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Study on the structure of SF fiber mats electrospun with HFIP and FA and cells behavior

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Abstract *Bombyx mori* silk fibroin (SF) fiber mats were prepared by electrospinning with the solvent of hexafluoroisopropanol (HFIP) and formic acid (FA). The average diameters of SF fiber mats observed by SEM were 2.0 and 0.3 μm when different solvent, HFIP and FA, were used. Fourier transform infrared and X-ray diffraction were employed to study the secondary structure of the SF fiber mats; the results showed that the electrospin solvent not only affect the secondary structure of as-spun SF fiber mats, but also indirectly affect the structure transition of SF fiber mats post-treatment with ethanol. And the SF fiber mats electrospun with FA showed more β-sheet structure before and after ethanol treatment. The differential thermal analysis curve indicated that the solvent of HFIP or FA had a weak effect on the thermal properties of SF fiber mats. To assay the cytocompatibility and cell behavior on the SF fiber mats, cell attachment, spreading, and proliferation of normal human epidermal fibroblasts (NHEF) seeded on the scaffolds was studied. The results indicated that the SF fiber mats support NHEF attachment and growth on SF fiber mats in vitro, and no difference between the SF fiber mats electrospun with HFIP and FA was observed. In this article, a desired morphology and secondary structure of SF fiber mats could be prepared by choosing different electrospinning solvent.

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Introduction

Silks are naturally occurring protein polymers produced by a wide variety of insects and spiders [1, 2] that have been used clinically as sutures for centuries [3]. The domesticated silkworm (*Bombyx mori*) silk is mainly composed of SF and sericin, which is about 25–30% of the silk cocoons mass. The degummed silk is being reassessed as biomaterial scaffolds due to its unique mechanical properties [4], and recent studies clarifying biodegradability, good biocompatibility, minimal inflammatory reaction, and noncytotoxicity [5–8].

Ultra-fine SF fiber mats prepared by electrospinning regenerated SF solution are architecturally similar to the structure of the extracellular matrix (ECM), which is beneficial to medical applications, such as wound dressings and scaffolds for tissue engineering. Upon electrospinning and treatment with methanol [9, 10], ethanol [11, 12], and water vapor [5, 13], the SF mats are predominately β-sheet structures, which is insoluble in water. The electrospun ultra-fine fiber mats can mimic the structure and biological function of native ECM proteins, which provide mechanical support and regulate cell activities. It has been reported that SF fiber mats were effective in the cell attachment, spreading, and proliferation of normal human keratinocytes, fibroblasts, and bone marrow stromal cells [5, 9, 13–15].

The water vapor-treated SF fiber mats showed good cellular compatibility in comparison with traditional methanol-treated ones [13]. In the present study, we first studied the effect of different electrospin solvent of FA [9, 16–18] and HIFP [5, 13, 19], which have been employed to electrospin SF by many researchers before on the diameter and structure of electrospun SF fibers, and on adhesion and proliferation of normal human epidermal fibroblasts (NHEF) cultured on SF ultra-fine fibers.

Materials and methods

Preparation of regenerated SF fiber mats

Raw silk fibers were degummed twice with 0.5% (w/w) $NaHCO_3$ solution at 100 °C for 30 min and then rinsed thoroughly with deionized water to remove glue-like sericin proteins. The extracted silk was then dissolved in 9.3 M LiBr solution at 60 °C, followed by dialysis with cellulose tubular membranes (molecular weight cut-off, 8,000–14,000) against distilled water for 3 days. The SF membrane was prepared by spread the SF solution on the polyethylene plastic board and dried under the room temperature.

Silk fibroin solutions with 10% concentration were prepared by dissolving the regenerated SF membranes in 98% FA for 3 h and in hexafluoroisopropanol (HFIP) for 1 week. In the electrospinning process, a voltage of 10 kV was applied to the stainless needle and a distance of 10 cm between the syringe tip and the collecting target were employed.

Ethanol treatment of SF fiber mats

Silk fibroin fiber mats electrospun with different solvent were immersed in 75/25 (v/v) ethanol/water for 30 min to induce crystallization of SF, and then dried in vacuum at room temperature for 24 h.

