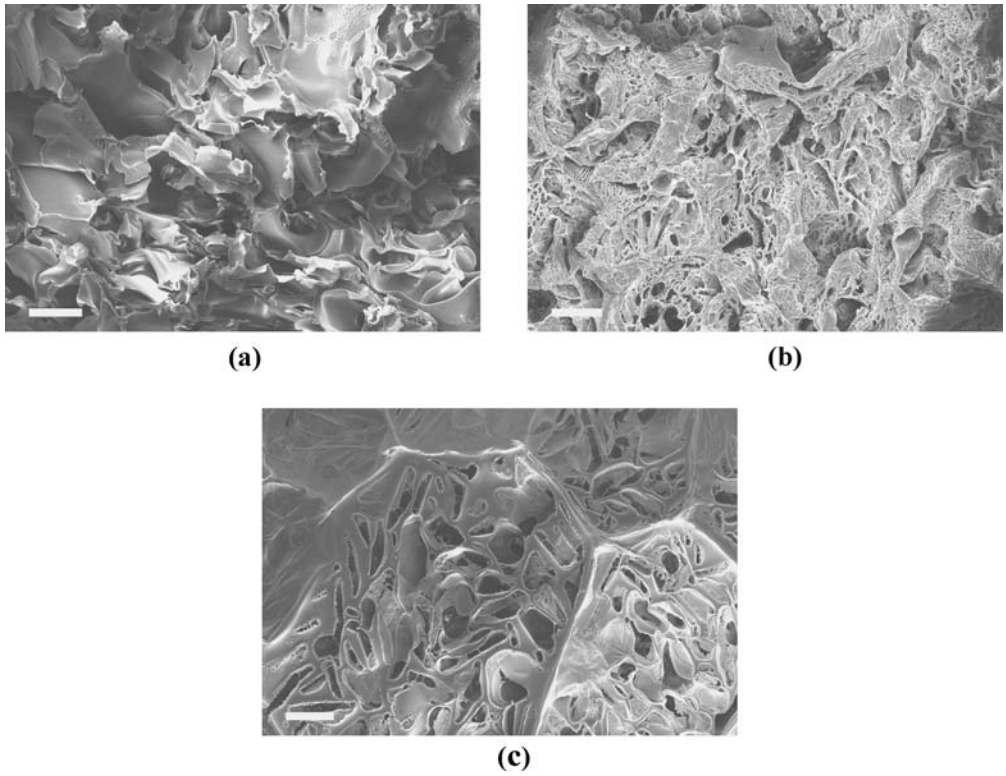

Fig. 2 SEM images of the cross section and surface structure of silk scaffold from 8% solution: (a), (b) the cross section morphology (scale bar, 100 μm), and (c) the surface morphology (scale bar, 100 μm).

