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Three-dimensional fibroin/collagen scaffolds derived from aqueous solution and the use for HepG2 culture

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Abstract

Although three-dimensional fibroin scaffolds have been prepared with freeze–drying method, these scaffolds still cannot meet the requirements of tissue engineering. In this article, a new process is described to form fibroin-based porous scaffolds with controllable structure and morphological features. When collagen was added to fibroin solution, the viscosity of the blend solution increased because of the interaction between fibroin and collagen, and then it restrained the unwanted fibroin leaf formation in freezing process that generally appeared in the previous fibroin scaffold preparation. With methanol treatment, the fibroin/collagen scaffolds became water-stable, following the transition from random and α-helix to β-sheet conformation. The aqueous-fibroin porous scaffolds had highly homogeneous and interconnected pores with pore sizes ranging from 127 to 833 μm, depending on the fibroin concentration. The porosity of scaffolds was $>90\%$, and the yield strength and modulus were up to 354 ± 25 kPa and 30 ± 0.1 MPa, respectively, when the blend solution, containing 20% collagen, maintained 4% fibroin concentration. Adhesion, spreading and proliferation of HepG2 cells on fibroin and fibroin/collagen blend scaffolds were also observed to investigate the biocompatibility. Scanning electron microscopy (SEM) and MTT analyses demonstrated that the adding of collagen evidently facilitated HepG2 attachment and proliferation in vitro. These new fibroin based three-dimensional scaffolds provided much more excellent properties due to the greatly improved control of pore size, the uniform pore distribution, the hydrophility, the mechanical properties and the biocompatibility compared with those of reported three-dimensional fibroin scaffolds.

Keywords: Fibroin; Collagen; Freeze–drying method

1. 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, the porous matrices should have enough mechanical stability to support cell adhesion and expansion and degrade at a rate comparable with new tissue growth. It should also have a high porosity and interconnected pores to provide sufficient opportunity for cell migration and expansion [3–5].

Silk fibroin produced by the silkworm, *Bombyx mori*, has been used commercially as biomedical sutures for decades [6,7]. Because of its impressive biological compatibility and mechanical properties, silk fibroins have also been explored for many other biomedical applications including osteoblast, hepatocyte and fibroblast cell support matrixes and for ligament tissue engineering [8–11]. Therefore, the preparation of fibroin porous scaffold, having high porosity and interconnected pores, has become one of the major challenges in tissue engineering. Several methods including fiber bonding [12], solvent casting/particulate leaching [4,13], three-dimensional printing [14], gas forming [15], freeze–drying [16], and phase separation [17] have been developed to generate porous three-dimensional scaffolds from natural and synthetic polymers. In these methods, freeze–drying, gas forming and salt leaching have been developed to fabricate porous silk fibroin scaffolds. For example, Mingzhong Li et al. [16] prepared porous fibroin scaffold with freeze–drying method, however, the porosity of fibroin scaffolds prepared by this method was below 70%, which was unsuited for tissue engineering. Rina Nazarov et al. [18] and Ung-Jin Kim et al. [19] have achieved three-dimensional matrices from 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and aqueous solutions respectively by salt leaching method. But the pore distribution and the mechanical properties should be further improved to facilitate the use in tissue engineering.

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In the present study, we sought to develop a new preparation strategy for silk fibroin scaffolds by freeze–drying method. Collagen, one of the most biocompatible natural polymers, was added to fibroin solution and then interacted with fibroin to restrain the unwanted fibroin aggregation in the preparation processes. Through this strategy, it is very easy to prepare fibroin-based porous scaffolds with uniform pore distribution, controllable pore size and functional features.

2. Experimental section

2.1. Materials

Bombyx mori silkworm silk was purchased from Yi Xian raw silk factory in China. The bovine collagen type I gel containing 1% collagen was supplied by Medical and Health Biological Company in Beijing, China.

2.2. Preparation of regenerated fibroin solution

Bombyx mori silk fibroin was prepared just as described in our earlier procedure [20,21]. 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 aqueous solution. The final fibroin concentration was about 3–4 wt%, which was determined by weighing the remaining solid after drying.

2.3. Three-dimensional scaffold fabrication by freeze–drying method

Various fibroin/collagen blends in water were prepared by adding different ratios of collagen gel into fibroin aqueous solutions. The blending ratios of fibroin/collagen were 100:0, 90:10 and 80:20 (wt%), respectively. When heated up to 50–60 °C with mild stir, the collagen gel dissolved in fibroin solutions. The aqueous solution of fibroin and collagen was concentrated at 50–60 °C with mild stir until the fibroin concentration was up to 3–4 wt%. These solutions were put into the polystyrene Petri dishes and then frozen in refrigerator at −20 °C for 12 h. The ice/silk composites were dried with a freeze-dryer, leaving a porous matrix. After the porous matrices were obtained, they were immersed in methanol for about 1 h to induce crystallization and water-stability. The insoluble fibroin/collagen three-dimensional scaffolds were then prepared following methanol evaporating at room temperature.

