Preparation of Insoluble Fibroin Films Without Methanol Treatment

Qiang Lv, Chuanbao Cao, Ying Zhang, Xilan Ma, Hesun Zhu

Research Center of Material Science, Beijing Institute of Technology, Beijing 100081, China

ABSTRACT: Insoluble fibroin films were prepared without methanol treatment. A 15 wt % fibroin solution was obtained through concentration, and then, the insoluble film was achieved by the adjustment of the drying temperature and rate. These films were examined through Fourier transform infrared–attenuated total reflection, X-ray diffraction, and scanning electron microscopy to determine the structure of the silk fibroin. The physical and mechanical properties were investigated to discover the feasibility of using these films as biomedical materials. When fibroin films were dried above 60°C, abundant β-sheet crystals existed in the fibroin films, and many globule-containing micelles aggregated and interacted with each other, which resulted in excellent mechanical properties of the regenerated fibroin films in the wet state. Interestingly, the amide III band of the random coil structure in the fibroin films dried at 70 and 80°C was shifted to a lower frequency, 1228 cm^{-1}, which meant that a partly orientated structure formed. This may have also affected the mechanical properties of the fibroin films. The tensile strength and breaking elongation of the films dried at 70°C were 29.8 MPa and 59.6%, which is distinctly superior to fibroin films treated with methanol. If the drying temperature was raised to 60°C, the stability of the films in water was also excellent. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 2168–2173, 2005

Key words: conformational analysis; mechanical properties; structure

INTRODUCTION

Silk fibroin is the protein that forms the filaments of the silkworm and has been recognized as a potential new biomaterial;[1–3] therefore, the development of silk reprocessing strategies is an important component in the development of new biomaterials. Although the influences of casting temperature,[4,5] drying rate,[6] solvent,[7] and heat treatment[8,9] on the molecular conformation have been extensively studied, insoluble regenerated fibroin films have still not been achieved directly through the adjustment of the processing conditions. Until now, insoluble fibroin films have been obtained with methanol treatment.[10,11]

In a recent article, Jin and Kaplan[12] reported that the formation of emulsion and micellar structures from aqueous solutions was the first step in the process to control water and protein–protein interactions. These micelles subsequently aggregated into larger globules and gel-like states as the concentration of silk fibroin increased, while the solubility was maintained because of hydrophilic regions of the protein interspersed among the larger hydrophobic regions. This was a critical factor that made silk fibroin insoluble through physical shearing or stretching structural transitions. When one considers that an increase in casting temperature is also favorable to the formation of a β-sheet conformation, drying concentrated fibroin solution at a higher temperature may achieve the insoluble fibroin films directly.

In this study, insoluble fibroin films were obtained directly without methanol treatment. X-ray diffraction (XRD), IR spectroscopy, and scanning electron microscopy (SEM) were used to analyze the structural changes of silk fibroin. The mechanical properties were also studied to determine the effect of drying temperature. In addition, the stability of fibroin films was determined by their weight loss in water for 24 h.

EXPERIMENTAL

Preparation of fibroin films

Bombyx mori silk was immersed in 0.5 wt % (w/w) Na_2CO_3 at 100°C for 60 min to remove the sericin component; then, fibroin fibers were dissolved in a $CaCl_2/H_2O/EtOH$ solution (molar ratio = 1:8:2) at 80°C. The fibroin solution was filtered and dialyzed against distilled water for 3 days to yield a fibroin water solution. After a 15 wt % fibroin solution was

obtained through concentration at 45–50°C with stirring, it was cast on polystyrene petri dishes and dried at 40, 50, 60, 70, and 80°C (labeled as SF40, SF50, SF60, SF70, and SF80, respectively) to prepare the film samples.

Measurements

The swelling ratios of the fibroin films were calculated with the following equation:

$$\text{Swelling ratio } (\%) = (W_1 - W_2)/W_2 \times 100$$

where W_1 is the weight of the swollen samples and W_2 is the weight of the dry samples. The weight of the completely dried sample was measured directly, and that of the samples swollen in distilled water at 37°C for 24 h was measured after the removal of excess water on the surface.