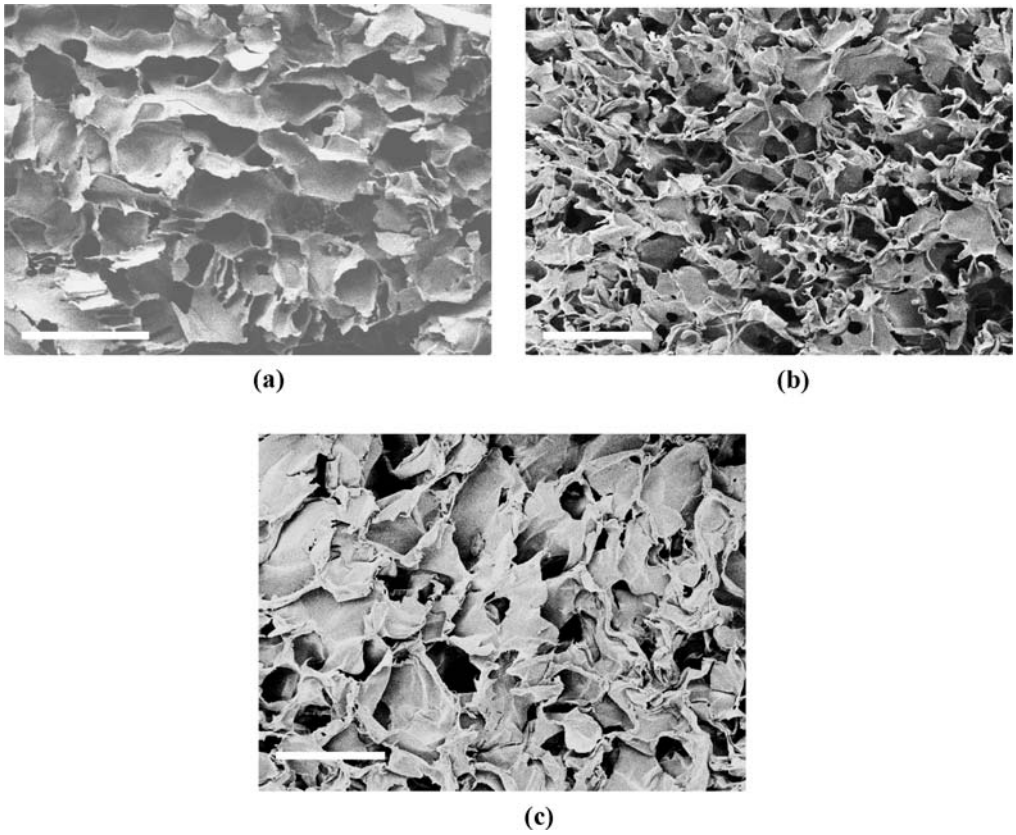

Fig. 3 SEM images of the cross section and surface structure of silk scaffold from 6% solution: (a) the cross section morphology (scale bar, 300 μm), and (b), (c) the surface morphology (scale bar, 300 μm).

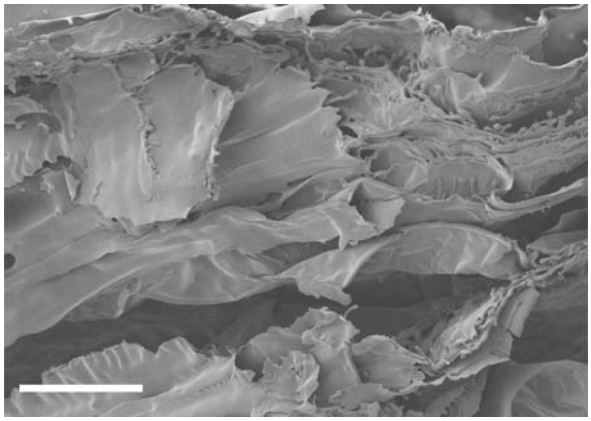

Fig. 4 SEM images of silk scaffold from 4% solution (scale bar, 300 μm).

aggregated to nearly separated sheets. The scaffold was brittle. If some action were applied to the scaffold, it easily became the separated sheets.

Morphology of scaffold prepared by freeze drying/ foaming technique

When the novel method, freeze drying/foaming technique was used to prepare fibroin scaffold, the morphology of fibroin scaffolds evidently changed. As shown in Figs. 5 and 6, the general pore size became 120 ± 30 μm and 109 ± 20 μm when fibroin concentrations were 8% and 12%, respectively. On the other hand, the flake-like layer formed on the surface of the scaffold prepared with freeze drying method from 12% and 8% fibroin solution also disappeared in fibroin scaffolds following the introduction of freeze drying/foaming technique. The results indicated that preparation of fibroin scaffolds suitable for tissue engineering become available in 6%–12% fibroin concentrations.

Porosity

The porosity of fibroin scaffolds prepared increased with the decrease of fibroin concentration (Table 1). The porosity was $87.6 \pm 2.0\%$ when fibroin concentration was 6%, while the

Table 1 Porosity and density of the scaffolds from different fibroin concentration

Fibroin concentration (wt%)	ε(%)[a]	d[b]	Pore size (μm)
12	75 ± 2.0	196 ± 8	81 ± 63
8	77.7 ± 1.6	148 ± 5	88 ± 80
6	87.6 ± 2.3	91.4 ± 2	151 ± 40

[a]Porosity.
[b]Density(mg/ml).

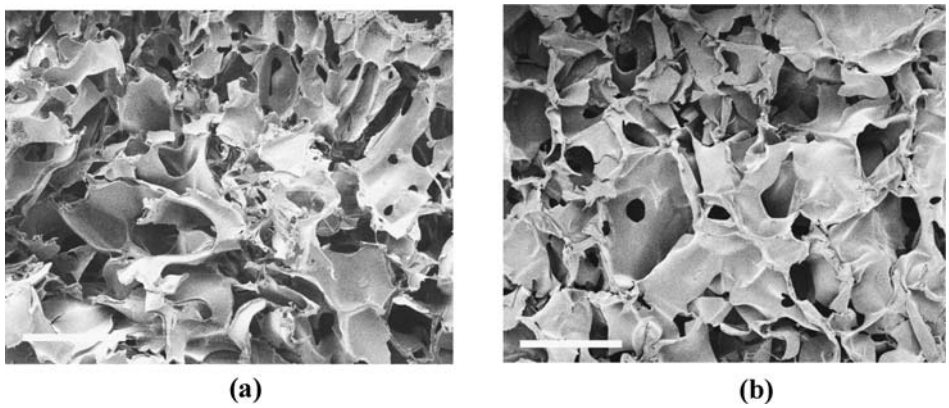

Fig. 5 SEM images of the cross section and surface structure of silk scaffold prepared from 8% solution when the thawing time at 20°C was 3 min: (a) the cross section morphology (scale bar, 300 μm), and (b) the surface morphology (scale bar, 300 μm).

