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Preparation of 3-D porous fibroin scaffolds by freeze drying with treatment of methanol solutions

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In this study, silk scaffolds with appropriate porous structures were prepared by adjusting solution concentrations and providing treatment with methanol solutions in the way of freeze drying. The effects of the preparation conditions on the microstructures and properties of the scaffolds were discussed. Fibroin solutions with different concentrations of 4, 6, 8, 10 wt% were used respectively to prepare the scaffolds. The effects of the addition of 20 vol% methanol before or after freeze drying to the 4 wt% fibroin solution were investigated. As demonstrated by Scanning Electron Microscope (SEM), the fibroin scaffolds prepared without methanol had porous microstructures composed of thin sheets, and the sizes of the pores decreased with the increase of the fibroin solution concentrations, while the scaffolds prepared in the presence of methanol showed porous microstructures formed by fine-particle aggregates. The porosities and mechanical properties of the prepared fibroin scaffolds under different conditions were tested. The crystalline structures and conformations of the fibroin scaffolds were detected by Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD).

fibroin, porous scaffold, freeze drying, methanol

With the development of life science and material science, a novel discipline has emerged, named tissue engineering. It has attracted much attention of the scientists nowadays since it can serve to rehabilitate and reconstruct the wound area. Scaffolds, cells and growth factors are the three essential factors in tissue engineering. Among them, biocompatible and degradable scaffold is one of the crucial factors to the industrialization of tissue engineering projects. In order to provide enough space and surface areas for the cells to migrate into and adhere to the scaffolds, as well as to provide good mechanical properties, a 3-D porous structure of the scaffold with appropriate pore size and porosity is desired.

Silk fibroin has been reported to be a kind of promising biomaterial with good biocompatibility. It has been used as surgical suture[1−3], the protective material for burn wounds[4], artificial skins[5,6] and the substitute for articular cartilage lesions[7], etc. Epidermal cells, fibroblasts and chondrocyte can grow well on the fibroin[7−9].

Recently, some studies have been reported on the preparation of porous fibroin scaffolds in different ways, such as freeze drying, salt leaching and foaming[10]. Among them, freeze drying method is a simple and effective way. Scaffolds made of fibroin without further treatment usually have leaf or sheet like morphologies with relatively smooth surfaces, which is unfavorable for the cells to adhere[11,12]. Additionally, the connectivity of the pores in these sheet-like structures is not good enough for the cells to migrate. On the other hand, it is reported[13] that treatment with methanol could change the confirmation of the fibroin, which could re-

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sult in the change of the structure and morphology of the fibroin scaffold.

In this study, 3-D silk scaffolds with appropriate porous structures were prepared by freeze drying method. The effects of the fibroin solution concentration, the freezing temperature and the addition of methanol during the preparation on the microstructures and properties of the scaffolds were discussed.

1 Experimental

1.1 Preparation of the fibroin solution

The *Bombyx mori* domestic silk was dissolved in the 0.05% Na_2CO_3 solution at 100℃ for 60 min. Then the silk was rinsed thoroughly with water to obtain the fibroin fiber. The fibroin solution was obtained by dissolving the fibroin fiber in the mixing solution of $CaCl_2$∶CH_3CH_2OH∶H_2O (molar ratio of 1∶2∶8) for 60 min at 80℃. The obtained solution was dialyzed against deionized water to remove excess calcium chloride and ethanol. The deionized water was changed every 4 h for 3 d. And then, the fibroin solution was concentrated at 40℃ to get fibroin solutions with different concentrations of 4, 6, 8, 10 wt% respectively.

1.2 Preparation of 3-D porous fibroin scaffolds

The processing procedures and the nominating of the samples are listed as follows:

1. The solutions obtained at room temperature above were put in a freezer at −16℃ before they were lyophilized for 48 h to get the porous scaffolds. The samples were nominated as 4/0/0, 6/0/0, 8/0/0 and 10/0/0 corresponding to the fibroin solution concentrations of 4%, 6%, 8% and 10% respectively.

2. The 4% fibroin solution was mixed with 20% methanol (volume percentage to the fibroin solution). The followed freeze drying process was the same as listed above. This sample was labeled as 4/20/0.

3. When samples 4/0/0 and 4/20/0 immerged in the methanol solution and were lyophilized again, two new porous scaffolds were obtained, which were labeled as 4/0/20 and 4/20/20 respectively.

