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Preparation of three-dimensional fibroin/collagen scaffolds in various pH conditions

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Abstract Three dimensional (3-D) fibroin/collagen scaffolds are the novel fibroin based scaffolds derived from aqueous solution. In this article, we investigated the effect of pH on the formation of fibroin/collagen scaffolds. In the range of pH from 4 to 8.5, the fibroin/collagen scaffolds with good porous structures can be prepared using freeze-drying method, which would facilitate the adding of other biopolymers. The structures of different fibroin-based scaffolds were investigated with FTIR and DSC, which indicated that the interaction of fibroin and collagen affected the methanol-induced transformation of fibroin from random-coil to β-sheet conformation. The mechanical properties were also studied. The results mean that all the fibroin-based scaffolds prepared in various pH values had better mechanical properties than other reported fibroin scaffolds. Since the fibroin/collagen scaffolds can be prepared in the range of pH from 4 to 8.5, it is very easy to prepare different multifunctional scaffolds such as fibroin/collagen/chitosan scaffolds and fibroin/collagen/heparin scaffolds in acidic or neutral conditions. These new fibroin-based blend materials extend the range of biomaterial properties that can promote the use in biomedical applications such as drug release and tissue engineering.

Introduction

Silk fibroin produced by the silkworm, *Bombyx mori*, has been used commercially as biomedical sutures for decades. 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 supporting matrixes and for ligament tissue engineering [1–9]. Therefore, the formation of 3-D fibroin-based scaffolds has become one of the major challenges in tissue engineering.

Recently, four fabrication technologies—freeze-drying [10], salt leaching [11, 12], gas forming processing [13] and freeze-thaw treatment of a fibroin aqueous solution in the presence of water-miscible organic solvents [14] have been reported to form the porous 3-D fibroin structure. Although formed porous structures, the mechanical properties of these scaffolds should be further improved to satisfy the requirements of different biomedical applications. More importantly, the rigorous processes made it impossible to add other bioactive materials in these scaffolds to achieve specific bio-functions.

In our previous research, through adjusting the interactions between fibroin and collagen to restrain the formation of separate fibroin sheets in scaffolds, fibroin/collagen scaffolds with controllable pore size and stronger mechanical strength have also been prepared by freeze-drying method [15]. The growths of different cells in fibroin/collagen scaffold are also better than that in fibroin scaffold, which indicates that the fibroin/collagen scaffold would become a promising matrix for tissue engineering. Furthermore, since the fibroin/collagen scaffolds are derived from aqueous solution, many other bioactive materials, such as heparin, growth factors, chitosan, and hyaluronate, might be directly blended with fibroin/collagen solution to obtain the hybrid scaffolds with specific functions. However, the dissolution of these materials requires different pH values, so the effect of pH on the formation of scaffolds should be studied to confirm

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whether different hybrid scaffolds could be prepared directly from aqueous solutions.

This paper presents the effect of pH on the morphologies of fibroin/collagen scaffolds. It further reports the changes of structural and mechanical properties of the sponges. The studies could help the understanding of the interaction between fibroin and collagen, and also open a door to prepare more complex and useful fibroin-based scaffolds.

Experimental section

Materials

Bombyx mori 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.

Preparation of regenerated fibroin solution

Bombyx mori silk fibroin was prepared just as described in our earlier procedure [16, 17]. Silk was boiled for 1 h in an aqueous solution of 0.5 wt% Na_2CO_3 and then rinsed thoroughly several times with distilled water to extract the sericin proteins. The rinsed silk was dried at 80 °C for 12 h to obtain fibroin protein. The degummed silk protein (10 g) was dissolved in $CaCl_2/H_2O/CH_3CH_2OH$ solution (mole ratio 1:8:2, 100 mL) at 80 °C. Then the fibroin solution was filtered and dialyzed against distilled water for 3 days to remove $CaCl_2$ and CH_3CH_2OH. After filtrating with filter paper, we obtained the final fibroin aqueous solution with concentration of about 3–4 wt% which was determined by weighting the remaining solid after drying.

3-D scaffold fabrication at different pH values

According to our previous research [15], fibroin/collagen blend (weight ratio, 80:20) in water was prepared by adding collagen gel into fibroin aqueous solutions. 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%. By adding some content of 1 mol/L HCl or 1 mol/L NaOH, the pH values of the solution were adjusted to 4, 5.5, 7 and 8.5, respectively. These solutions were put into the polystyrene *Petri* dishes and then frozen in refrigerator at –20°C for 12 h. The blends were dried with a freeze-dryer, forming a porous matrix. After the porous matrices were shaped and dried, they were treated with 100% methanol for about 1 h to make them water-stability.

