Effect on Properties of Regenerated Silk Fibroin Fiber Coagulated with Aqueous Methanol/Ethanol

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ABSTRACT: Random coil silk fibroin membrane was designed to dissolve with HFIP, and regenerated silk fibroin fibers were obtained by wet spinning. The effect of coagulants (methanol and ethanol) on structure, mechanical properties, and actinomyces biodegradability of regenerated silk fibroin fiber was investigated through X-ray diffraction, IR spectra, and TGA. The results showed that the effect on the structure and properties of regenerated silk fibroin fibers coagulated by methanol and ethanol was negligible, but the difference in actinomyces biodegradability *in vitro* was obvious. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 53–59, 2007

Key words: methanol; ethanol; coagulating agent; regenerated silk fibroin fiber; mechanical properties; biodegradability; wet spinning

INTRODUCTION

Bombyx mori silk is a kind of protein fiber consisting of 18 amino acid, such as glycin, alanine, serine, etc. Silk fibroin has nontoxic, nonirritability, and nonstimulation to the organism.[1,2] Recent studies showed that silk fibroin has the same good absorption as collagen to animal cells cultivated *in vitro*, and has important effect to keep cell functions.[3]

It has been 40 years to research regenerated silk fibroin fiber. For example, in 1989, Ishizaka et al. dissolved silk fibroin with H_3PO_4 in the laboratory and got regenerated silk fibroin fiber by wet spinning with $(NH_4)_2SO_4$ or Na_2SO_4 as coagulating agent. The fiber was drawn and spun in methanol, with the highest strength 2.2 g/d and the elongation is 10%.[4] In 1996, Matsumoto reported that the regenerated silk fibroin fiber could be spun by the self-dialyzing method.[5] In 1998, Liivak reported that the regenerated silk fibroin fiber could be spun by dissolving silk fibroin in HFIP.[6] In 2002, Yao reported that he dissolved silk fibroin in HFA to spin regenerated silk fibroin fiber.[7] In above reports, generally methanol was used as coagulating agent of wet spinning. However, methanol is a moderate toxic solvent, which may harm nerve and blood vessel and vapors at normal temperature. Because of the anaesthetic and prominent accumulating effect, it may harm optic nerve and retina. On the contrary, ethanol is little toxic, and slightly stimulating to ocular mucous membrane. In this article, methanol and ethanol were respectively used as coagulating agent of regenerated silk fibroin fiber spun by HFIP process under the same conditions. The effect of the coagulants on structure and mechanical properties was studied, through X ray diffraction, IR spectra, TGA, and mechanical property test. The differences in biodegradable properties were also investigated to provide basis for using as biomedical material.

MATERIALS AND METHODS

Materials

Waste *Bombyx mori*, silk, $NaCO_3$, methanol, ethanol, LiBr, HFIP (ACROS company) and actinomyces (SIGMA company).

Main experiment apparatus

Infrared spectrophotometer (Alpha Centausi Company, USA): He-Ne laser was used to collect samples, and the range of wavelength was set as 400–4000 cm^{-1}; OLYMPUS-BX40 laser Raman spectrum meter; D/MAX-3A X ray diffraction meter (Physics electric machine company, Japan), with 40 KV tube voltage and 30 mA tube current, CuK_α radiation was used with diffraction angle $2\theta = 2°–45°$; TGA7 analysis meter (Perkin-Elmer company, USA), testing condition was nitrogen flux at 30 mL/min, at a heating rate of 20°C/min and temperature 60–380°C; domestic 81-2 type magnetic constant temperature beater;

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German Leica BME biomacroscope; and Instron electronic strength tester (3365), etc.

Preparation of silk fibroin

Raw *B. mori* silk consists of several proteins mainly fibroin and sericin. To remove sericin, raw *B. mori* silk fibers was degummed with 0.5 (W/W) Na_2CO_3 solution at 100°C for 30 min ,then rinsed with water (at about 60°C). All the processes repeated three times, but the third time washed with distilled water.