4. In order to investigate the effect of the freezing temperature on the structures of the scaffolds, the procedure of putting the fibroin solutions obtained at room temperature in the freezer at −16℃ in preparing samples 4/0/0 and 4/20/0 was substituted by immersing the solutions in liquid nitrogen. These two samples were nominated as 4/0/0N and 4/20/0N respectively.

1.3 Characterization

Powder X-ray diffraction patterns were recorded at a scanning rate of 2°/min by using a D8-Discover automatic diffractometer with Cu K_α radiation (λ=1.5418 Å). The tube voltage was 40 kV and the tube current was 40 mA. Infrared spectra were taken using a NICOLET 560 Fourier transform IR spectrometer in the range of 4000−400 cm^{-1}. SEM images were taken on a JSM-6301F scanning electron microscope operating at 20 kV. Mechanical properties were tested on the WDW3020 electronic universal testing machine equipped with 0.1 kN load cell at room temperature. The crosshead speed was 0.5 mm/min, and an initial pressure of 2 N was exerted. Then the compressive modulus and the compressive strength were worked out based on the obtained data. The density and porosity of the silk scaffolds were measured by liquid displacement[10]. Hexane was used as the displacement liquid since it is a nonsolvent for silk and is able to easily permeate through the scaffold, not causing swelling or shrinkage, unlike ethanol. The porosity (ε) of the foam is expressed as $\varepsilon = (V_1 - V_3)/(V_2 - V_3)$, where V_1, the original volume of hexane; V_2, the total volume of the hexane and the hexaneimpregnated scaffold; V_3, the residual hexane volume after removing the hexaneimpregnated scaffold.

2 Results and discussion

2.1 Influences of the fibroin concentration on 3-D porous scaffolds

The 3-D scaffolds prepared by freeze-drying method have uniform morphologies macroscopically. SEM images of the cross sections of the samples prepared from various fibroin concentrations are shown in Figure 1. It can be seen that all the samples had porous structures composed of leaf-like sheets. The pore sizes of samples 4/0/0, 8/0/0 and 10/0/0 are in the ranges of 47.22−78.64, 32.55−70.83 and 30.48−56.50 μm respectively. And the corresponding porosities of these scaffolds were 86.7%, 82.1% and 79.4%. It can be figured out that with the increase of the fibroin concentration, the size of the pores decreased, while the density of the pores increased. It is reported[14] that the movement of the water molecules during the freezing process was resisted with the network formed by the fibroin molecules so that smaller ice participles were formed at higher fibroin concentra-

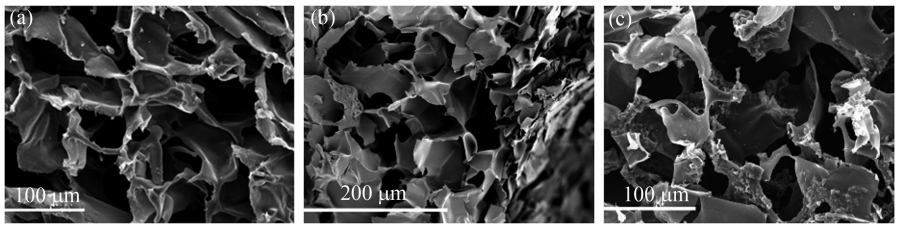

Figure 1 SEM images of the cross sections of the samples prepared from various fibroin concentrations. (a) 4%; (b) 8%; (c) 10%.

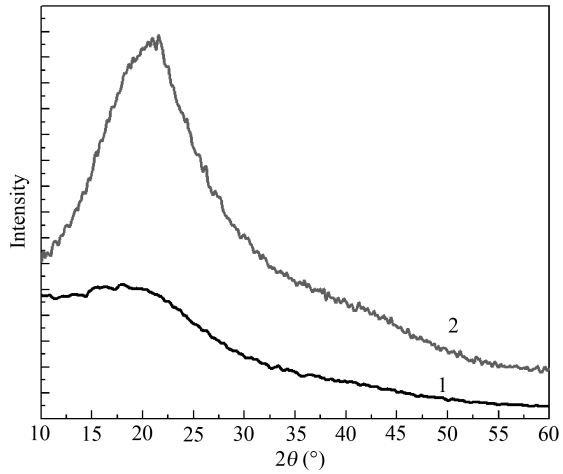

Figure 2 X-ray diffraction patterns of the samples prepared from different fibroin concentrations. 1, 4/0/0; 2, 10/0/0.