2.4. Characterization

2.4.1. Scanning electron microscopy (SEM)

The freeze–dried fibroin/collagen scaffolds were fractured in liquid nitrogen using a razor blade and then sputter coated with gold. The morphology of the scaffolds was observed with JEOL JSM-6460LV SEM (Japan). The pore sizes were determined by measuring random samples of 20 pores from the SEM images.

2.4.2. Porosity

The porosity of the scaffolds was measured by mercury intrusion porosimetry (Atopore III 9510, Micromeritics, American). To determine the porosities, it was assumed that the shape of the pores was cylinder. The contact angle of mercury is 130°, and the surface tension of mercury is 0.485 N m^{-1}.

2.4.3. ATR-FTIR

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

2.4.4. Swelling properties

The swelling properties were obtained according to the previous method [19]. Fibroin/collagen scaffolds were immersed in distilled water at room temperature for 24 h. After excess water was removed, the wet weight of the scaffold (W_s) was determined. Samples were then dried in an oven at 65 °C under vacuum overnight and the dry weight of scaffolds (W_d) was determined. The water take-up of the scaffold was calculated as follows:

$$\text{Water take} - \text{up} = \frac{(W_s - W_d)}{W_d}$$

2.4.5. Mechanical properties

The compression modulus of the scaffold was evaluated on an Instron-6022 instrument with a 0.1 kN load cell at room temperature. The cross-head speed was set at 2 mm min^{-1}. Four samples were evaluated for each composition. Cylinder-shaped samples were 9 mm in diameter and 10–20 mm in height. Since the scaffolds were ductile and sponge-like in behavior, the yield strength rather than the compress strength was used to express the mechanical property in this study.

2.5. Cell culture

HepG2 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 2 mg ml^{-1} sodium bicarbonate, and 100 μg ml^{-1} penicillin/streptomycin. The cells were cultured in 37.5 cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2. Confluent monolayers were split by treatment with sterile phosphate-buffered saline (PBS) and 0.05% trypsin/EDTA solution, and the culture medium was replaced every 3 days.

Samples of fibroin and fibroin/collagen blend scaffolds were cut into circular discs suitably sized (diameter 14 mm, height 3 mm) for 24-well tissue culture plate wells. The circular matrices were sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively three times with

sterile PBS. Before cell culturing, scaffolds were pre-wetted by immersion in DMEM for 12 h in the 37 °C incubator.

Cells were trypsinized, counted, and plated at a density of 2×10^5 cells cm^{-2} into the pre-wetted matrices that were placed in 24-well culture plates precoated with 0.3% poly-HEMA to prevent cell attachment to the tissue culture polystyrene surface. After cultured for 2 and 10 days, the seeded fibroin scaffold and fibroin/collagen blend scaffold were immediately rinsed in 0.2 M sodium cacodylate buffer, fixed in Karnovsky fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate) overnight at 4 °C. Fixed samples were dehydrated through exposure to a gradient of alcohol and allowed to air dry in a fume hood. After sputter-coated with gold, samples were examined with a scanning electron microscope (SEM, JSM-35C, JEOL, Japan).

2.6. MTT assay

MTT assay is a quantitative colorimetric assay for mammalian cell survival and cell proliferation. It is an indirect method for assessing cell growth and proliferation, since mitochondria oxidize the MTT solution, giving a typical blue–violet end-product, O.D. value of 490 nm can be quantified to cell number.

Briefly, HepG2 cells were cultured inside the samples ($n=4$) of fibroin and fibroin/collagen blend scaffolds for 5 and 10 days, then the culture medium was replaced with serum free culture medium containing thiazolyl blue (MTT) (0.5 mg ml^{-1}). Cultured for 4 h, the samples were transferred to 2 ml plastic tubes. Tubes were centrifuged for 5 min at 8000 rpm, and then the supernatant was aspirated. After DMSO was added into each tube, samples were cut into pieces and disintegrated using a Microbeater. Tubes were centrifuged at 8000 rpm for 10 min. The solution of each sample was aspirated into a microtiter plate and the absorbance at 490 nm was measured on a SS-3000 Immunoanalyser.

3. Results and discussion

3.1. Preparation of aqueous-derived scaffolds

The fibroin/collagen porous scaffolds were prepared using a freeze–drying method that has been widely used in the preparation of porous scaffolds with other polymers. Rina Nazarov [18] and Mingzhong Li [16,22] both prepared porous three-dimensional scaffolds from regenerated silk fibroin with freeze–drying method, but these scaffolds prepared by lyophilization were unsuitable for cell culture. As shown in these studies, fibroin has the spontaneity of forming leaf or sheet morphology in the freezing process, which made fibroin scaffolds frangible. If the concentration of fibroin solutions was increased to restrain the sheet formation, the pore size would become too small to satisfy a minimum requirement of tissue engineering. So, it is a dilemma to prepare the appropriate three-dimensional fibroin scaffolds with the freeze–drying method.