Characterization

The morphology of collected SF fiber mats were observed using scanning electron microscope (SEM, Hitach S-520, Japan) at 20 °C, 60 RH, samples were mounted on copperplate and sputter-coated with gold layer 20–30-nm thick prior to imaging, diameters of the fiber mats were acquired from SEM images randomly collected, using German Leica BME biomacroscope, for each sample, the diameter was the average of 100 measurements;

Fourier transform infrared (FTIR) spectra were obtained using a Magna spectrometer (NicoLET5700, America) in the spectral region of 400–4,000 cm^{-1}, the powdered electrospun SF fibers were pressed into potassium bromide (KBr) pellets prior to data collection;

X-ray diffraction(X'PERT PRO MPD, PANalytical Company, Holland) was operated at 40 kV tube voltage and 40 mA tube current, CuK_α radiation was used with diffraction angle $2\theta = 2°–45°$, the scanning rate is 2°/min with powdered electrospun SF fibers.

Thermogravimetry/differential thermal analysis (TG-DTA, PE-SII, America) conditions were nitrogen flux at 30 mL/min, heating rate at 10 °C/min, and temperature range from 40 to 500 °C and the samples weight is about 5 mg.

Cells and cell culture

Primary NHEF were established from the explant cultures of foreskins, which were excised from patients undergoing surgery with their consent. The cells that proliferated outwardly from the explants culture were continuously cultured in Dulbecco's modified Eagle's medium (DMEM) (containing 1% Antibiotic antimycotic solution: 10,000 IU streptomycin (per ml), 10 μg/mL ampicillin, 9 g/L NaCl) supplemented with 10% fetal bovine serum (FBS).

The SF fiber mats were collected onto the glass sheet (2 cm × 2 cm), which were placed into culture dish after post-treatment. The cells were seeded on the SF fiber mats and empty glass sheet in each well at a density of 1×10^5 cells/well. The medium was replenished every 3 days.

MTT assay

The cell proliferation on the SF fiber mats was assessed with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) at different culture periods up to 8 days.

In brief, the MTT solution was added to each cultured sample and further incubated for 4 h for MTT formazan formation. The purple formazan on each sample was extracted with dimethyl sulfoxide (DMSO), and the optical density of each extraction solution was measured with an automatic microplate reader (Molecular Devices, USA) at a wavelength of 540 nm. Triplicate readings were taken for each sample.

Statistical analysis

The data were presented as means ± SD. One-way analysis of variance (ANOVA) was performed in SPSS software. A p-value less than 0.05 was considered statistically significant.

Results and discussion

Morphology of electrospun SF fiber mats

The morphology of the SF fiber mats was varied with the different electrospinning solvents of HFIP and formic acid (FA), as showed in Fig. 1. The rod-like as-spun fiber had a smooth surface and rounded cross section. Fibers adhesion and bifurcation were observed because of incompletely solvent evaporation and jet disruption. The average diameters of the SF fiber mats electrospun with HFIP and FA were 2.0 and 0.3 μm, respectively. It is reported that under the same condition, the viscosity of SF–HFIP solution is

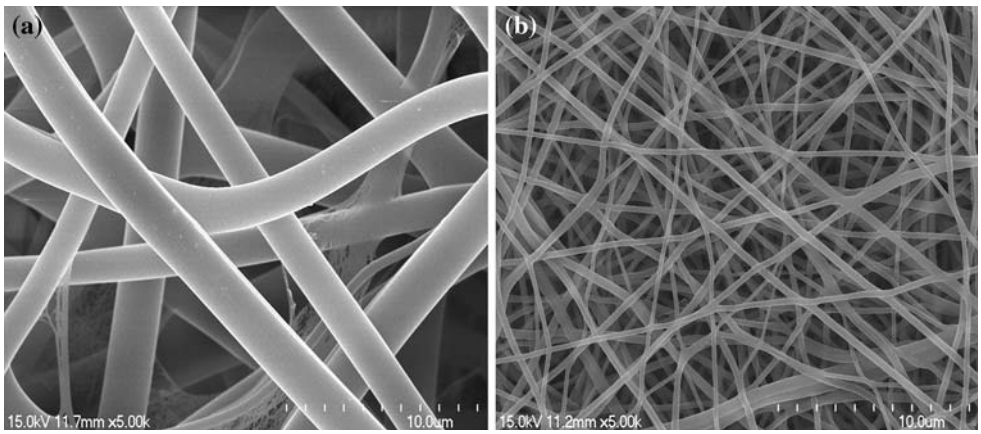

Fig. 1 SEM micrograph of electrospun SF fiber mats: **a** electrospun with HFIP; **b** electrospun with FA

six times of SF–FA solution [9, 13]. The higher viscosity of solution may result in the hard jet extension and disruption during the electrospinning process, and that must be one of the major reasons for the diameter difference of SF fiber mats electrospun with different solvents.