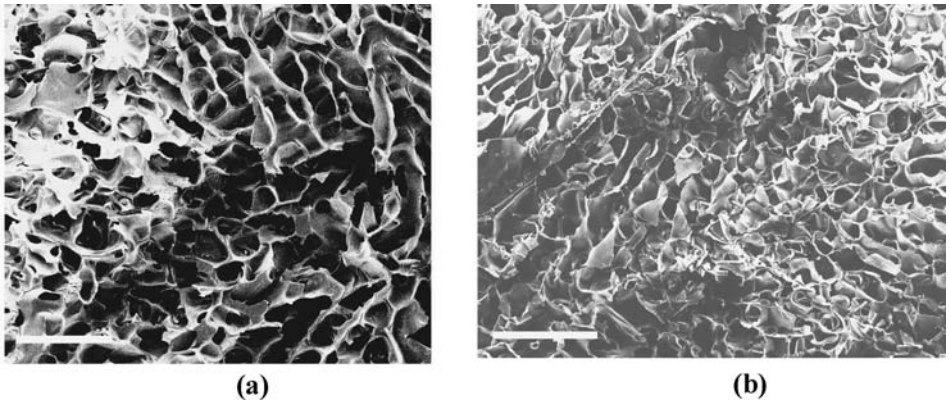

Fig. 6 SEM images of the cross section and surface structure of silk scaffold prepared from 12% solution when the thawing time at 20°C was 8 min: (a) the cross section morphology (scale bar, 300 μm), and (b) the surface morphology (scale bar, 300 μm).

Table 2 Porosity and density of the fibroin scaffolds with freeze drying/foaming technique

Fibroin concentration (wt%)	Thawing time (min)	ε(%)[a]	d[b]	Pore size (μm)
12	8	85.8 ± 4%	140 ± 7.5	109 ± 20
8	3	96.9 ± 3.6%	87.7 ± 2.6	120 ± 30

[a]Porosity.
[b]Density (mg/ml).

porosities of scaffolds obtained from 8% and 12% fibroin solution were both under 80%.

Although the porosities of scaffolds obtained from 8% and 12% fibroin solution were both under 80% through lyophilization, the porosity of scaffold obtained from 12% fibroin solution evidently increased from 75% to 85.8% while the porosity of scaffold from 8% fibroin solution increased from 77.7% to 96.9% when freeze drying/foaming technique was used in preparation of fibroin scaffold (Table 2).

ATR-FTIR

Since ATR-FTIR spectra of fibroin scaffold can be influenced by surface morphology, FTIR spectra of the scaffold obtained from 6% fibroin solution can not be obtained. Moreover, the difference of spectra between fibroin scaffolds was unsuitable to examine the slight structure change following the decrease of fibroin concentration. However, from FTIR spectra shown in Fig. 7, the β-sheet structure was still observed for all samples immersed in methanol with peaks at 1614 and 1505 cm^{-1}. The results indicated that methanol really induced the β-sheet structure formation and made the three-dimensional scaffold insoluble in water.

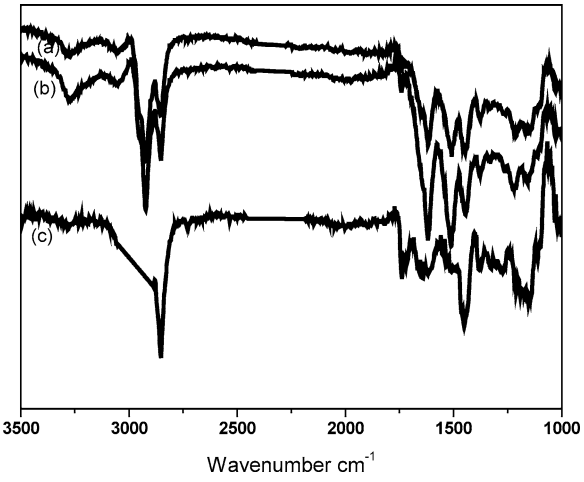

Fig. 7 ATR-FTIR of silk fibroin scaffold from different concentrations: (a) fibroin concentration 20%, (b) fibroin concentration 12% and (c) fibroin concentration 8%.