In this study, the dilemma was solved by adding collagen into fibroin solution. Because collagen and fibroin both are the excellent biocompatible natural proteins, they easily interacted with each other in aqueous solution to restrain the unwanted fibroin aggregation. As shown in Table 1, when 10 and 20% collagen were added into fibroin solution, the shear viscosity increased from 5 to 6.8 and 9.8 mPa s, respectively. More interesting, the above fibroin/collagen blend solution could become gel at 4 °C, which indicated the interaction between fibroin and collagen. On the other hand, because the interaction between fibroin and collagen also affected the crystal transformation from random conformation to β-crystal with methanol treatment, the content of collagen in fibroin/collagen scaffolds was studied as well. Table 2 shows the collagen contents used in the study. When the collagen content was above 50%, the fibroin/collagen scaffolds became soluble in water even after methanol treatment. So, only the fibroin/collagen scaffolds having 10 and 20% collagens were extensively investigated in this study.

Table 1
Influence of collagen content on the viscosity of fibroin/collagen blend solutions containing 4 wt% fibroin

	Collagen content (%)		
	0	10	20
Shear viscosity (mPa s)	5±0.1	6.8±0.3	9.8±0.17

Values are average±standard derivation ($N=3$).

3.2. Morphology

Fig. 1 shows SEM images of freeze–dried scaffolds prepared from fibroin/collagen blend solutions and different silk fibroin concentrations. Scaffolds prepared from 4% fibroin solution formed the separated sheets, which made the scaffolds frangible (Fig. 1(a)). When fibroin concentration increased to 6%, the three-dimensional porous scaffold was successfully prepared using freeze–drying method, but the matrices were also generally composed of sheets (Fig. 1(b)) which induced the poor mechanical properties. Interestingly, following the adding of collagen, the fibroin/collagen scaffolds prepared from blend solutions containing 4% fibroin showed highly interconnected porous structures, in which no sheet was formed. The results indicate that collagen really prevent fibroin from forming sheets.

Table 3 shows actual pore sizes in the different scaffolds. In studies of Rina Nazarov et al. [18] and Ung-Jin kim et al. [19], fibroin scaffolds prepared by salt leaching method both had the interconnected porous network with an average pore size of above 350 μm. In contrast, the pores in fibroin scaffold from 6% concentration were about 151±40 μm with irregular sheet structure. When 10 and 20% collagen was added into 4%

Table 2
Water-stability of fibroin/collagen scaffolds with different collagen content after methanol treatment

	Collagen content (%)			
	0	10	20	50
Water-stability	Insoluble	Insoluble	Insoluble	Soluble

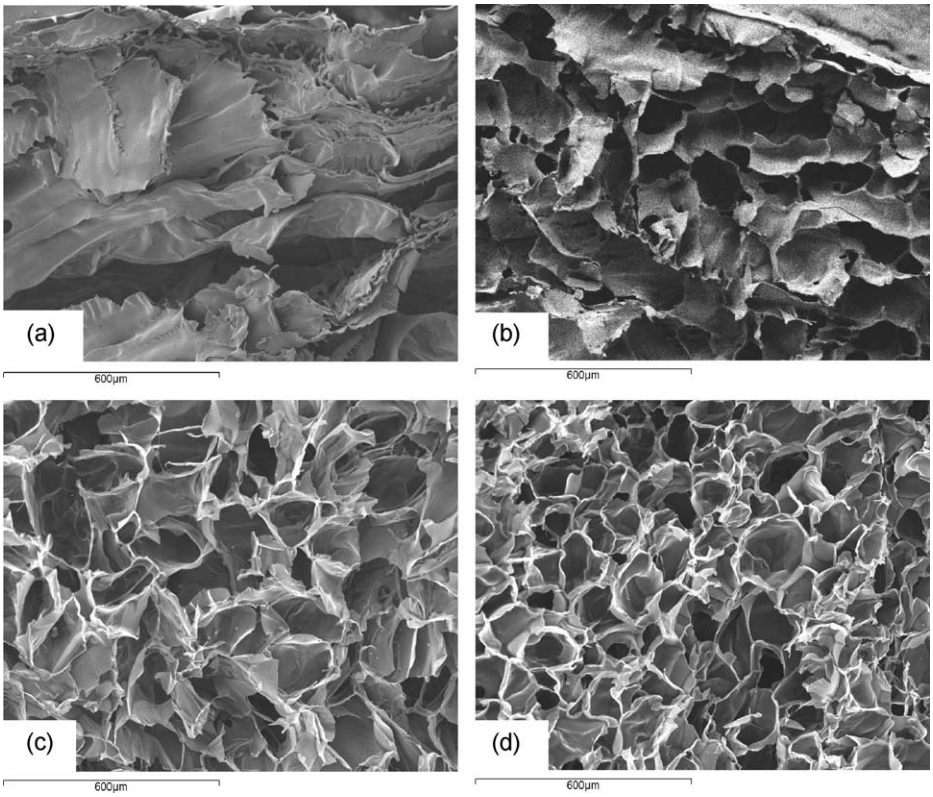

Fig. 1. SEM images of porous scaffolds prepared from fibroin and fibroin/collagen aqueous solutions. (a) 4 wt% fibroin; (b) 6 wt% fibroin; (c) fibroin/collagen scaffolds containing 10% collagen from 4 wt% fibroin solution; and (d) fibroin/collagen scaffolds containing 20% collagen from 4 wt% fibroin solution.

fibroin solution, the pores in blend scaffolds were changed to 139.7 ± 34 and 127.3 ± 30 μm, respectively. More importantly, the pores in blend scaffolds were highly interconnected with the typical reticulate structure rather than irregular sheets. Considering that the decrease of pore size will increase the surface area and mechanical properties, the scaffolds made up of fibroin and collagen would become more propitious for tissue engineering than fibroin scaffolds prepared with salt leaching method.