Pure silk fibroin without sericin was obtained by boiling the raw *Bombyx mori* silk without impurity in 0.5% (W/W) Na_2CO_3 solution (weight rate of silk to solution = 1 : 20) for 30 min, then the silk was rinsed two times in water at more than 60°C. All the processes repeated three times.

Preparation of spun solution

Put dry pure silk fibroin in a ternary solvent system of LiBr/ethanol/H_2O at constant temperature 70°C for more than 4 h, then pour the solution into dialysis tube, of which the molecular weight of cut-off current was 10,000 D, to be dialyzed for 3 days and nights in flowing and distilled water. The regenerated SF film was obtained by casting from the aforementioned solution onto a polystyrene plastic plate, dried at room temperature. Then, pure spun solution of more than 10% (W/W) was obtained after dissolving the film into HFIP solution, filtering the solution from stainless steel net, and eliminating the foam at vacuum.

Wet-spinning process of regenerated fibroin SF fiber

The regenerated SF as-spun fiber was spun by wet-spinning process, respectively taking methanol and ethanol as coagulant, through the diffusion to each other and phase separation of solvent and coagulant. Finally, two types of regenerated SF fibers coagulated by different solvents were obtained after drawing and setting.

Measurement of enzyme degradation of regenerated SF fibers *in vitro*

Degradation experiment for the silk fibers was carried out with actinomyces enzyme bought from Sigma at pH 7.0 and temperature 37°C. The enzyme was dissolved in phosphonate buffer solutions of pH 7.0 to form solutions containing 5 unit actinomyces enzyme per milliliter. The regenerated and native SF fibers were respectively put in the enzyme solutions with a liquor ratio of SF fibers to the solution 1 : 20 (w/w). Pour the solutions in many test tubes, seal tubes with films, and then put them in water bath at constant temperature 37°C. Take one out from each type every 5 days per time. Wash fibers from tubes with water and heat to a constant weight in oven at 105°C. The remained tubes were changed with new enzyme solutions, respectively. Repeat the above process for several times and obtain fibers at different degrading time .The degradability (D) was calculated as follows:

$$D = (w_1 - w_2)/w_1 \times 100\,\%$$

where w_1 is weight of dried regenerated SF fibers and w_2 is weight of dried regenerated SF fibers after having been degraded for certain days.

RESULTS AND DISSCUSSION

IR spectrogram analysis

According to the position of absorption peak of amide I, amide II, and amide V on IR spectra curve, the conformation of protein molecule can be analyzed and judged. Qian and Yao[8,9] considered that the characteristic absorption bands were 1625–1640 cm^{-1} (amide I), 1515–1525 cm^{-1} (amide II), 1265 cm^{-1} (amide V), and 1265 cm^{-1} (amide III) attributed to β-sheet conformation, 1650 cm^{-1} (amide I), 1545 cm^{-1} (amide II), 1240 cm^{-1} (amide III), and 600 cm^{-1} (amide V), because of α-helix conformation, and 1650–1660 cm^{-1} (amide I), 1535–1545 cm^{-1} (amide II), 1235 cm^{-1} (amide III), and 650 cm^{-1} (amide V) because of random coil conformation, by measuring IR absorption spectrum bands of SF molecule conformation having typical β-sheet and random coil conformation. Figures 1–5 are the IR spectrograms of regenerated SF drown fibers, regenerated SF as-spun fiber, and native SF fiber coagulated by methanol and ethanol.