Characterization

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).

Attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR)

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

Differential scanning calorimetry (DSC)

The thermal behavior of each scaffold was determined by means of differential scanning calorimetry (DSC) using a DSC 2910 TA Instruments (USA). According to other's research [18], the measurements were carried out in the range of 25–400 °C under nitrogen at a scanning rate of 20 °C/min. Since the initial parts of DSC were unstable, the following DSC results were shown in the range of 50–400 °C.

Mechanical properties

The mechanical properties of the scaffold were evaluated on an Instron-6022 instrument with a 0.1 KN load cell at room temperature. The cross-heat speed was set at 2 mm min^{-1}. The samples were 9 mm in diameter and 10–20 mm in height.

Results and discussion

Morphology

The preparation of fibroin porous scaffold, having high porosity, controllable pore sizes and excellent mechanical properties, is still a major challenge in tissue engineering. Fibroin usually self-assembles separate sheets in freeze-drying process, which make it difficult to prepare porous structure. In our previous research [15], it has found that collagen could form hydrogen bands with fibroin to prevent the formation of separate sheets in freeze-drying process. When containing 20% collagen, the porous fibroin/collagen scaffolds are easily prepared with freeze-drying method. Considering that the pH conditions could markedly

influence the formation and intensity of hydrogen bands of proteins, the interactions between fibroin and collagen possibly changed in various pH environments, and then influence the formation of porous structures.

To investigate the effects of pH on the formation of scaffolds, the pH ranged from 8.5 to 4 using HCl solutions or sodium hydroxide. Figure 1 shows SEM images of freeze-dried fibroin/collagen scaffolds prepared from blend solutions in the range of pH from 4 to 8.5. When the pH value is 7, the fibroin/collagen scaffold shows highly interconnected and homogeneous porous structure. When the pH value is adjusted to 4 or 8.5, although the unevenness of the structures increases, 3-D porous scaffolds could still be successfully prepared. The results indicate that it is possible to prepare fibroin-based scaffolds with synergistic properties through adding other biopolymers or proteins to the fibroin/collagen aqueous systems in different pH environments.

In order to confirm the feasibility, heparin and chitosan was added to fibroin/collagen blend solutions in acidic and neutral environment respectively. Figure 2 shows the SEM images of two different fibroin/collagen scaffolds containing 1 wt% heparin and 10 wt% chitosan, respectively. It can be seen that fibroin/collagen scaffolds containing heparin and chitosan both maintain the highly interconnected porous structures. Although it is necessary to further investigated the influence of other biopolymers on the formation and properties of porous structure, the above results have confirmed that fibroin/collagen blend solutions really acted as a novel multicomponent approach wherein other biopolymers can be added in fibroin and collagen blend solutions to generate different scaffolds with synergistic properties.

Structural analysis

Structure changes of fibroin/collagen scaffolds derived from different pH conditions were determined by ATR-FTIR. The reported peaks of the β-sheet conformation appear at 1,625 cm^{-1}(amide I), 1,520 cm^{-1} (amide II), while the typical peaks of random conformation are at 1,647 cm^{-1} (amide I), 1,545 cm^{-1} (amide II), and the peaks of α-form (silk I) are at 1,658 and 1,652 cm^{-1} [19–21]. Figure 3 shows the ATR-FTIR spectra of methanol-treated fibroin/collagen scaffolds prepared from different pH environments. The spectra of collagen and fibroin films with methanol treatment were also shown as the contrast. The spectra of fibroin show β-sheet conformation (1,620, and 1,515 cm^{-1}), with some α-form structure (1,652 cm^{-1}), and collagen shows amide peaks at 1,636 and 1,545 cm^{-1}. Some researchers have confirmed that methanol treatment has little effect on collagen when fibroin and collagen are mixed at the molecular level [22]. After methanol treatment, all the scaffolds come from different pH environments have the similar spectra, showing the absorption bands associated with β-sheet and α-form conformations (1,652, 1,628 and 1,510 cm^{-1}). Because of the adding of collagen, collagen amide peaks (1,636 and 1,545 cm^{-1}) also appear in the spectra.