Fourier transform infrared (FTIR) spectra were obtained with a System 2000 spectrometer (Perkin Co., Wellesley, MA) in the spectral region 1800–900 cm^{-1}. The XRD curve was obtained with a D-Max-2400 diffractometer (Rigaku Co., Japan) and Cu Kα radiation at a wavelength of 1.5418 Å. The scan speed was 0.5°/min, and the range was $2\theta = 5$–35° under 30 kV and 20 mA. SEM of fibroin films was carried out with a HITACHI-3500 (Japan).

The tensile strength and elongation at break in the wet state was measured with an Instron 6022 machine (Canton, MA) after the sample was soaked in water for over 24 h and the excess water on the surface was removed. The experimental conditions were as follows: extension rate = 10 mm/min and dimension of the sample = 4 × 15 × 0.15 mm.
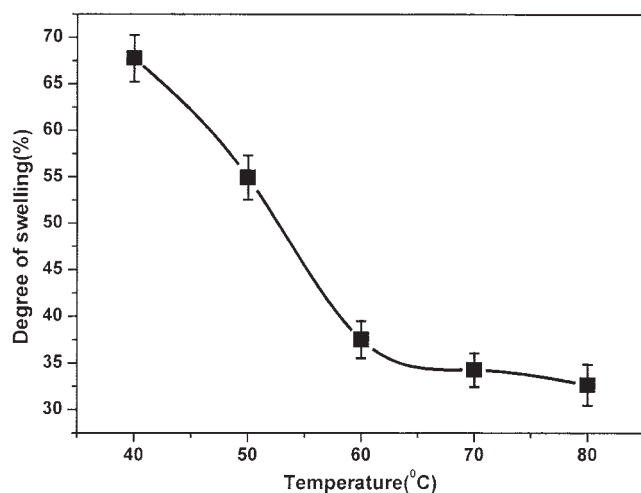

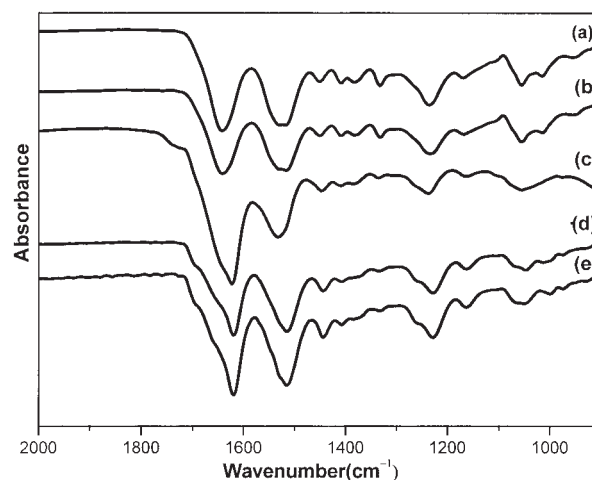**Figure 1** Relationship of the degree of swelling of the fibroin films with the drying temperature.

Figure 2 FTIR spectra of silk fibroin films prepared at various temperatures: (a) 40, (b) 50, (c) 60, (d) 70, and (e) 80°C.

RESULTS AND DISCUSSION

Swelling

The degree of swelling of the fibroin films was measured in terms of the equilibrium swell ratio (Fig. 1). When one considers the possible applications in artificial skin and wound dressings, the degree of swelling is one of the important factors in the determination of the usefulness of biomaterials. The swelling ratio of the fibroin films decreased from 67.7 to 32.6% when the drying temperature was increased from 40 to 80°C. The reason was that the increase in the temperature facilitated fibroin transformation from a random conformation to a β-sheet crystal. When the drying temperature was higher than 60°C, however, the degree of swelling was not much different than that of fibroin films dried at 60°C. The reason for this was there were lots of β sheets and relatively low random conformation in the samples.