In the figures of IR spectrogram, the characteristic absorption peak of amide was observed indicating that regenerated SF fibers coagulated by ethanol and native SF fiber have α-helix conformation, whereas

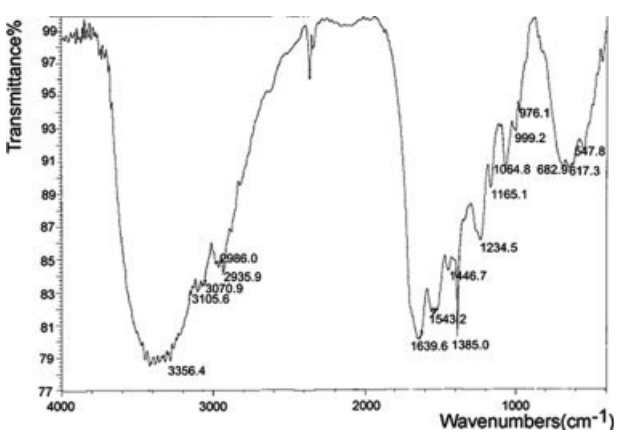

Figure 1 IR spectrogram of regenerated SF fiber coagulated by methanol.

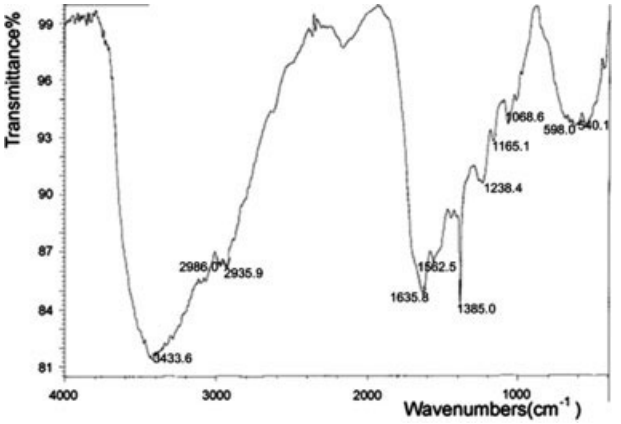

Figure 2 IR spectrogram of regenerated SF as-spun fiber coagulated by methanol.

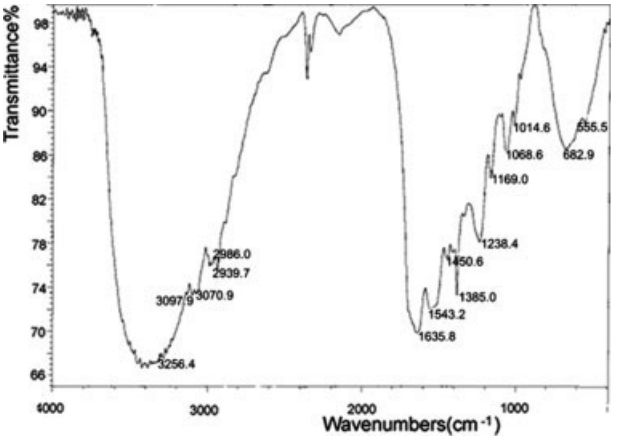

Figure 4 IR spectrogram of regenerated SF as-spun fiber coagulated by ethanol.

regenerated SF as-spun fiber and regenerated SF drawn fiber respectively coagulated by methanol and ethanol all have random coil conformation. Nearby 1701.3 cm^{-1}, there is a characteristic peak, corresponding to β-sheet conformation in native SF fiber. The characteristic absorption peak of amide II, was also observed, reflecting that the β-sheet conformation obviously exists in native SF fiber, and α-helix conformation exists in regenerated silk fibroin fiber and its as-spun fiber. At the characteristic absorption peak of amide V, the characteristic absorption peak of regenerated silk fibroin fiber and its as-spun fiber are similar to native SF fiber, all have β-sheet conformation, but β-sheet conformation of the latter two fibers are not obvious. Moreover, regenerated silk fibroin fiber coagulated by ethanol has α-helix conformation at characteristic absorption peak of amide I and amide II. It is implicated that the characteristic absorption peak of regenerated silk fibroin fiber and its as-spun fiber are similar to native SF fiber, and have β-sheet conformation. But β-sheet conformation of regenerated SF fiber is not obvious, that is the reason may be it still contains some random coil peptide chains.

Laser Raman spectrum analysis

Raman scatter is generated when molecule is irradiated by incident ray and produce induced dipole. It is related to the polarizing capability of molecules. However, when the vibration or rotation energy level of the molecules varies, the absorbing spectrum will be generated. So, Raman spectrum can be used as supplement of FTIR spectrum in the study of materials structure.