FTIR spectroscopy

The major conformations of SF are random coil, α-helix, β-sheet, and β-turn. IR is a powerful and common tool for the molecular conformations investigation, especially in the study of silk protein structure [20]. FTIR was employed to study the structure of the fibroin in the fibers mats electrospun with different solvents, HFIP and FA, as shown in Fig. 2. The FTIR spectrums of a and b showed almost same absorption bands, which were summarized in Table 1.

Silk fibroin fiber mats treated with aqueous ethanol solution showed characteristic absorption bands at 1,630, 1,530, 1,235, 1,068, 700 cm^{-1}, which are related to the

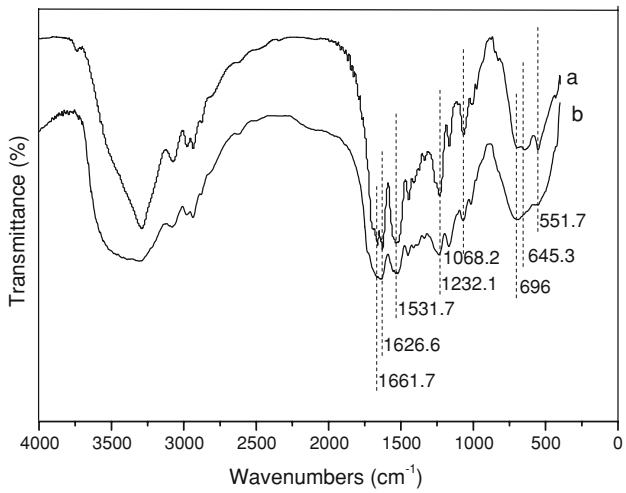

Fig. 2 FTIR spectra of the SF fiber mats electrospun with: (a) HFIP and (b) FA

β-sheet conformation and at 1,661, 645.3, 551.7 cm^{-1} due to α-helix or random coil conformation [13, 18, 20]. Differences appeared in the characteristic absorption peaks of the amide I and amide V of the SF fiber mats. Peaks of β-sheet and α-helix or random coil conformation coexisted in the amide I and amide V of SF fiber mats electrospun with HIFP, but the amide I and amide V of SF fiber mats electrospun with FA only showed β-sheet conformation.

The secondary structure of SF could be analyzed quantitatively through an established technique which had been reported before [21, 22]. Table 2 showed the proportion of each secondary structure of electrospun SF fiber mats. β-Sheet content in SF–FA fiber mats was more than that in SF–HFIP fiber mats before and after ethanol treatment. Interestingly, after ethanol treatment, the proportion of random coil, α-helix, and β-turn (sample b) all decreased and at the same time, β-sheet proportion increased; the proportion of β-sheet, random coil, α-helix (sample a) all increased and at the same time β-turn proportion decreased. It demonstrated that the fiber mats electrospun with FA contain more β-sheet conformation contents before and after ethanol treatment than that electrospun with HIFP. It has reported that the solvent FA can induce conformation change from α-helix or random coil to β-sheet [23, 24]. Furthermore, it is easier to induce structural transition of SF fiber mats electrospun with FA than that electrospun with HFIP.

X-ray diffraction

The molecular conformations of the SF fiber mats electrospun with different solvents are different as proven above. So, the crystalline structure was likely different as different electrospinning solvents were used. Figure 3 showed the X-ray diffraction pattern of the SF fiber mats electrospun with HFIP and FA. The similarities between the patterns of two different electrospinning solvents strongly suggest structural similarity of the fiber mats

Table 1 IR absorption bands of the SF fiber mats electrospun with: (a) HFIP and (b) FA

	AmideI (cm^{-1})		AmideII (cm^{-1})	AmideIII (cm^{-1})	AmideIV (cm^{-1})	AmideV (cm^{-1})		
a	1661.7	1626.6	1531.7	1232.1	1068.2	696	645.3	551.7
b	1636.7		1533.5	1235.9	1068.9	686.9		

Table 2 Proportion of each secondary structure (a and a′ are SF-HFIP fiber mats before and after ethanol treatment; b and b′ are SF-FA fiber mats before and after ethanol treatment)