FTIR–attenuated total reflection spectra

Because typical absorption bands are sensitive to the molecular conformation of fibroin, many researchers have used IR spectra to determine fibroin conformation.[5,7,13–18] As shown in Figure 2, the fibroin film dried at 40 and 50°C showed absorption bands at 1642 cm^{-1} (amide I) and 1235 cm^{-1} (amide III), which were attributed to the α/random coil structure. As the drying temperature was increased up to 60°C, the absorption bands characteristic to the antiparallel β-sheet form, 1620 and 1260 cm^{-1}, were clearly observed. When the films were dried at 70 and 80°C, besides the bands at 1620 and 1260 cm^{-1}, the absorption band of amide II was also changed from 1530 to 1515 cm^{-1}.
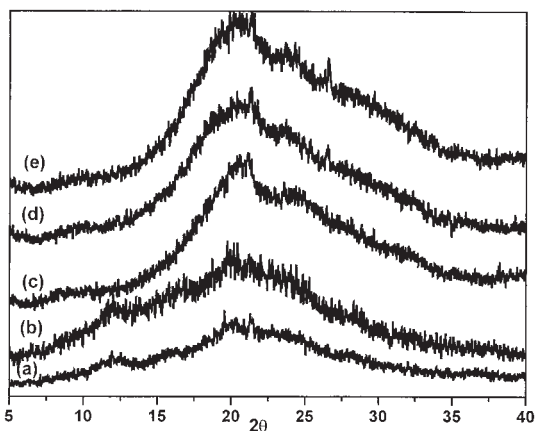
Figure 3 XRD patterns of the fibroin films prepared at different temperatures: (a) 40, (b) 50, (c) 60, (d) 70, and (e) 80°C.

These data showed that the conformational transition from the α/random coil to an antiparallel β-sheet form occurred by the increase in the drying temperature, which was partly in agreement with the investigation by Tretinnikov.[5] However, the result differed with that of Tretinnikov in that the intensity of the 1620-cm^{-1} bands still increased instead of decreased when the drying temperature was raised from 50 to 70°C. This difference may have been due to the effect of fibroin concentration because Tretinnikov dried fibroin films at 2.5–2.8 wt %, whereas we obtained fibroin films from a 15 wt % fibroin solution. Although the amide III bands at about 1235 cm^{-1} still existed in the fibroin films dried at different temperatures, which indicated that the random conformations did not all transform into β-sheet formations with increasing drying temperature, the SF70 and SF80 films had a peak at 1228 cm^{-1}, which was shifted to a lower frequency compared to the results of the other fibroin films. This suggests that the random coil structure in SF70 and SF80 has a more partly orientated structure than that of films dried at other temperatures, which may be one of the reasons that SF70 and SF80 had excellent mechanical properties in the wet state.

XRD

XRD curves of the regenerated fibroin films dried at different temperatures are shown in Figure 3. The films dried at 40 and 50°C could be characterized by the presence of three peaks at 12, 20.5, and 23.7°, corresponding to the α-helix crystalline spacing of 7 Å and the β-sheet crystalline spacings of 4.3 and 3.75 Å,[12,19] respectively. These results exhibited the copresence of α-helix and β-sheet structures. When the films were dried above 60°C, a new peak at 9.0°, which corresponded to the β-sheet crystalline spacing of 9.8

Å,[12] appeared, and the peak at 12° disappeared. The results indicate that α-helix form was transformed to an antiparallel β sheet with increasing drying temperature, and the α-helix crystalline almost disappeared when the drying temperature was raised above 60°C.

Surface characteristics

Figure 4 shows the characteristic surfaces of the films dried at different temperatures. Putthanarat et al.[20] reported that four major classes, including particles, grains, nanofibrils, and an irregular morphology, existed in fibroin films. When the drying temperature increased, the samples exhibited larger grains and larger, more densely packed nanofibrils. In this study, the morphology of the silk films dried above 50°C was mainly composed of grains. This may be because the concentrated fibroin solution (15 wt %) favored the formation of grains. When the drying temperature was raised to 70 and 80°C, the grains grew larger and congregated each other, which had a similar morphology with fibroin films that were prepared by Hyoung-Joon Jin through methanol treatment.

Jin and Kaplan[12] suggested that globule-containing micelles were obtained by increases in the fibroin concentration. Then, the physical shear elongated and aligned the globule-containing micelles and initiated the final structural transition to the insoluble crystalline β sheet to establish the basis for the high-strength and tough silk fibers. In our research, the increases in drying temperature promoted the transformation from the α-helix to β-sheet form. More importantly, when the drying temperature was raised above 60°C, the unusual round morphologies existed in the fibroin films, which were similar to the morphology of fiber spun from a pure fibroin solution (16 wt % fibroin).[12] This suggests that the increase in drying temperature had a similar effect on the fibroin morphology with spinning. A possible reason is that the increase in drying temperature enhanced the activity of micelles in the concentrated fibroin solution and made the fibroin transform into a more stable state. In addition, the samples exhibited a larger and more densely packed aggregation of the globule-containing micelles with increasing drying temperature. This may have induced the improvement in the mechanical properties of the fibroin films in the wet state. However, because the morphology of SF70 was more regular and well-proportioned than that of SF80, the SF70 films had the best mechanical properties, rather than the SF80 films, in the wet state.