Raman spectrogram is a curve with Raman displacement as *X*-axis and the strength of Raman scatter light as *Y*-axis. Laser Raman spectrum can reflect the characteristic vibration band of peptide bond, main chain, and side chains of protein molecules, then the conformational character can be analyzed by the characteristic Raman peak of peptide bond.[10] In the laser Raman spectrum, multipeptide and protein molecule chain have several amide bands, especially at the wavenumber lower than 1700 cm^{-1}, each amide char-

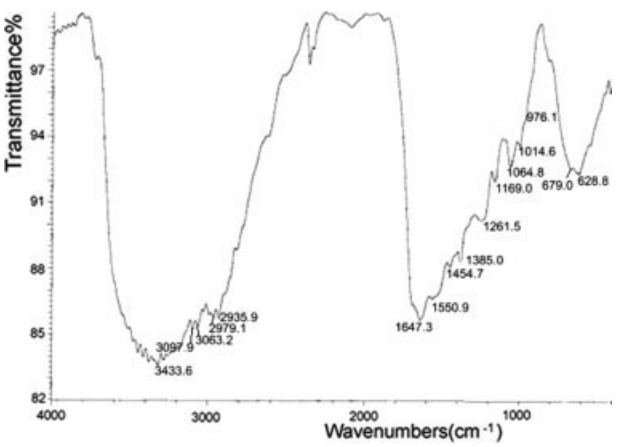

Figure 3 IR spectrogram of regenerated SF fiber coagulated by ethanol.

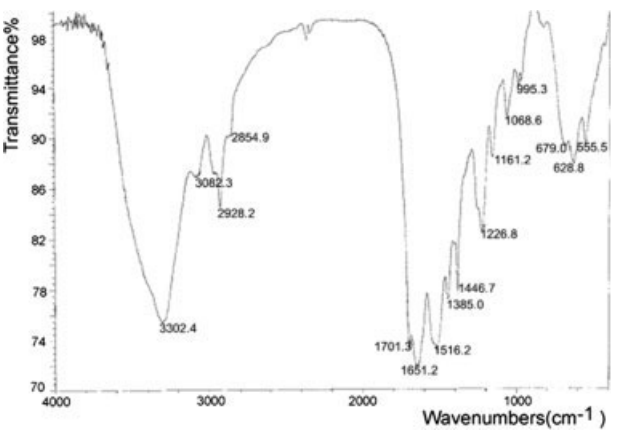

Figure 5 IR spectrogram of native SF fiber.

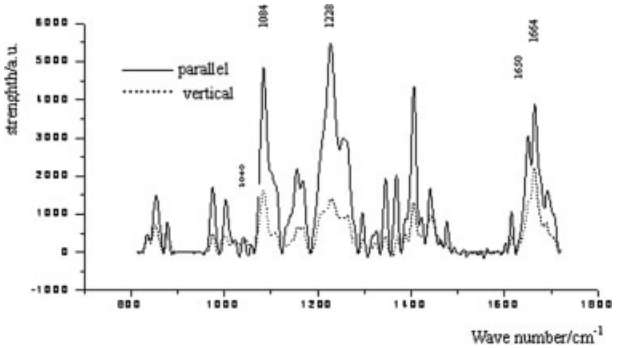

Figure 6 Laser Raman spectrum of regenerated SF fiber coagulated by methanol.

acteristic peak has close relationship with the conformation of protein. And their wave numbers at 1665–1680 cm^{-1} (amide I), 1230–1245 cm^{-1} (amide III), and 1020–1060 cm^{-1} (other sensitive peak), attribute to β-sheet conformation, 1645–1658 cm^{-1} (amide I), 1264–1310 cm^{-1} (amide III), and 890–945 cm^{-1} (other sensitive peak), attribute to α-helix conformation, and 1660–1666 cm^{-1} (amide I) and 1242–1250 cm^{-1} (amide III) attribute to random coil conformation.[11]