Sample	β-sheet	Random coil	α-helix	β-turn
a	35.0	19.3	5.9	40.0
a′	41.2	21.0	17.0	20.8
b	36.3	19.2	17.5	27.0
b′	51.2	12.7	14.9	21.3

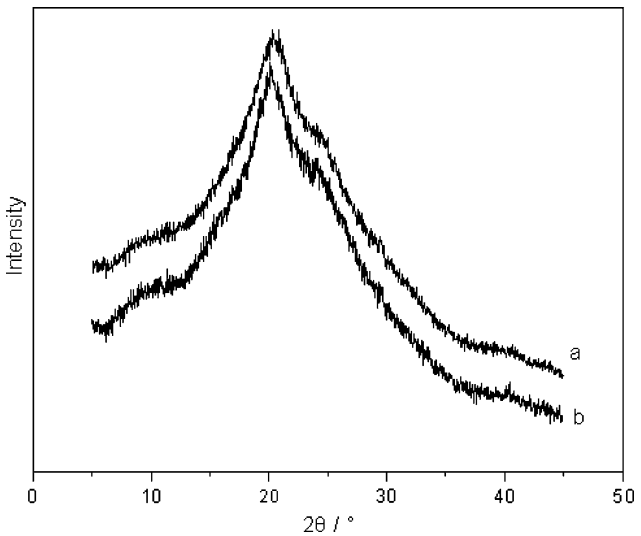

Fig. 3 X-ray diffraction pattern of the SF fiber mats electrospun with: (a) HFIP and (b) FA

electrospun with HFIP and FA. They were characterized by a strong diffraction peaks at 20.1° attributed to silk II and a weak shoulder peak at 23.2° due to silk II too [25], corresponding to the spacing of 4.41 and 3.83 Å, respectively. The difference was in the patterns and shapes, which were characterized by a more sharp diffraction peak of fiber mats electrospun with FA compared to that electrospun with HIFP. It demonstrated that the crystalline structure of the fiber mats after ethanol treatment was mainly composed of silk II, but compared to sample a, sample b contained more silk II contents and had a higher crystallinity. The crystallinity of the SF fiber mats calculated by the method of Herman [26], were 26.6 (sample b) and 19.7% (sample a), respectively.

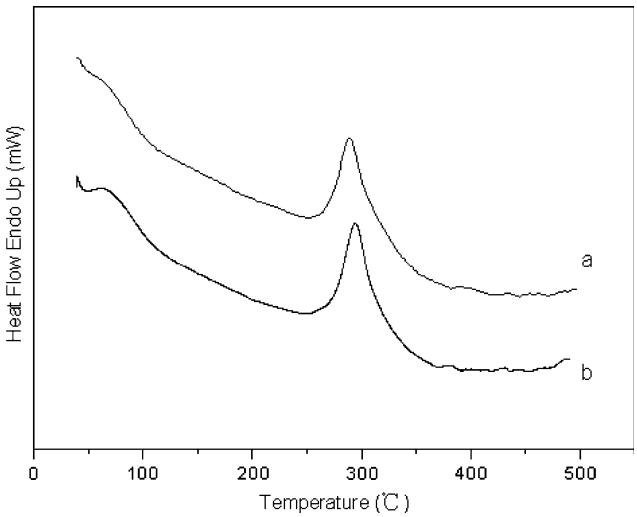

Fig. 4 DTA curves of the SF fiber mats electrospun with: (a) HFIP and (b) FA

DTA analysis

The thermal property of SF fiber mats electrospun with FA or HFIP was studied by means of DTA measurements. The curve a and b both only had an endothermic peak, at 289.7 and 294.0 °C, which is due to the thermal decomposition of the SF with unoriented β structure [27], as shown in Fig. 4. The solvent of FA and HFIP has a weak effect on the thermal property of SF fiber mats, which is mainly composed of β-sheet structure.

Cells attachment and proliferation

Scaffold materials for tissue engineering are required to promote cell adhesion, growth, and to maintain normal states of cell differentiation. The present study proved that the SF fiber mats show a difference in fiber diameter and structure when electrospun with different solvent. These differences may affect the cytocompatibility of SF fiber mats. Therefore, a study on the cytocompatibility of SF fiber mats electrospun with different solvent is especially valuable in relation to its possible application as biomaterials for tissue engineering.