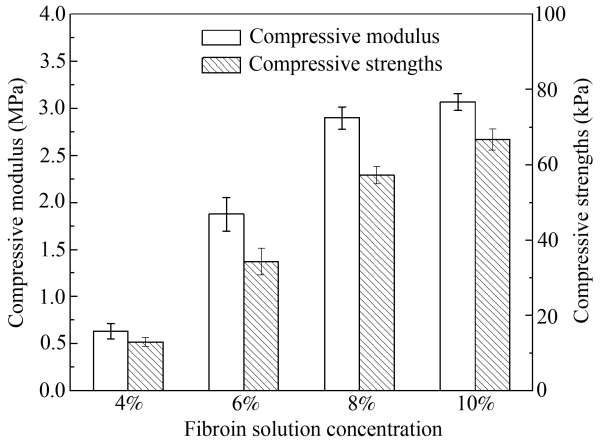

Figure 3 Mechanical properties of the samples prepared from different fibroin concentrations.

tion, which produced smaller pores and higher density after freezing process. This was consistent with our experimental results.

The conformation and the crystalline structure of samples 4/0/0 and 10/0/0 were examined by XRD and the diffraction patterns are shown in Figure 2. The diffraction peak at $2\theta = 20.96°$, corresponding to the reflection of (201) plane of β-sheet crystalline structure[13], was much sharper for sample 10/0/0 than sample 4/0/0. This indicated a better β-crystalline structure for sample 10/0/0.

The mechanical properties of the porous samples prepared from different fibroin concentrations are listed in Figure 3. Samples 4/0/0, 6/0/0, 8/0/0 and 10/0/0 had compressive strength of 13.04, 34.36, 57.25 and 66.84 kPa and compressive moduli of 0.63, 1.88, 2.90 and 3.07 MPa respectively. It was illustrated that both the compressive strength and the compressive modulus would rise considerably as the fibroin concentration increased from 4% to 10%. The rise of the mechanical properties could be attributed to the increased pore wall sites induced by the decreased pore size as observed in SEM images, which would give more paths for distribution to the applied stress and function as a barrier against crack propagation[14].

2.2 Influences of methanol treatment

When methanol was added, the morphologies of the samples changed a lot as shown in Figure 4. Compared with the smooth surface and the uniform pore structure in sample 4/0/0, sample 4/20/0 showed a more connective porous structure composed of coarse sheets with small holes on them. Although the compressive strength and compressive modulus, which are 1.10 kPa and 0.07 MPa respectively, are lower than those of 4/0/0, the porous structure of 4/20/0 looks better than 4/0/0. This may provide a better candidate scaffold for more cells to adhere to and migrate into it. The morphology of sample 4/0/20 was quite different from the morphologies of samples 4/0/0 and 4/20/0. Solid structure with concavities of diameter around 100 μm was observed in sample 4/0/20, and nanopores of diameter of several hundred nanometers were dispersed in the solid structure homogeneously. In sample 4/20/20, most of the leaf-like sheets were maintained, while many particles of diameter of several micrometers formed clusters on the surface of the sheets.

The structures of the samples were investigated by FTIR spectra shown in Figure 5. The intensity of the

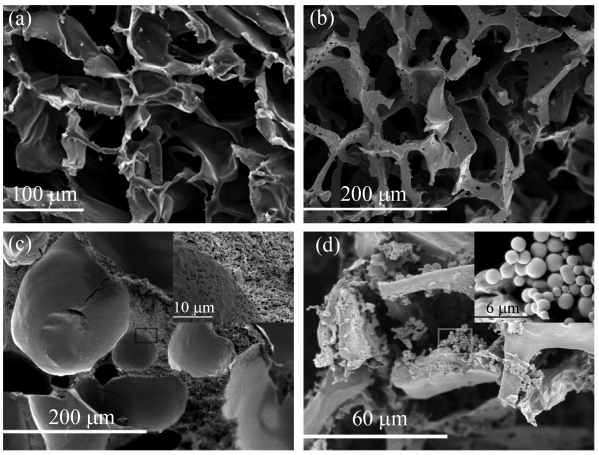

Figure 4 SEM images of fibroin with and without the treatment of methanol (20% *v/v*). (a) 4/0/0; (b) 4/20/0; (c) 4/0/20; (d) 4/20/20. The insets in (c) and (d) are the magnified pictures taken from the area in the square.