As shown in Figures 6–8 laser Raman spectrums of regenerated SF fiber coagulated by methanol and ethanol, and native SF fiber, the spectra of parallel orientation and vertical orientation are different, because that fiber molecular chain possess anisotropy. Referring the wave-number of each characteristic peak to the Raman spectra character of typical conformation,[11] it can be concluded that these three silk fibroin fibers mainly have β-sheet and α-helix conformation. The absorption at 1665–1680 cm^{-1} (amide I), attributes to β-sheet conformation, 1660–1666 cm^{-1} (amide I) to α-helix conformation, and 1645–1658 cm^{-1} (amide I) to random coil conformation. So at amide I, β-sheet conformation coexists with random coil conformation in native SF fiber, whereas α-helix coexists with random coil in regenerated silk fibroin fiber coagulated by methanol and ethanol. It is indicated that at amide I native SF fiber had compact structure and good crystallinity, but regenerated silk fibers had

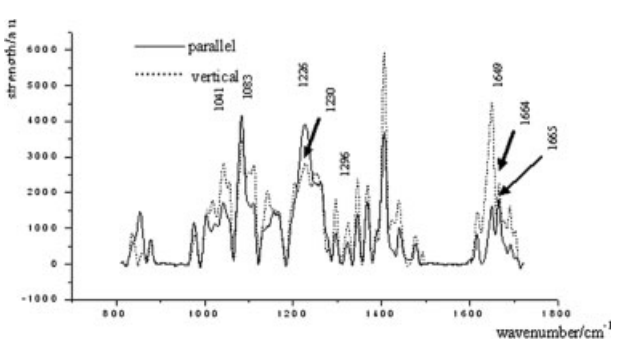

Figure 7 Laser Raman spectrum of regenerated SF fiber coagulated by ethanol.

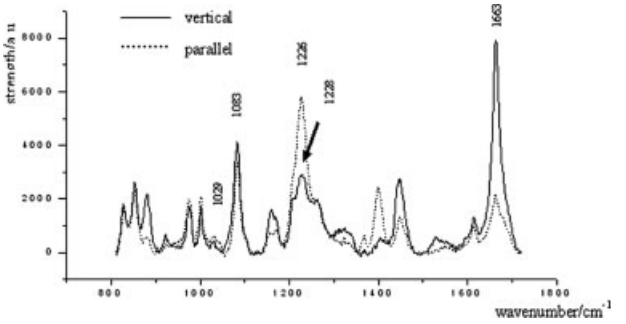

Figure 8 Laser Raman spectrum of native SF fiber.

loose structure. Nearby amide III position, all three silk fibers appeared stronger peak at 1226–1230 cm^{-1} because of β-sheet and β-rotational angle conformation. Moreover, the Laser Raman spectrogram of regenerated SF fibers had certain peak number nearby 1296–1297 cm^{-1} (α-helix conformation), whereas native SF fiber had not. This implicated that at amide III, native SF fiber had β-sheet conformation, while β-sheet coexisted with α-helix conformation in regenerated SF fibers, and all three fibers had β-sheet conformation at 1020–1060 cm^{-1} (other sensitive peak).

X-ray diffraction

X-ray diffraction is an important method to study the morphology of fiber. Figure 9 shows X-ray diffraction diagrams of regenerated SF fiber and native SF fiber.