Mechanical properties

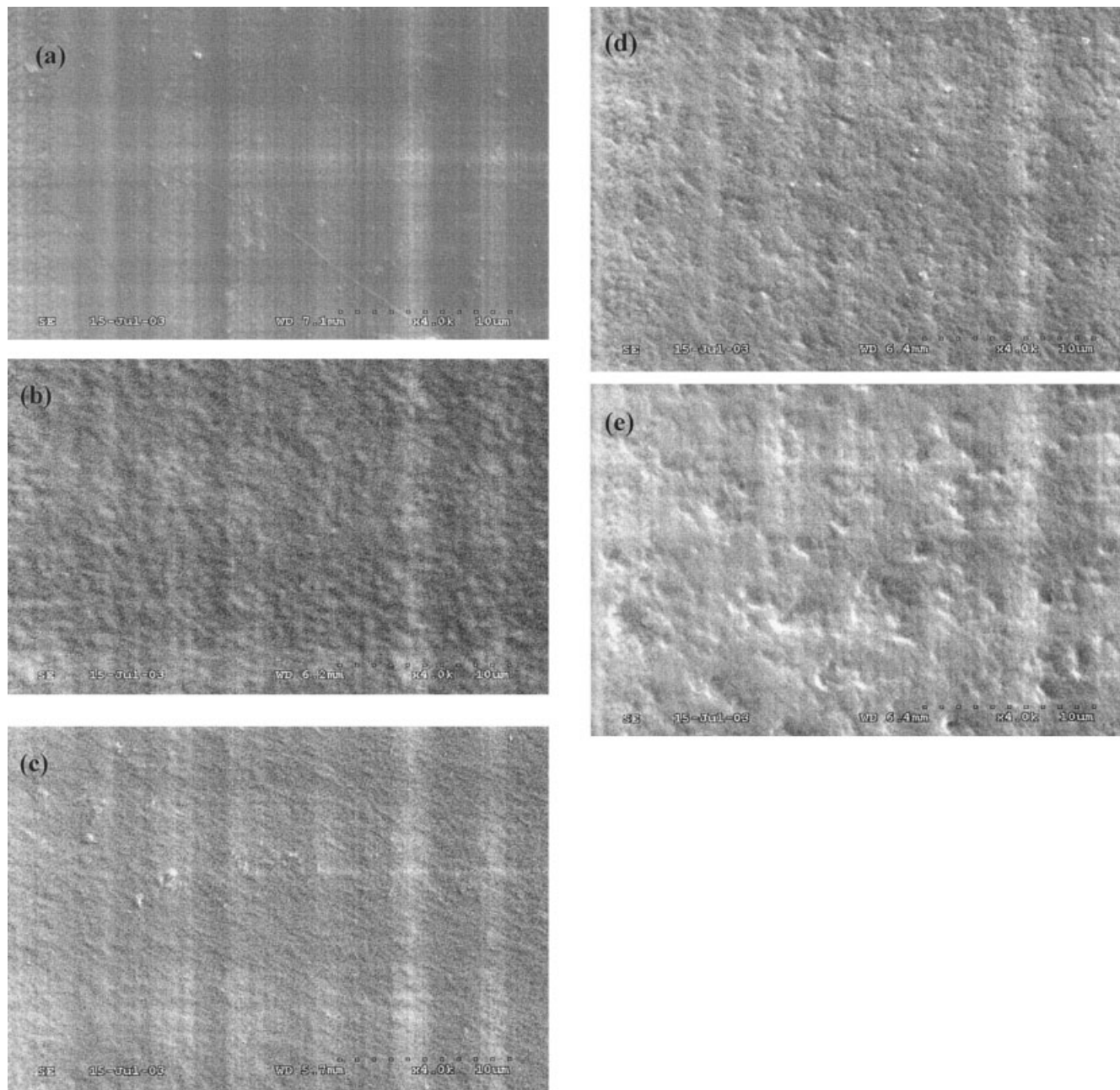The mechanical properties in the wet state are of primary importance for the determination of the

Figure 4 SEM photographs of the fibroin films prepared at different temperatures: (a) 40, (b) 50, (c) 60, (d) 70, and (e) 80°C.

performance of materials expected to undergo various types of stresses during use. In our study, the stress–strain curves were obtained in the wet state.