To observe and compare the biological properties of SF fiber mats, NHEF were seeded onto SF fiber mats. Photographs of proliferating NHEF adhered to the SF fiber mats

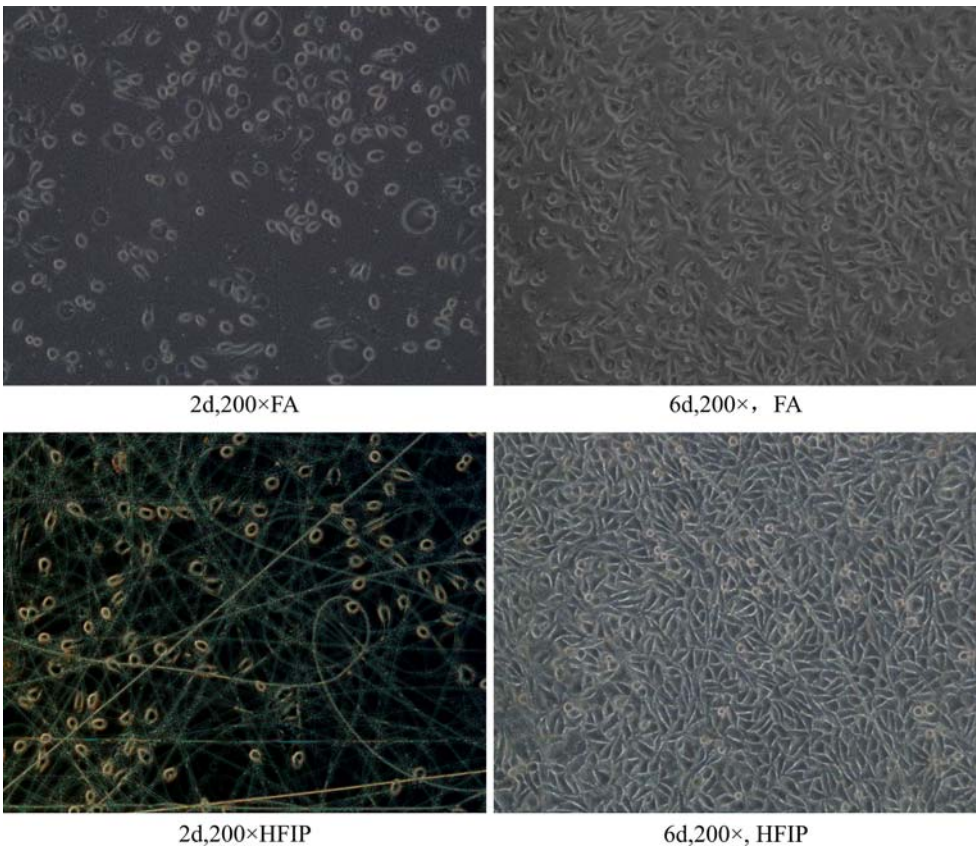

Fig. 5 Photographs of NHEF adhered to SF fiber mats

were taken and used for the attachment assay. Figure 5 shows representative pictures of cells adhered to the SF fiber mats after 2 and 6 days culture. The SF fiber mats electrospun with FA had a thin diameter (0.3 μm), so NHEF adhered onto several fibers; on the contrary, the SF fiber mats electrospun with HFIP had a coarse diameter (2.0 μm), so NHEF could adhere onto single fibers. A relatively low level of cell attachment of NHEF after 2 days culture was compared with 6 days culture, it suggested that the SF fiber mats support NHEF adhesion and proliferation. The proliferation of NHFF cells on the SF fiber mats assayed in different culture periods within 8 days was shown in Fig. 6. The results showed that the cell numbers were increased in accordance with culture periods. MTT assay results indicated that the three groups exhibited no observed significant difference in the cell viability during the 8 days culture time ($p < 0.05$). These results may indicate that the electrospinning solvent of FA and HFIP would not affect the cytocompatibility of SF fiber mats, and the two kinds of electrospun SF fiber mats showed generally similar adhesion activity under our conditions compared to the empty glass sheet control.

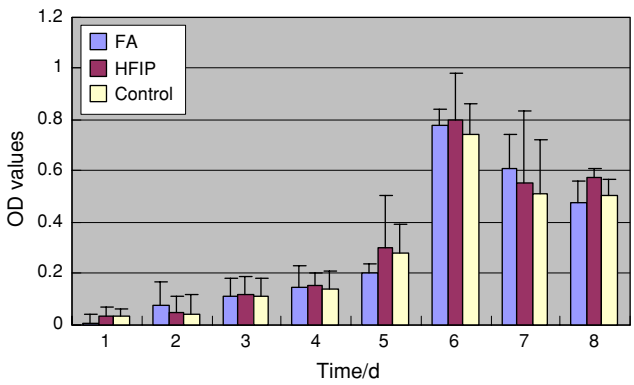

Fig. 6 The viability of NHEF cells on the SF fiber mats assayed by MTT

Conclusions

The SF fiber mats were prepared with different solvent of HFIP and FA. SEM, FTIR, X-ray diffraction, and TG-DTA were employed to study the morphology, secondary structure, and thermal properties.