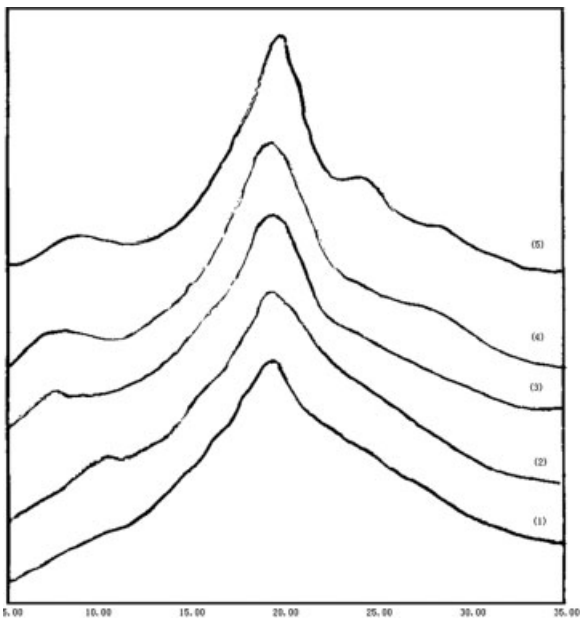

Figure 9 X-ray diffraction spectrum. (1) As-spun fiber coagulated by ethanol, (2) as-spun fiber coagulated by methanol, (3) regenerated SF fiber coagulated by ethanol, (4) regenerated SF fiber coagulated by methanol, (5) native SF fiber.

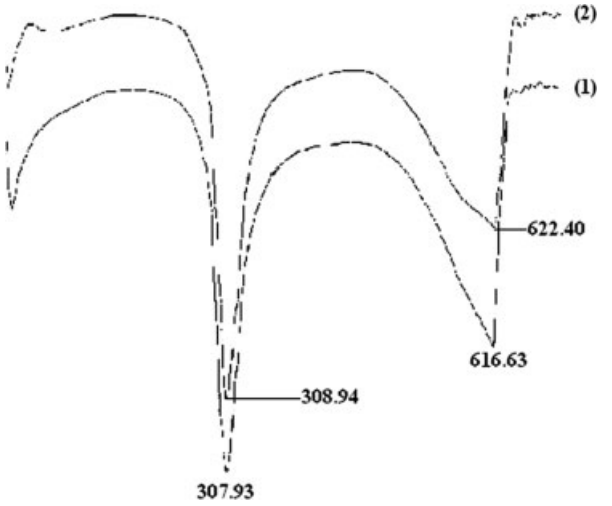

Figure 10 TGA spectrum of regenerated SF fiber. (1) Regenerated SF fiber coagulated by ethanol. (2) Regenerated SF fiber coagulated by methanol.

Table I shows the crystallinity and orientation degree of regenerated SF fibers coagulated by different methods and the crystallinity and orientation degree of native SF fiber, by X-ray diffraction method.

Through measuring the crystallinity and orientation degree by X-ray diffraction method, it was found that the spun solution began to coagulate and crystallize as it got into the alcohol coagulating bath, so the as-spun SF fiber had also higher crystallinity. But since the regenerated as-spun SF fibers were too brittle, it was difficult to measure the orientation degree. The crystallinity of regenerated silk fibroin fiber was lower than that of the native silk.

Thermogram analysis

There were two thermal decomposition peak values for regenerated SF fibers coagulated by ethanol, 307.93 and 616.63°C respectively. As shown in Figure 10, both of them were smaller than these of corresponding regenerated SF fibers by methanol. So, it is indicated that crystallinity of the former was slightly larger than the latter, but the difference was not obvious.

TGA data of regenerated SF fiber and native SF fiber were shown in Table II. On the basis of TGA, the characteristic temperatures of regenerated SF fiber coagulated by methanol and ethanol were compared with that of native SF fiber. Since the boost in decomposition temperature can reflect the increase of the crystallinity, it was clear that the crystallinity of the regenerated SF fiber was lower than that of the native fiber. There was no significant difference in decomposition temperature between regenerated drawing SF fiber and as-spun SF fiber, coagulated by either methanol or ethanol. The difference in crystallinity was also little.

TABLE I
Crystallinity and Orientation Degree

Samples	Crystallinity (%)	Orientation degree (%)
Regenerated as-spun SF fiber coagulated by methanol	44.0	/
Regenerated SF fiber coagulated by methanol	46.2	76.0
Regenerated as-spun SF fiber coagulated by ethanol	47.2	/
Regenerated SF fiber coagulated by ethanol	48.3	77.8
Native SF fiber	61.5	87.1

Mechanical properties of regenerated SF fiber

Fineness of regenerated SF fiber

The images of regenerated SF fibers were photographed by biomicroscope with electronic video camera. The pels of current magnified 1 mm region were demarcated by image processing software. By calculating the radial pels of the sample image and the diameter of each sample were obtained.