On the other hand, since the adding of collagen increases the viscosity of solution and then restrains the unwanted fibroin aggregation in freezing process, the three-dimensional scaffolds with different pore sizes can be easily prepared by adjusting the solution concentration. Fig. 2 shows SEM images of fibroin/collagen scaffolds containing 20% collagen prepared from 2.5 and 3% fibroin concentrations. These scaffolds still had the interconnected porous network with an average pore size of 833 ± 87 and 319 ± 70 μm, respectively. The results indicate that the fibroin/collagen scaffolds with different pore sizes could be easily prepared by freeze–drying method to meet the different application requirements.

3.3. Structural analysis

Structural changes in the fibroin/collagen scaffolds containing 20% collagen were determined by ATR-FTIR. Fig. 3 shows the ATR-FTIR spectra of fibroin/collagen scaffolds containing 20% collagen before and after methanol treatment. The spectra of collagen and fibroin scaffolds prepared from 6% concentration without methanol treatment were also shown as the contrast. Before methanol treatment, the spectra of fibroin scaffold show characteristic random conformation peaks at 1641 and 1530 cm^{-1} [23] and collagen shows amide peaks at 1628 and 1523 cm^{-1}, but the fibroin/collagen scaffold just shows one peak at 1601 cm^{-1}, which indicates that the peaks of amide I and II overlap in the scaffold. The result means that there are some interactions between fibroin and collagen. After methanol treatment, the amide peaks move to 1623 and 1517 cm^{-1}, the characteristic peaks of silk II [24–31], which means the conformational transition from random coil to β-sheet. The doublets at 1230 and 1260 cm^{-1} are assigned to amide III; the signal at 1260 cm^{-1} is associated with β-sheet conformation and the signal at 1230 cm^{-1} is associated with random-coil conformation. By comparing the intensities of the pair of component bands at 1260 and 1230 cm^{-1}, we could both qualitatively and quantitatively analyze the cystallinity degree of fibroin [32,33]. Table 4 shows the crystallinity degree of the different scaffolds by FTIR analysis. After methanol

Table 3
Measured pore sizes (μm) of different fibroin based scaffolds

Collagen content in scaffolds (%)	Silk fibroin concentration (wt%)			
	2	3	4	6
0	–	–	–	151 ± 40
10	–	–	139.7 ± 34	–
20	833 ± 87	319 ± 70	127.3 ± 30	–

Values are average ± standard derivation ($N = 20$).

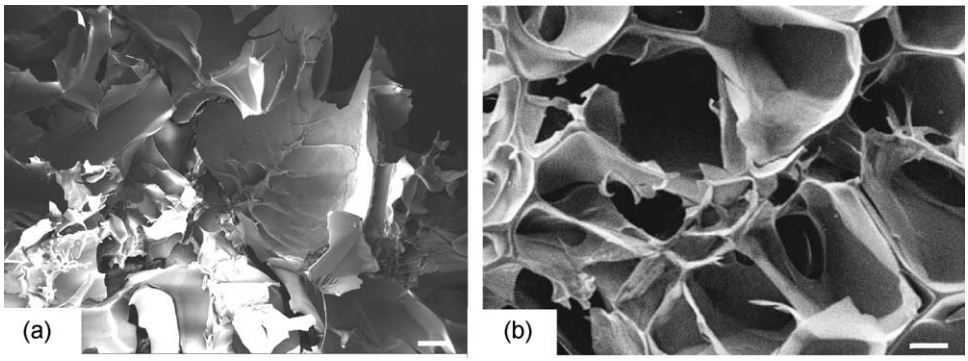

Fig. 2. SEM images of porous fibroin/collagen scaffolds containing 20% collagen prepared from fibroin/collagen aqueous solutions. (a) 2 wt% fibroin concentration; (b) 3 wt% fibroin concentration. Scale bar = 200 μm (a) and 100 μm (b).

treatment, the crystallinity increased from 55.4 to 60.8%. The result indicates that methanol treatment facilitates the transformation from random conformation into β-sheet crystal after collagen was added.