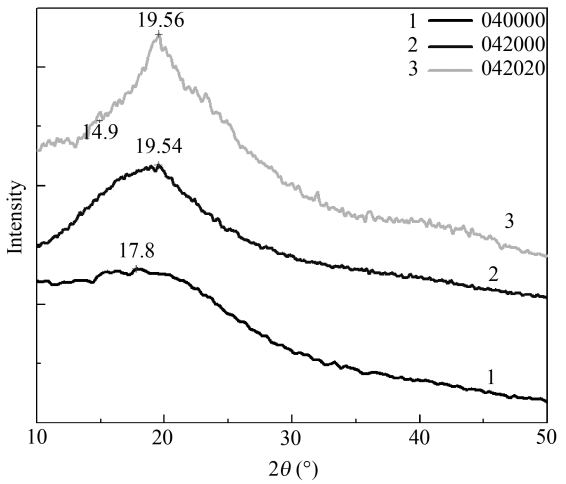

Figure 6 X-ray diffraction patterns of the samples with or without the treatment of methanol. 1, 4/0/0; 2, 4/20/0; 3, 4/20/20.

absorption bands due to amine I (1620—1660 cm^{-1}), amine II (1525—1545 cm^{-1}) and amine III (1230—1266 cm^{-1}) of fibroin increased from sample 4/0/0 to 4/20/0 and 4/20/20, which demonstrated that the addition of methanol would result in higher crystalline degree[13,15,16]. This result was also examined by XRD. As shown in Figure 6, sample 4/20/20 exhibited a sharper and stronger diffraction peak at $2\theta = 19.56°$ than 4/0/0, which further certified a better crystalline structure with the addition of methanol.

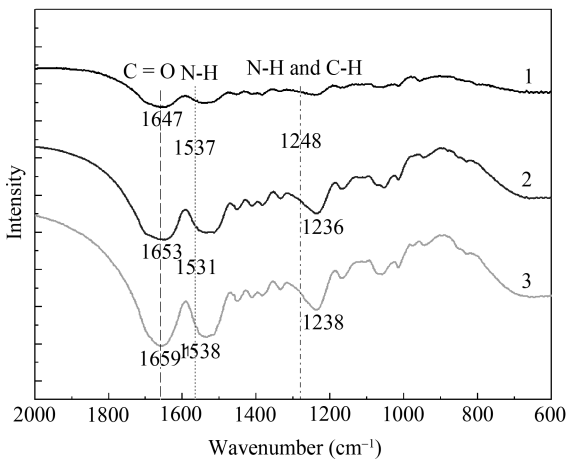

Figure 5 FTIR spectra of the samples. 1, 4/0/0; 2, 4/20/0; 3, 4/20/20.

The difference in the morphologies and structures of different samples can be explained by the influences of methanol solution on the conformation and structure of fibroin molecules. It has been reported[13] that the addition of hydrophilic methanol to the fibroin solution could make the fibroin molecular chains interact strongly with each other within a narrow range and be rearranged in a regular array to form a crystalline structure, and this crystallization change would result in the formation of different morphological structures. This provided reasonable explanations for the different morphologies observed in our experiments. As we found out during the experiments, 4/0/0 can be dissolved in aqueous solution, while the solubility of 4/20/0 decreased a lot because the fibroin has formed crystalline structure due to the addition of methanol. Then, the solid structure of 4/0/20 could be attributed to the collapse of porous structure in 4/0/0 caused by the dissolution of fibroin scaffold in 20% *v/v* methanol solution, while 4/20/20 could maintain the sheet-like structure in 4/20/0 mostly. Meanwhile, the change into better crystalline structure might make the appearance of fine particle aggregates[13] in sample 4/20/20.

2.3 Influences of the freezing temperature

The effects of the freezing temperature on the morphology of the scaffolds were also studied. SEM images of the cross sections of samples 4/0/0N and 4/20/0N are shown in Figure 7. Compared with the uniform pore structure in sample 4/0/0, sample 4/0/0N showed denser structure composed of flat sheets arranged in a preferential direction. In contrast with sample 4/20/0, an aggregate structure of spherical particles of diameter 1—2 μm was formed in sample 4/20/0N. This illustrated that freezing temperature could affect the morphology of scaffolds more greatly when the samples were treated with methanol solution. The easy-sliding in the layered structure of sample 4/0/0N and the spherical composi-