The fineness of the fibers coagulated by methanol was one time larger than the fibers by ethanol under the same spinning conditions. This indicated that at faster velocity of coagulating in ethanol bath, the drawing force made the as-spun silk to have larger drawing ratio.

Mechanical properties of regenerated silk fibroin fiber

Table III showed mechanical properties of regenerated SF fiber measured by INSTRON electronic strength tester. Table III showed little difference between regenerated SF fibers coagulated by methanol and those coagulated by ethanol. However, the breaking strengths of the regenerated SF fibers were

TABLE II
Decomposition Temperature of Regenerated SF Fiber Coagulated by Methanol and Ethanol

Samples	Decomposition temperature (°C)
Regenerated as-spun SF fiber coagulated by methanol	302.85
Regenerated SF fiber coagulated by methanol	308.94
Regenerated as-spun SF fiber coagulated by ethanol	301.83
Regenerated SF fiber coagulated by ethanol	307.93
Native SF fiber	331.09

TABLE III
Mechanical Properties of SF Fiber Coagulated by Methanol and Ethanol

Properties samples	Coarseness (dtex)	Break strength (cN/dtex)	Elongation at break (%)
Regenerated SF fiber coagulated by methanol	110.18	0.83	17.46
Regenerated SF fiber coagulated by ethanol	49.08	0.82	25.04
Native SF fiber	17.11	2.56	9.30

40% lower than that of the native fiber, and their breaking elongation was two to three times higher than that of the native silk. This was consistent with that the findings on the crystallinity and orientation degree.

It seems that the mechanical properties will be increased by improving the spinning process of the regenerated fiber. But the macromolecule of SF fiber will be surely degraded in dissolving process, so it is difficult for the regenerated fiber to surpass the native fiber in mechanical properties. It is also similar for crystallinity and orientation degree.

Enzyme degradation of regenerated SF fiber *in vitro*

Experiments to study the degradation of regenerated silk fibroin fiber were also carried out by actinomyces *in vitro*.

The moisture regain of regenerated SF fiber coagulated by methanol was 11.55%, that of regenerated silk fibroin fiber coagulated by ethanol was 11.20%, and that of native SF fiber was 10.74%.

Figure 11 showed the degradation rate curves of regenerated SF fibers coagulated by methanol or ethanol *in vitro*. The regenerated SF fiber coagulated by methanol was treated by phosphate buffer without enzyme after 30 days; its degradation rate was 1.98% while the fiber coagulated by ethanol was 1.97%.

As shown in Figure 11, actinomyce degradation rate of regenerated SF fiber coagulated by ethanol *in vitro* was lower than fiber being coagulated by methanol for 20 days. But after 20 days, actinomyces degradation rate of fibers coagulated by ethanol *in vitro* increased; on 30th day, it reached to 37.16%. However, actinomyce degradation rate of fiber coagulated by methanol *in vitro* was only 14.41%. It was clear that regenerated SF fiber coagulated by ethanol not only had good safety during spinning process, but also had larger degradation rate. The actinomyce degradation rate *in vitro* of native SF fiber was 10.7%, lower than that of the regenerated on 30th day.

DISCUSSION

The solubility parameter of methanol was $\delta_p = 6.0$, and that of ethanol was $\delta_p = 4.3$.[12] The coagulating velocity of ethanol was faster than methanol during wet spinning process of regenerated silk fibroin fiber. So under the same processing conditions, the as-spun silk coagulated by ethanol had better drawing effect, its regenerated silk fibroin fiber had small fineness, but its breaking elongation was a little larger. However, the mechanical properties of regenerated SF fiber coagulated by ethanol were similar to that of the fiber coagulated by methanol.