Figure 5 shows the tensile strength of fibroin films produced at different temperatures. When fibroin films were prepared at 40 and 50°C, the tensile strengths of films were very weak, only about 0.94 and 1.53 MPa, respectively. However, when fibroin films were dried above 60°C, the tensile strength increased sharply, and the maximum value obtained at 70°C was 29.8 MPa. Compared with the results reported by Kweon et al.[21] (4.5 MPa and elongation at break = 10%), the strengths of the fibroin films dried above 60°C were distinctly superior to fibroin films treated with methanol. The elongation at break of the fibroin films is shown in Figure 6. Although the silk fibroin film was very brittle in dry conditions, the elongation increased as the film absorbed water in the wet state. From 40 to 80°C, the elongations at break were 21.9, 29.3, 28.8, 58.6, and 44.7%, respectively. Interestingly, the breaking elongation of the films also had the maximum value when the fibroin films were dried at 70°C. The results indicate that the fibroin films produced at higher drying temperatures had better mechanical properties than those treated with methanol.
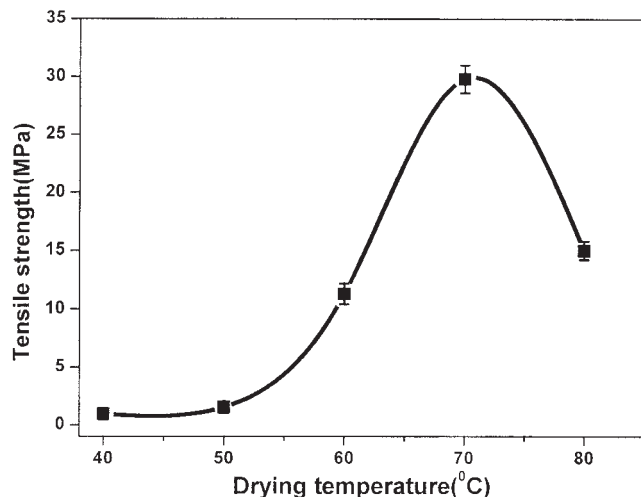
Figure 5 Relationship of the tensile strength of the fibroin films with the drying temperature.

The mechanical properties of fibroin films are quite important from a practical point of view. In our study, fibroin films with excellent mechanical properties were easily obtained through increased drying temperatures.

Stability in water

Because most biomaterials are used in the wet state, their stability in water is of importance for performance. In this study, weight changes were investigated after the fibroin films were immersed in water at 37°C for 24 h and dried at 80°C for 24 h. As shown in Figure 7, the weight losses of the fibroin films dried at 40, 50, 60, 70, and 80°C were 35.0, 15.1, 2.5, 0.08, and
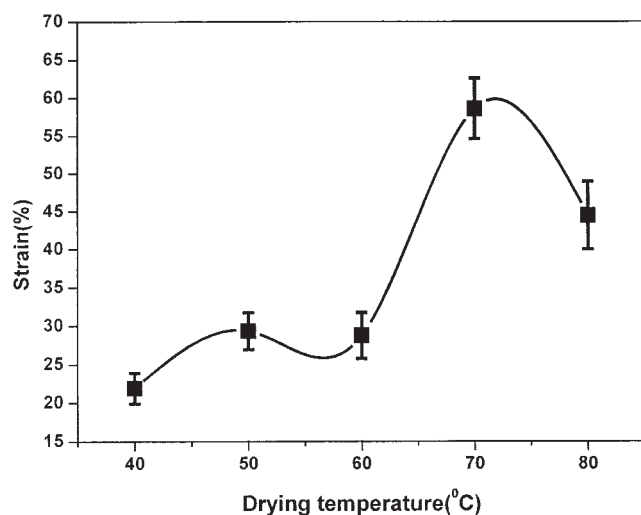