1. The morphology of SF fiber mats was affected greatly by the solvent of FA and HIFP.
2. Effect of solvent of FA and HFIP on the secondary structure of regenerated SF fiber mats was also observed by FTIR and X-ray diffraction. The results

showed that the electrospin solvent not only affect the secondary structure of as-spun SF fiber mats, but also indirectly affect the structure transition of SF fiber mats post-treatment with ethanol. And the SF fiber mats electrospun with FA showed more β-sheet structure before and after ethanol treatment.

3. The thermal properties of SF fiber mats were not affected by the different solvent obviously.
4. SF fiber mats electrospun with FA or HFIP all promote NHEF attachment and proliferation, and no difference was found between the two groups.

We believed that suitable morphology and secondary structure of SF could be prepared by choosing different solvent, and the different morphology of the SF fiber mats has little effect on NHEF adhesion and proliferation.

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References

1. Kaplan DL, Adams WW, Farmer B, Viney C (1994) American Chemical Society Symposium Series, vol 544, p 2
2. Vollrath F, Knight DP (2001) Nature 410:541
3. Moy RL, Lee A, Zalka A (1991) Am Fam Physician 44:2123
4. Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen JS, Lu H, Richmond J, Kaplan DL (2003) Biomaterials 24:401
5. Park KE, Jung SY, Lee SJ, Min BM, Park WH (2006) Int J Biol Macromol 38:165
6. Santin M, Motta A, Freddi G, Cannas M (1999) J Biomed Mater Res 46:382
7. Meinel L, Hofmann S, Karageorgiou V, Kirker-Head C, McCool J, Gronowicz G, Zichner L, Langer R, Vunjak-Novakovic G, Kaplan DL (2005) Biomaterials 26:147
8. DalPra I, Freddi G, Minic J, Chiarini A, Armato U (2005) Biomaterials 26:1987
9. Min BM, Lee G, Kim SH, Nam YS, Lee TS, Park WH (2004) Biomaterials 25:1289
10. Ishida M, Asakura T, Yokoi M, Saito H (1990) Macromolecules 23:88
11. Zuo BQ, Wu ZY, Yan C, Sha XY (2004) J Mater Sci Eng 6:842
12. Zuo BQ, Wu ZY (2006) Chin J Clin Rehabil 10:168
13. Min BM, Jeong L, Lee KY, Park WH (2006) Macromol Biosci 6:285
14. Min BM, Jeong L, Nam YS, Kim JM, Kim JY, Park WH (2004) Int J Biol Macromol 34:281
15. Jin HJ, Chen J, Karageorgiou V, Altman GH, Kaplan DL (2004) Biomaterials 25:1039
16. Ayutsede J, Gandhi M, Sukigara S, Micklus M, Chen HE, Ko F (2005) Polymer 46:1625
17. Sukigara S, Gandhi M, Ayutsede J, Micklus M, Ko F (2003) Polymer 44:5721
18. Park WH, Jeong L, Yoo DL, Hudson S (2004) Polymer 45:7151
19. Zarkoob S, Eby RK, Remeler DH, Hudson SD, Ertley D, Adams WW (2004) Polymer 45:3973
20. Zhou W, Chen X, Shao ZZ (2006) Prog Chem 11:1514
21. Byler DM, Susi H (1986) Biopolymers 25:469
22. Jackson M, Mantsch HH (1995) Crit Rev Biochem Mol Biol 30:95
23. Um IC, Kweon HY, Hudson S (2001) Int J Biol Macromol 29:91
24. Ha SW, Tonelli AE, Hudson SM (2005) Biomacromolecules 6:1722
25. Ki CS, Lee KH, Baek DH, Hattori M, Um IC, Ihm DW, Park YH (2007) J Appl Polym Sci 105:1605
26. Weidinger A, Hermans PH (1961) Makromol Chem 50:98
27. Tsukada M, Freddi G, Kasai N (1994) J Polym Sci 32:1175