3.4. Porosity and swelling properties

The porosity and water-binding ability of the scaffolds are important features to evaluate biomaterial properties for tissue engineering. The porosity of fibroin scaffold prepared from 6% fibroin concentration was about 87.6%. When 10 and 20% collagen was added to fibroin solution, respectively, both of the fibroin/collagen scaffolds had above 90% porosities, and the porosity increased with the decrease of fibroin concentration (Table 5). Since the adding of collagen increases the solution viscosity, and makes it possible for the three-dimensional fibroin/collagen scaffolds to be formed from low fibroin concentration, the increase of porosity of the scaffolds could be attributed to the decrease of fibroin concentration. The water take-up increased significantly with the first adding of collagen, and increased slightly with the increase of collagen content. Also, the water take-up changed slightly with the decrease of fibroin concentration (Table 6). The results indicate that the adding of collagen greatly improved the hydrophilicity, likely due to the difference in hydrophilicity of the two types of proteins [34], but the effect may become small after the content of collagen reached some degree, which should further be investigated in the subsequent research. On the other hand, when collagen was added into fibroin scaffold, fibroin concentration had just a slight effect on the hydrophilicity of the scaffolds.

3.5. Mechanical properties

The structure of freeze–dried scaffolds was foam-like and very porous. All samples of fibroin/collagen scaffolds exhibited ductile and sponge-like behavior. Table 7 shows the mechanical properties of fibroin scaffold and fibroin/collagen scaffolds. The yield strength rather than the compressive

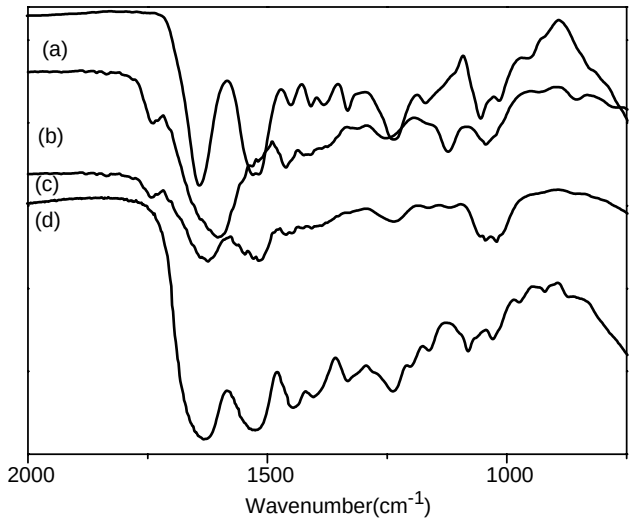

Fig. 3. ATR-FTIR spectra of (a) fibroin scaffold; (b) fibroin/collagen scaffold containing 20% collagen without methanol treatment; (c) fibroin/collagen scaffold containing 20% collagen with methanol treatment; and (d) collagen.

Table 4
Crystallinity degree of fibroin/collagen scaffolds

Sample	Crystallinity degree $A_{1260}/(A_{1230}+A_{1260})$
Fibroin/collagen scaffold	55.4%
Fibroin/collagen scaffold with methanol treatment	60.8%

A_{1260} represents the peak area at 1260 cm^{-1} and A_{1230} represents the peak area at 1230 cm^{-1}.

Table 5
Porosity (%) of different fibroin based scaffolds

Collagen content in scaffolds (%)	Silk fibroin concentration (wt%)			
	2	3	4	6
0	–	–	–	87.6 ± 2.0
10	–	–	93.4 ± 0.3	–
20	97.7 ± 0.5	93.8 ± 1.2	93.4 ± 0.1	–

Values are average ± standard derivation ($N = 3$).

Table 6
Water take-up of different fibroin based scaffolds

Collagen content in scaffolds (%)	Silk fibroin concentration (wt%)			
	2	3	4	6
0	–	–	–	63.1±2.4%
10	–	–	120.4±10.7%	–
20	130.2±5.3%	123.5±4.0%	122.6±7.4%	–

Values are average±standard derivation ($N=3$).

Table 7
Mechanical properties of fibroin scaffolds from 6% concentration and fibroin/collagen scaffolds with different content

Mechanical properties	Collagen content (%)		
	0[a]	10[b]	20[b]
Yield stress (kPa)	20±1	310±10	354±25
Compressive modulus (MPa)	0.43±0.055	10±0.1	30±0.1

Values are average±standard derivation ($N=4$).
[a] Fibroin concentration was 6 wt%.
[b] Fibroin concentration was 4 wt%.

strength is used to express the mechanical properties of fibroin scaffolds.

The fibroin scaffold prepared from 4% fibroin concentration was so fragile that the mechanical properties could not be obtained. Prepared from the 6% fibroin concentration, the three-dimensional scaffolds were formed. However, the yield strength of the scaffold obtained from 6% fibroin solution was only 20±1 kPa. When 10 and 20% collagen were added to scaffolds, the yield strength of fibroin/collagen scaffolds was 310±10 and 354±25 kPa, respectively. The yield stress of fibroin/collagen scaffolds was 15 times larger than that of fibroin scaffold. Many other researches indicated that the decreased pore size and the increased thickness of pore wall could bring on the higher compressive strength and modulus [18,19]. In addition, it has also been reported that more uniform pore distributions in scaffolds improved mechanical properties of the polymer matrices [35]. Considering that the fibroin scaffold prepared from 6% concentration and the fibroin/collagen scaffolds from 4% concentration have the similar pore sizes, the significant increase of strength should be mainly attributed to the uniform reticulate structure of fibroin/collagen scaffolds. Because the fibroin scaffold prepared from 6% concentration was generally composed of sheets, the pore distribution is not uniform, and the fibroin matrices deformed at a low stress.