With regard to biodegradation, the fibers coagulated by ethanol were superior to the fibers coagulated by methanol; moreover, they could avoid the larger toxicity of methanol. So in biomedical material field, ethanol can be used instead of methanol as spinning concreting solvent.

CONCLUSION

This study can be concluded as follows:

1. Under the same formation conditions, the fineness of regenerated SF fiber coagulated by ethanol was about half of that of the fiber coagulated by methanol, but their mechanical properties were similar.
2. There was certain difference between regenerated SF fiber coagulated by methanol spun with EFIP dissolving method and fibers coagulated by ethanol. Fibers coagulated by ethanol had α-helix conformation at characteristic peak of amide I and amide II.
3. On 30th day, actinomyce degradation rate of regenerated SF fiber coagulated by ethanol reached 37.16% *in vitro*, while fibers coagulated by methanol were just 14.41%.
4. Ethanol can be used as spinning coagulating bath instead of methanol, when adopting wet

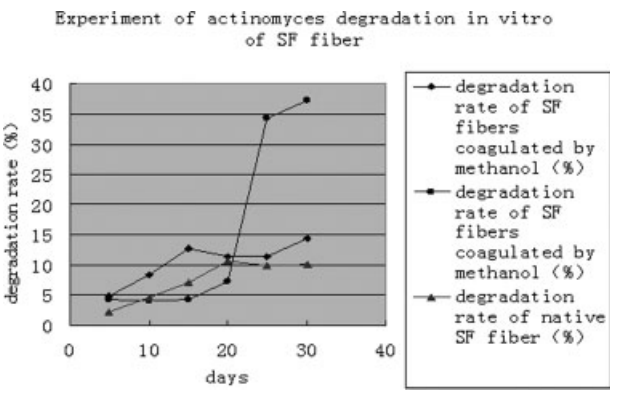

Figure 11 Actinomyces degradation rate curve *in vitro* of regenerated silk fibroin fiber coagulated by methanol and ethanol.

spinning method to spin regenerated silk fibroin fiber.

References

1. Ning, L.; Xue, M.; Zhang, Y.; Sun, J.; Li, Y. W.; Huang, H. N.; Gu, G. Z.; Qian, Y. F.; Meng, A. Y.; Li, C. B.; Zhao, S. Y. China J Mod Med 1999, 5, 1.
2. Wu, Z. Y. Mater Res 2001, 2, 50.
3. Wu, H. T.; Zhong, C. P.; Gu, Y. D. China J Reparative Reconstr Surg 2000, 14, 301.
4. Hiroko, I.; Watanabe, Y.; Ishida, K.; Fukumoto, O. J Srric Sci Jpn 1989, 58, 87.
5. Matsumoto, K; Uejima, H.; Iwasaki, T.; Sano, Y.; Sumino, H. J Appl Polym Sci 1996, 60, 503.
6. Liivak, O.; Blye, A.; Shah, N.; Jelinski, L. W. Macromolecules 1998, 31, 2947.
7. Yao, J.; Masuda, H.; Zhao, C.; Asakura, T. Macromolecules 2002, 35, 6.
8. Qian, G. D.; Yao, Y. L. J Suzhou Inst Silk Text Technol 1983, 4, 26.
9. Silk Reeling Chemistry. Edited by Suzhou Institute of Silk Textile Technology and Zhejiang Institute of Silk Textile Technology. J China Textile Publishing Company: Beijing, China, 1996; p 62.
10. Pan, J. L. Application of Laser Raman Spectrum on Organism Chemistry. Chemical Industry Publishing Company: Beijing, China, 1986.
11. Shao, Z. Z.; Wu, D.; Li, G. X.; Pent, L. W.; Yu, T. Y.; Zheng, S. D. China J Light Scattering 1995, 1, 2.
12. Dong, J. Z.; Sun, T.; Gu, D. Z. Synthetic Fiber Production Technology. Textile Industry Publishing Company: Beijing, China 1984; p 286.