Table 1 shows the relative contents of α-form, β-sheet and random conformation in methanol-treated fibroin and

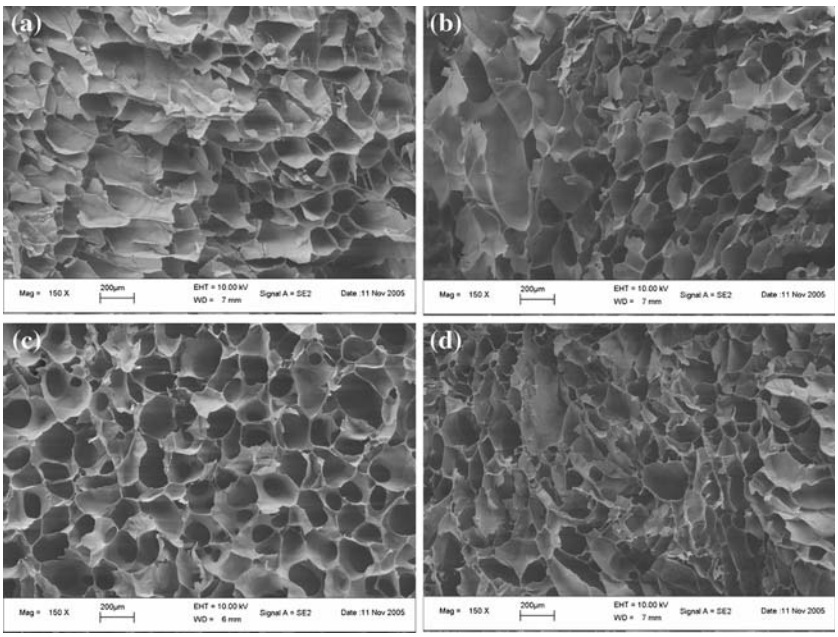

Fig. 1 Morphology of methanol treated Fibroin/collagen scaffolds derived from different pH values: (**a**) pH = 4, (**b**) pH = 5.5, (**c**) pH = 7 and (**d**) pH = 8.5

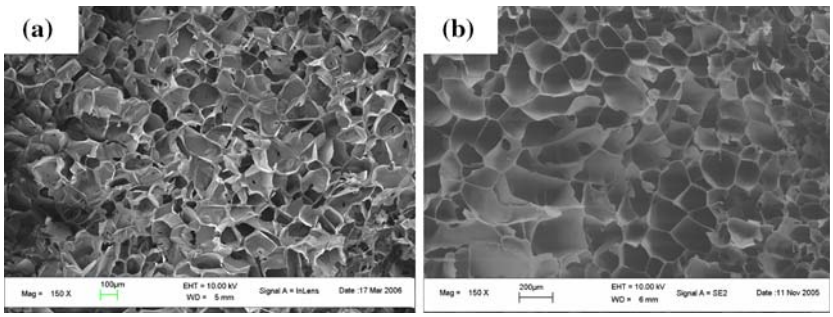

Fig. 2 Morphology of methanol-treated (**a**) Fibroin/collagen/heparin scaffolds and (**b**) Fibroin/collagen/chitosan scaffold (fibroin/collagen/heparin scaffold contains 1 wt% heparin, pH = 7, while fibroin/collagen/chitison contains 10 wt% chitosan, pH = 4)

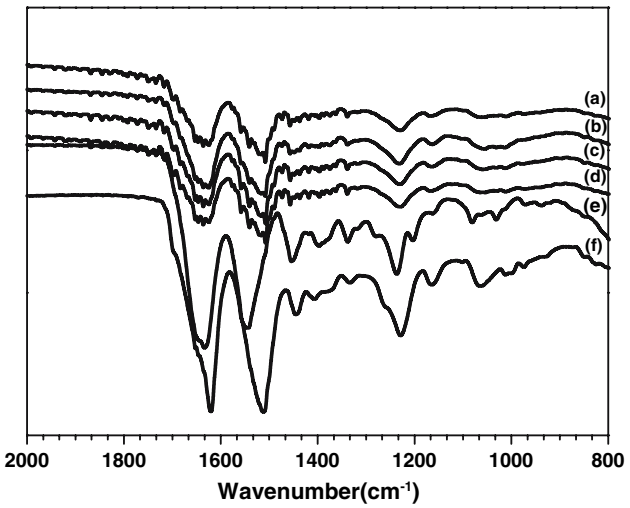

Fig. 3 ATR-FTIR spectra of methanol-treated fibroin/collagen scaffolds derived from different pH values: (**a**) pH = 4, (**b**) pH = 5.5, (**c**) pH = 7 and (**d**) pH = 8.5. Methanol-treated collagen (**e**) and fibroin (**f**) films are used as the contrast