The compressive modulus of fibroin and fibroin/collagen scaffolds was also investigated. The fibroin scaffold prepared from 6% concentration had the low compressive modulus (430±55 kPa), while the modulus of fibroin/collagen scaffolds was 10±0.1 and 30±0.1 MPa, respectively. These results agree with the uniform pore distribution effects described above, comparing the even pore morphologies of the scaffolds generated from fibroin and collagen blend solution (Fig. 1). The mechanical properties of fibroin scaffolds prepared by salt leaching method have been investigated by Ung-Jin Kim et al. [19]. In their reports, the largest compressive modulus was below 3.5 MPa. So, the mechanical properties of fibroin/collagen scaffolds prepared from freeze–drying method were greatly improved compared with the fibroin scaffold prepared with salt leaching method. Moreover, as shown in Fig. 1, the fibroin/collagen scaffolds were more homogeneous in overall microstructure than those derived from salt leaching method, which should conduce to the increase of mechanical properties. The above results mean that the fibroin/collagen scaffolds might be more appropriate for tissue engineering.

3.6. Cell culture

The proliferation of HepG2 in fibroin scaffold and fibroin/collagen blend scaffold cultured for 5 and 10 days was compared by MTT assay. The data are shown in Fig. 4. The number of HepG2 cells is relatively stable in both fibroin and fibroin/collagen scaffolds. More importantly, the proliferation of cells in fibroin/collagen scaffolds markedly increased compared to that in fibroin scaffold. The results indicate that the fibroin/collagen scaffold has better biocompatibility than the fibroin scaffold.

SEM was also used to confirm the biocompatibility of different scaffolds. Fig. 5 reveals that HepG2 cells cultured for 2 days adhered on the surface of scaffolds without proliferation. After 10 days, it can be seen that the cells proliferated and distributed better in fibroin/collagen scaffold than in fibroin scaffold. Furthermore, the HepG2 cells re-established cell–cell contacts and formed aggregates in fibroin/collagen scaffold, which meant that fibroin/collagen scaffold was more propitious to the growth of HepG2. Although the biocompatible studies in this research were relative preliminary and the cell, HepG2, is not the best choice for studying the hepatic tissue engineering, the results have indicated that fibroin/collagen scaffold was more suitable to be used in tissue engineering, especially in liver tissue engineering.

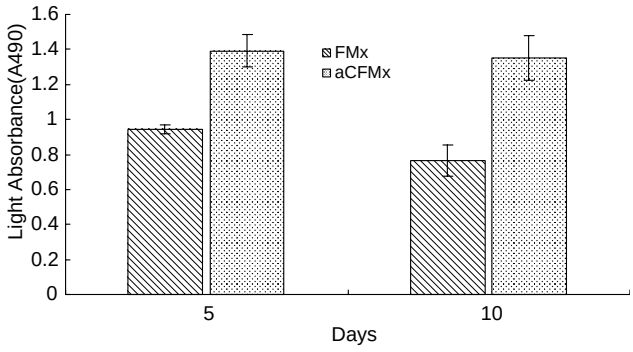

Fig. 4. MTT assay after the HepG2 cells cultured in fibroin and fibroin/collagen scaffolds for 5 and 10 days (FMx: fibroin scaffold, aCFMx: fibroin/collagne scaffold).