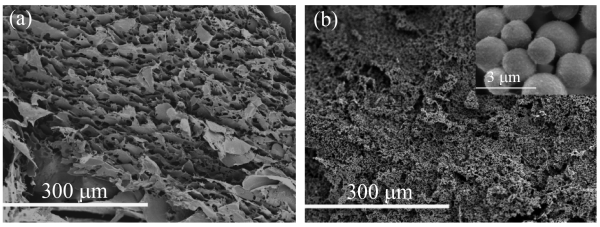

Figure 7 SEM images of the cross sections of the samples. (a) 4/0/0N; (b) 4/20/0N. The inset in (b) is the magnified picture taken from the area in the square.

tion of sample 4/20/0N led to much less compressive strength and compressive modulus than 4/0/0 and 4/20/0. Jin et al.[13] had illuminated that at a higher cooling rate, the formation of aggregates would be favored. As a result, smaller ice crystals would be formed in minute voids occupied by water, and consequently, smaller pores would be left after freezing process. This experiment confirmed that the freezing temperature and methanol-induced structural changes could affect the formation of fine particle aggregates of silk fibroin.

3 Conclusions

3-D silk scaffolds with porous structures were prepared by adjusting solution concentrations and providing treatment with methanol solutions in the way of freeze drying. Samples prepared at 4 wt% fibroin solution concentration can have uniform porous structures with appropriate pore size for cell growth, and good mechanical properties for use as scaffolds. Surface coarseness and pore connectivity of the scaffold, which should be beneficial for the cell adhesion and migration, may be improved at the expense of mechanical properties when the samples are treated with methanol. Then, fibroin scaffold with proper porous structures and properties may be prepared by adjusting the preparation conditions and parameters based on the requirements of application.

1 Minoura N, Tsukata M, Nagura M. Physico-chemical properties of silk fibroin membrane as a biomaterial. Biomaterials, 1990, 11(6): 430
2 Asakura T. Structural characteristics of silk fibroin and the application as enzyme-immobilized material. Bioindustry, 1987, 4: 878
3 Chen K, Iura K, Takano R, et al. Effect of fibroin administration on the blood cholesterol level of rats loaded with cholesterol. Sericuet Sci Jpn, 1993, 62(1): 56－60
4 Wu C, Tian B, et al. Properties and applications of wound protective membrane made from fibroin. In: Third International Silk Conference, Suzhou, China, 1996
5 Demura M, Asakura T. Porous membrane of Bombyx mori silk fibroin: structure characterization, physical properties and application to glucose oxidase immobilization. Membr Sci, 1991, 59(1): 39－52
6 Tsukada M, Freddi G, Minoura N, et al. Preparation and application of porous silk fibroin materials. J Appl Polym Sci, 1994, 54(4): 507－514
7 Sally R, Frenkel, Paul E. DI Cesare. Scaffolds for articular cartilage repair. Ann Biomed Engin, 2004, 32(1): 26－34
8 Marcelo C L, Kim Y G, Kaine J L, et al. Stratification, specialization, and proliferation of primary keratinocyte cultures. Evidence of a functioning in vitro epidermal cell system. Cell Biol, 1978, 79: 356－370
9 Minoura N, Aiba S, Higuchi M. Attachment and growth of fibroblast cells on silk fibroin. Biochem Biophys Res Commun, 1995, 208(2): 511－516
10 Nazarov R, Jin H -J, Kaplan D L. Porous 3-D scaffolds from regenerated silk fibroin. Biomacromolecules, 2004, 5(3): 718－726
11 Mitchell N, Shepard N. The resurfacing of adult rabbit articular cartilage by multiple perforations through the subchondral bone. J Bone Joint Surg, 1976, 58A: 230－233
12 Poole A R, Kojima T, Yasuda T, et al. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res, 2001, 391: 26－33
13 Jin N, Young H P. Morphology of regenerated silk fibroin: Effects of freezing temperature, alcohol addition, and molecular weight. J Appl Polym Sci, 2001, 81(12): 3008－3021
14 Li M Z, Wu Z Y, Zhang C S, et al. Study on porous silk fibroin materials. II. Preparation and characteristics of spongy porous silk fibroin materials. J Appl Polym Sci, 2001, 79(12): 2192－2199
15 Qian J H, Liu Y C, Liu H Y, et al. An amperometric new methylene blue N-mediating sensor for hydrogen peroxide based on regenerated silk fibroin as an immobilization matrix for peroxidase. Anal Biochem, 1996, 236(2): 208－214
16 Liu Y C, Qian J H, Liu H Y, et al. Blend membrane of regenerated silk fibroin, poly(vinyl alcohol), and peroxidase and its application to a ferrocene-mediating hydrogen peroxide sensor. J Appl Polym Sci, 1996, 61(4): 641－647