Table 1 The relative contents of random, β-sheet and α-form conformations in the ATR-FTIR spectra of methanol-treated fibroin and fibroin/collagen scaffolds

Samples	pH values	Relative contents (%)		
		Random coil	β-sheet	α-form
Fibroin/collagen	4	30.0	46.6	23.4
Fibroin/collagen	5.5	29.9	46.4	23.7
Fibroin/collagen	7	28.1	40.8	31.1
Fibroin/collagen	8.5	29.6	46.5	23.9
Fibroin	7	24.0	59.6	16.4

fibroin/collagen scaffolds according to the amide I component bands. Compared with fibroin, fibroin/collagen scaffolds have more α-form and random conformations and less β-sheet content. The results indicate that the interaction of fibroin and collagen affects the methanol-induced transformation of fibroin from random-coil to β-sheet conformation. On the other hand, although the random conformation hardly changes in the range of pH from 4 to 8.5, fibroin/collagen scaffolds have the most α-form and least β-sheet conformation when pH value is 7, which indicates that the strongest interaction between fibroin and collagen takes place in neutral conditions. It means that the interaction of fibroin and collagen facilitates the formation of α-form conformation. The intensity of the interaction also changes following the variation of pH conditions.

In order to confirm the influence of collagen, the ATR-FTIR spectra of fibroin/collagen scaffolds without methanol treatment were also investigated when the scaffolds were derived from different pH environments. Since the results are similar when the scaffolds derived from acidic or basic conditions, only the results of fibroin/collagen scaffolds derived from pH values at 4 and 7 were shown in Fig. 4. The peaks of amide I and amide II overlap and form a peak at 1,603 cm^{-1} in neutral conditions, while become two peaks at 1,632 and 1,546 cm^{-1}. Considering that amide I band shifts to lower wave number during the formation of hydrogen bands, while amide II band shifts to higher wave number, the results indicate that the interaction between fibroin and collagen decreased in acidic conditions.

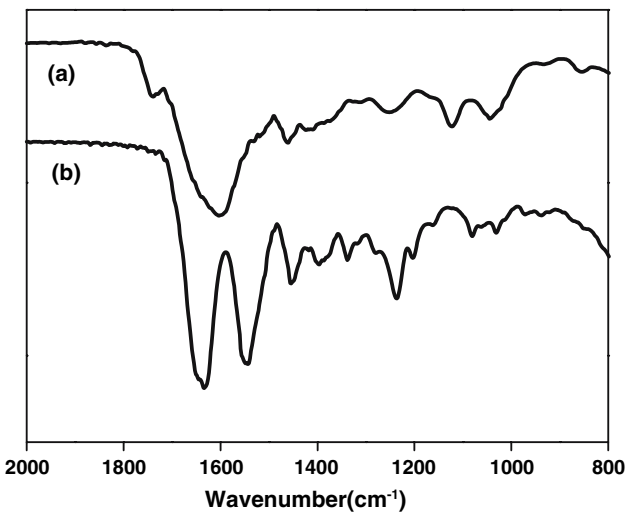

Fig. 4 ATR-FTIR spectra of fibroin/collagen scaffolds without methanol treatment. The scaffolds were derived from different pH values: (**a**) pH = 7 and (**b**) pH = 4

Thermal properties

The thermal properties of fibroin and collagen are very sensitive to moisture, which acts as a plasticizing agent to depress thermal transition temperatures [23]. Agarwal et al. reported that the glass transition temperature (Tg) of regenerated fibroin films could be shifted from 178 to 39 °C if the moisture content increased to 18 wt% [18]. The Tg of collagen also decreased dramatically from 217 °C for dry state to 0 °C for collagen with 25 wt% moisture [23]. Figure 5 displays DSC thermograms of fibroin/collagen scaffolds prepared from different pH environments. All the fibroin/collagen scaffolds have an endothermic peak below 100 °C, which might be due to the influence of moisture. More importantly, though the endothermic peaks become broader than that of fibroin scaffolds because of the effect of collagen, all the fibroin/collagen scaffolds had the prominent endothermic peaks at 281 °C, attributed to the thermal decomposition of silk fibroin with un-oriented β-sheet structure. The results confirm that the fibroin/collagen scaffolds prepared from different pH conditions have enough β-sheet content, which makes these scaffolds water-stable.