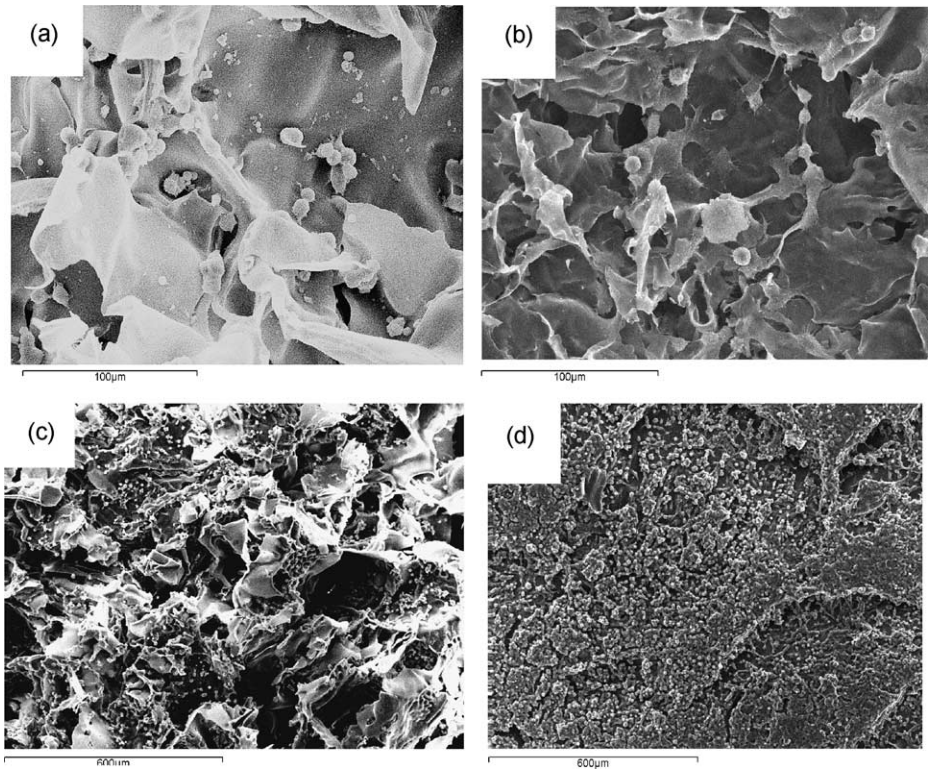

Fig. 5. SEM images of HepG2 cells cultured within fibroin scaffold and fibroin/collagen scaffold: (a) fibroin scaffold, 2 days; (b) fibroin/collagen scaffold, 2 days; (c) fibroin scaffold, 10 days; and (d) fibroin/collagen scaffold, 10 days.

Table 8
Mechanical properties of natural polymer scaffolds

Materials	Compressive strength (kPa)	Compressive modulus (kPa)	Reference
HFIP-derived silk[a]	175–250	450–1000	[18]
Aqueous-derived silk[a]	320	3330	[19]
Fibroin/collagen[b]	354 (yield strength)	30,000	This work
Collagen[b]	~15	~150	[36]
Chitosan[b]	~45	~750	[36]
Collagen/chitosan[b]	~30	~500	[36]

[a] Processed using forming or salt leaching, ratio of NH_4HCO_3 or NaCl to silk was 20:1.
[b] Processed by freeze–drying.

4. Conclusions

Porous fibroin-based scaffolds were prepared directly from fibroin and collagen blend aqueous solutions by a freeze–drying method. With methanol treatment, the scaffolds became water-stable, following the transition from random coil and α-helix conformation to β-sheet. Because the adding of collagen increased the solution viscosity and then restrained the unwanted fibroin aggregation, the three-dimensional scaffolds with high porosity, appropriate pore sizes and the uniform reticulate microstructure were easily prepared through adjusting the fibroin concentration. Compared to the mechanical properties of other porous natural biodegradable polymeric scaffolds (Table 8), the fibroin/collagen scaffolds offer more favorable mechanical properties. On the other hand, the biocompatibility is also greatly increased with the adding of collagen. Considered along with their biocompatibility, increased hydrophility and versatility in processing and chemistry, these fibroin-based biomaterials should offer new and important options to the needs related to tissue engineering in general.

Acknowledgements

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References

[1] Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920–6.
[2] Nerem RM, Sambanis A. Tissue engineering: from biology to biological substitutes. Tissue Eng 1995;1:3–13.
[3] Agrawal CM, Ray RB. Biodegrable polymeric scaffolds for musculoskeletal tissue engineering. J Biomed Mater Res 2001;55:141–50.