Figure 6 Relationship of the breaking strain of the fibroin films with the drying temperature.
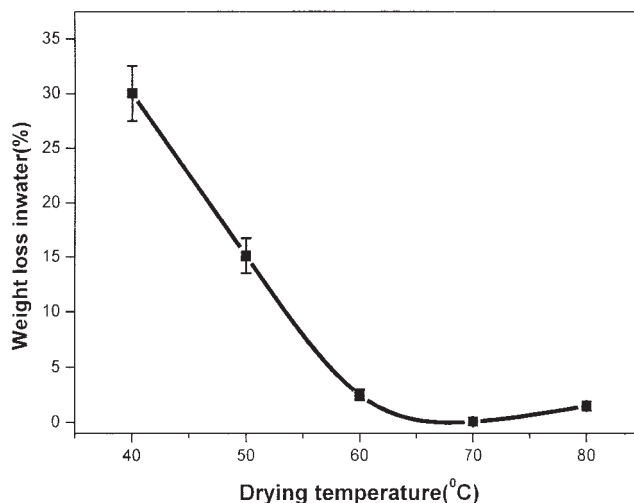

Figure 7 Relationship of the weight loss of the fibroin films immersed in water for 24 h at 37°C with the drying temperature.

1.5%, respectively. After immersion in water for 24 h, the fibroin films dried above 60°C retained a constant weight when they were immersed in water for another 10 days. The results indicate that the fibroin films dried above 60°C have excellent stability in water.

CONCLUSIONS

Insoluble fibroin films were prepared without methanol treatment, and their physical and mechanical properties were examined with various drying temperatures. When the concentrated fibroin solution was dried above 60°C, the fibroin films became insoluble, and abundant β-sheet crystals existed in films. The increase in the drying temperature induced the aggregation and interaction of globule-containing micelles, which may have made the mechanical properties of fibroin films in the wet state distinctly superior to fibroin films treated with methanol. Although the mechanism should be studied more extensively, fibroin films formed with this method were more useful and safer than those treated with methanol.

References

1. Altman, G. H.; Horan, R. L.; Lu, H. H.; Moreau, J.; Martin, I.; Richmind, J. C.; Kaplan, D. L. Biomaterials 2002, 23, 4131.
2. Demura, M.; Asakura, T. J Membr Sci 1991, 59, 39–52.
3. Sofia, S.; Mccarthy, M. B.; Gronowicz, G.; Kaplan, D. L. J Biomed Mater Res 2001, 54, 139.
4. Magoshi, J.; Nakamura, S. J Polym Sci Polym Phys Ed 1985, 23, 227.
5. Tretinnikov, O. N.; Tamada, Y. Langmuir 2001, 17, 7406.
6. Magoshi, J. Polymer 1977, 18, 643.
7. Mathur, A. B.; Tonelli, A.; Rathke, T.; Hudson, S. Biopolymers 1997, 42, 61.

8. Kweon, H.; Woo, S. O.; Park, Y. H. J Appl Polym Sci 2001, 81, 2271.
9. Freddi, G.; Monti, P.; Naguba, M.; Gotoh, Y.; Tsukada, M. J Polym Sci Part B: Polym Phys 1997, 35, 841.
10. Masuhiro, T.; Yoko, G.; Masaobu, N.; Norihiko, M.; Nobutami, K.; Giuliano, F. J Polym Sci Part B: Polym Phys 1994, 32, 961.
11. Norihiko, M.; Masuhiro, T.; Masanobu, N. Polymer 1990, 31, 265.
12. Jin, H.-J.; Kaplan, D. L. Nature 2003, 424, 1057.
13. Petrini, P.; Parolari, C.; Tanzi, M. C. J Mater Sci: Mater Med 2001, 12, 849.
14. Yoshimizu, H.; Asakura, T. J Appl Polym Sci 1990, 40, 1745.
15. Nam, J.; Park, Y. H. J Appl Polym Sci 2001, 81, 3008.
16. Tsuboi, Y.; Ikejiri, T.; Shiga, S.; Yamada, K.; Itaya, A. Appl Phys A 2001, 73, 637.
17. Noishiki, Y.; Nishiyama, Y.; Wada, M.; Kuga, S.; Magoshi, J. J Appl Polym Sci 2002, 86, 3425.
18. Wilson, D.; Valluzzi, R.; Kaplan, D. Biophys J 2000, 78, 2690.
19. He, S.-J.; Valluzzi, R.; Gido, S. P. Int J Biol Macromol 1999, 24, 187.
20. Putthanarat, S.; Zarkoob, S.; Magoshi, J.; Chenm, J. A.; Eby, R. K.; Stone, M.; Adams, W. W. Polymer 2002, 43, 3405.
21. Kweon, H.; Ha, H. C.; Um, I. C.; Park, Y. H. J Appl Polym Sci 2001, 80, 928.