Mechanical properties

The methanol-treated scaffolds prepared from different pH environments have the similar behavior with other aqueous-derived fibroin scaffolds, exhibiting ductile and sponge-like behavior. At the initial stage of strain, an elastic region was observed, followed by a peak stress. Then a nearly constant flow stress was observed, and finally, a densification region where the flow stress increased steeply in all samples.

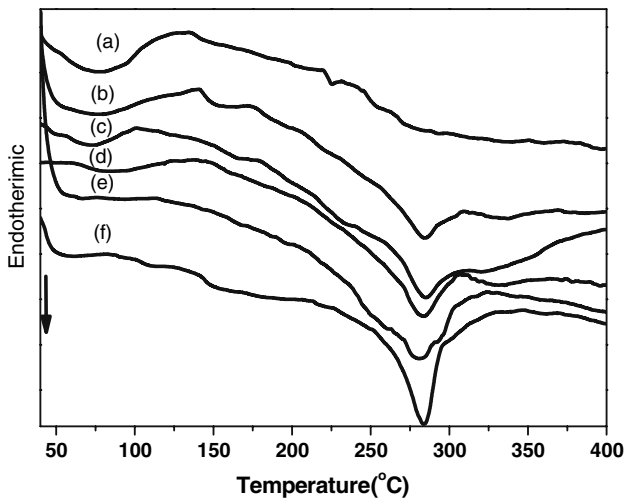

Fig. 5 DSC curves of methanol treated collagen (**a**), fibroin/collagen scaffolds derived from different pH values: (**b**) pH = 4, (**c**) pH = 5.5, (**d**) pH = 7, (**e**) pH = 8.5 and fibroin (**f**)

Table 2 Mechanical properties of methanol-treated fibroin/collagen scaffolds derived from different pH values

pH value	Compressive modulus (MPa)	Compressive strength (MPa)
4	6.50 ± 1.35	0.35 ± 0.04
5.5	9.96 ± 0.82	0.38 ± 0.02
7	12.2 ± 0.44	0.43 ± 0.01
8.5	9.91 ± 1.13	0.39 ± 0.02

Table 2 shows the mechanical properties of methanol-treated fibroin/collagen scaffolds. Fibroin/collagen scaffold derived from neutral solutions has the best mechanical properties. In acidic and basic conditions, the mechanical properties of fibroin/collagen scaffolds decrease following with the formation of some sheets. Many other researches indicated that more uniform pore distributions in scaffolds improved mechanical properties of the polymer matrices [11, 12]. Considering that some sheets appear in the fibroin/collagen scaffolds prepared in acidic and basic conditions, the increase of mechanical properties of the scaffold from neutral solution should be attributed to the uniform reticulate structure. On the other hand, it should be emphasized that all the fibroin/collagen scaffolds derived from our research have better mechanical properties compared with other reported fibroin scaffold (Table 3). The results indicate that all the fibroin/collagen scaffolds in our research might be useful in tissue engineering.

Conclusion

The fibroin/collagen scaffolds could be prepared in acidic, neutral, or basic conditions. Because the interaction of fibroin and collagen could be affected by the pH values, the morphology, thermal properties and mechanical properties of the scaffolds all diversified following the change of pH. The fibroin/collagen scaffolds derived from neutral solutions have best complex properties. In the range of pH from 4 to 8.5, we can successfully prepare the 3-D fibroin-based

Table 3 Mechanical properties of different fibroin-based scaffolds

Materials	Compressive strength (kPa)	Compressive modulus(MPa)	Reference
HFIP-derived silk	175–250	0.45–1	12
Aqueous-derived silk	320	3.33	11
Water-miscible organic-derived silk	–	<0.06	14
Fibroin/collagen	>350	>6.5	This work

scaffolds having better mechanical properties compared with other reported fibroin scaffolds. On the other hand, the method also opens a door to more complex and useful fibroin-based scaffolds by adding other bioactive polymers to the scaffolds.

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