[4] Liao CJ, Chen CF, Chen JH, Chiang SF, Lin YJ, Chang KY. Fabrication of porous biodegradable polymer scaffolds using a solvent merging/particulate leaching method. J Biomed Mater Res 2002;59:676–81.
[5] Zhang J, Wu L, Jing D, Ding J. A comparative study of porous scaffolds with cubic and spherical macropores. Polymer 2005;46:4979–85.
[6] Sofia S, McCarthy MB, Gronowicz G, Kaplan DL. Functionalized silk-based biomaterials for bone formation. J Biomed Mater Res 2001;54: 139–48.
[7] Pérez-Rigueiro J, Viney C, Llorca J, Elices M. Silkworm silk as an engineering material. J Appl Polym Sci 1998;70:2439–47.
[8] Unger RE, Wolf M, Peters K, Motta A, Migiaresi C, Kirkpatrick CJ. Growth of human cells on a non-woven silk fibroin net: a potential for use in tissue engineering. Biomaterials 2004;25:1069–75.
[9] Altman GH, Horan RL, Lu HH, Moreau J, Martin I, Richmond JC. Silk matrix for tissue engineered anterior cruciate ligaments. Biomaterials 2002;23:4131–41.
[10] Gotoh Y, Niimi S, Hayakawa T, Miyashita T. Preparation of lactose-silk fibroin conjugates and their application as a scaffold for hepatocyte attachment. Biomaterials 2004;25:1131–40.
[11] Ohgo K, Zhao C, Kobayashi M, Asakura T. Preparation of non-woven nanofibers of *Bombyx mori* silk, *Samia cynthia ricini* silk and recombinant hybrid silk with electrospinning method. Polymer 2003;44:841–6.
[12] Mikos AG, Bao Y, Cima LG, Ingber DE, Vacanti JP, Langer R. Preparation of poly(glycolic acid) bonded fibers structures for cell attachment and transplantation. J Biomed Mater Res 1993;27:183–9.
[13] Wake MC, Gupta PK, Mikos AG. Fabrication of pliable biodegradable polymer foams to engineer soft tissues. Cell Transplant 1996;5:465–73.
[14] Giordano RA, Wu BM, Borland SW, Cima LG, Sachs EM, Cima MJ. Mechanical properties of dense polylactic acid structures fabricated by three dimensional printing. J Biomater Sci Polym Ed 1996;8:63–75.
[15] Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, Langer R. Novel approach to fabricate porous sponges of poly(D, L-lactic-*co*-glycolic acid) without the use of organic solvents. Biomaterials 1996;17:1417–22.
[16] Li MZ, Wu ZY, Zhang CS, Lu SZ, Yan HJ, Huang D, et al. Study on porous silk fibroin materials. II. Preparation and characteristics of spongy porous silk fibroin materials. J Appl Polym Sci 2001;79:2192–9.
[17] Nam YS, Park TG. Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation. J Biomed Mater Res 1999;47:8–17.
[18] Nazarov R, Jin HJ, Kaplan DL. Porous 3D scaffolds from regenerated silk fibroin. Biomacromolecules 2004;5:718–26.
[19] Kim U-J, Park J, Kim HJ, Wada M, Kaplan DL. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. Biomaterials 2005;26:2775–85.
[20] Lu Q, Cao CB, Zhang Y, Ma XL, Zhu HS. Preparation and properties of insoluble fibroin films by novel method. Chem J Chin Univ 2004;25: 1752–5.
[21] Lv Q, Cao C, Zhang Y, Man X, Zhu H. The preparation of insoluble fibroin films induced by degummed fibroin or fibroin microspheres. J Mater Sci, Mater Med 2004;15:1193–7.
[22] Li MZ, Lu SZ, Wu ZY, Yan HJ, Mo JY, Wang LH. Study on porous silk fibroin materials. I. Fine structure of freeze dried silk fibroin. J Appl Polym Sci 2001;79:2185–91.
[23] Asakura T, Kuzuhara A, Tabeta R, Saito H. Conformation characterization of *Bombyx mori* silk fibroin in the solid state by high-frequency ^{13}C cross polarization-magic angle spinning NMR, X-ray diffraction, and infrared spectroscopy. Macromolecules 1985;18:1841–5.
[24] Petrini P, Parolari C, Tanzi MC. Silk fibroin–polyurethane scaffolds for tissue engineering. J Mater Sci, Mater Med 2001;12:849–53.
[25] Oleg NT, Yasushi T. Influence of casting temperature on the near-surface structure and wettability of cast silk fibroin films. Langmuir 2001;17: 7406–13.
[26] Mathur AB, Tonelli A, Rathke T, Hudson S. The dissolution and characterization of *Bombyx mori* silk fibroin in calcium nitrate–methanol solution and the regeneration of films. Biopolymer 1997;42:61–74.
[27] Hiroaki Y, Tetsuo A. The structure of *Bombyx mori* silk fibroin membrane swollen by water studied with ESR, ^{13}C-NMR, and FT-IR spectroscopies. J Appl Polym Sci 1990;40:1745–56.
[28] Jin N, Young HP. Morphology of regenerated silk fibroin: effect of freezing temperature, alcohol addition, and molecular weight. J Appl Polym Sci 2001;81:3008–21.
[29] Tsuboi Y, Ikejiri T, Shiga S, Yamada K, Itaya A. Light can transform the secondary structure of silk protein. Appl Phys A, Mater Sci Process 2001; 73:637–40.
[30] Yasutomo N, Yoshiharu N, Masahisa W, Shigenori K, Jun M. Mechanical properties of silk fibroin-microcrystalline cellulose composite films. J Appl Polym Sci 2002;86:3425–9.
[31] Wilson D, Valluzzi R, Kaplan D. Conformational transitions in model silk peptides. Biophy J 2000;78:2690–701.
[32] Shao J, Zheng J, Liu J, Carr CM. Fourier transform Raman and fourier transform infrared spectroscopy studies of silk fibroin. J Appl Polym Sci 2005;96:1999–2004.
[33] Bhat NV, Nadiger GS. Crystallinity in silk fibers: partial acid hydrolysis and related studies. J Appl Polym Sci 1980;25:921–32.
[34] Ma L, Gao C, Mao Z, Zhou J, Shen J, Hu X, et al. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials 2003;24:4833–41.
[35] Harris LD, Kim BS, Mooney DJ. Open pore biodegradable matrices formed with gas foaming. J Biomed Mater Res 1998;42:396–402.
[36] Kim SE, Cho YW, Kang EJ, Kwon IC, Bae LE, Kim JH. Three-dimentsional porous collagen/chitosan complex sponge for tissue engineering. Fibers Polym 2001;2:64–70.