Table 3 The yield strength of different scaffolds

Fibroin concentration (wt%)	Thawing time (min)	Yield strength (KPa)
6%	0	20 ± 2
8%	0	560 ± 32
8%	3	30 ± 1
12%	0	760 ± 57
12%	8	450 ± 6

Mechanical properties

The structure of freeze-dried scaffolds was foam like and very porous. Unlike fibroin scaffolds prepared by Rina Nazarov *et al.* [16], fibroin scaffolds prepared in our studies were much ductile, which make fibroin scaffold difficult to be broken under the compressive stress. So, the yield strength rather than the compress strength was used to express the mechanical property of fibroin scaffold.

As shown in Table 3, the yield strength of freeze-dried fibroin scaffolds increased following the increase of fibroin concentration. The yield strength of scaffold obtained from 6% fibroin solution was only 20 ± 1 KPa while that of scaffolds obtained from 8% and 12% were 560 ± 32 KPa and 760 ± 57 KPa respectively. When freeze drying/foaming technique was used to prepare scaffold in 8% solution with 3 min of thawing time, the yield strength decreased to 30 ± 1.2 KPa. Interestingly, the yield strength of scaffold obtained from 12% fibroin solution in 8 min of thawing time was 450 ± 10 KPa which is much higher than other fibroin scaffold having >100 μm diameter interconnected pores. The reasons for this difference in mechanical property may be the uniform distribution of pores within the scaffolds from 12% fibroin solution with freeze drying/foaming technique which lead to more consistent mechanical property and the differences in the pore sizes themselves.

Discussion

The pore size and porosity of fibroin scaffold can be affected by many process conditions, such as freezing temperature, concentration, freezing rate, and so on. Rina Nazarov [16] and Liming Zhong [14, 18] both prepared porous three dimensional scaffolds from regenerated silk fibroin with freeze

drying method and studied the effects of conditions. Although these three-dimensional fibroin scaffolds previous prepared with lyophilization were unsuitable for cell migration, the relationship between temperature and formation of pores was very useful for our research to obtain porous scaffold with >100 μm micron diameter pores using similar method. Just as previous report, the glass transition zone of fibroin in an aqueous solution is −20 to −34°C. The higher the freezing temperature above the glass transition the longer it will take for ice to form and grow. Therefore, the longer freezing time then the larger the pores [14, 16]. Moreover, fibroin has the spontaneity of forming leaf or sheet morphology in fibroin solution at freeze process. The lower the concentration of fibroin solution, the easier fibroin form large sheet and pore morphology. So, the concentration and the freezing temperature should be elaborately adjusted to make porous scaffold have the suitable pores for cell migration. Fig. 1 to Fig. 4 show the different morphology of fibroin scaffold with different concentrations at −20°C. The size of diameter increases following the decrease of fibroin concentration. When fibroin concentration decreased to 4%, fibroin formed the separate sheets rather than three-dimensional scaffold after lyophilization. Importantly, the three-dimensional fibroin scaffold, having pore structures with 87.6% porosity and >100 μm diameter, can be successfully prepared with freeze drying method when fibroin concentration was 6%. So freeze drying method was also a useful and simply method for formation of three-dimensional fibroin scaffold.

Although freeze drying method was really able to prepare fibroin scaffold used for tissue engineering, the morphology of scaffold was so sensitive to fibroin concentration that we had to confine fibroin concentration in a narrow scope which certainly increased the difficulty to control three dimensional fibroin scaffolds. Considering that Rina Nazarov *et al*. suggested the gas foaming were suitable method to prepare fibroin scaffold, we devised a novel method that blended freeze drying method and gas foaming to prepare fibroin scaffolds. When the ice/silk composites of different fibroin concentrations formed in −20°C, the composites were firstly placed in air at 20°C for several minutes before lyophilization to make the composites partly thaw rather than immediately lyophilized. Through adjusting the thawing time, the thawing portion and the ice portion achieve a balance, therefore, the pore size and porosity could both increase while the whole structure of ice/silk composites preserved in lyophilization. Although the effect of thawing time and temperature should further be investigated, our present study indicated that the freeze drying/foaming technique was a simple and useful method to prepare fibroin scaffold for tissue engineering.

Conclusion

The fibroin scaffolds having both high porosity and >100 micron diameter interconnected pores were firstly formed by freeze drying method. The porosity was 87.6% and the diameter was 151 ± 40 μm when fibroin solution was 6%. More importantly, a novel method, named as freeze drying/foaming technique, was devised to prepare fibroin scaffolds. The different fibroin scaffolds suitable for tissue engineering were succeeded to form from different fibroin concentrations. Through adjusting fibroin concentration and thawing time, the porosity, pore sizes and mechanical properties could be controlled. Considering that fibroin has the excellent biocompatibility and freeze drying relative methods were simple and gentle scaffold preparation methods, our research will inevitably facilitate the applications of fibroin in tissue engineering.

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