The preparation of insoluble fibroin films induced by degummed fibroin or fibroin microspheres

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Blending degummed fibroin (DF) or insoluble fibroin microspheres with concentrated fibroin solution, the insoluble films were obtained through drying the solution at 40–45 °C. The conformation of silk fibroin films was analyzed by infrared spectrum and X-ray diffractometry. The results demonstrated that β-sheet conformation increased rapidly when the degummed silk or insoluble microspheres blended with fibroin, while the pure SF membrane was mainly composed of α/random coil conformation when the other conditions kept same. This suggested that fibroin microspheres and degummed fibroin could induce the formation of β-sheet crystal and the insoluble films, without methanol after-treatment, could be obtained at approximately room temperature. Although the fibroin films blending with DF had many protuberances, the films containing fibroin microspheres had the smooth surface and could be used effectively in biotechnological materials and biomedical application.

Introduction

Silk fibroin has been extensively studied as a promising biomaterial because of its excellent biological compatibility [1–3]. However, the regenerated fibroin films, being soluble in water, are not suitable for use by themselves. The current applied method which made fibroin insoluble in water was methanol treatment in which methanol was poisonous and the treated fibroin films had poor mechanical properties [4–6]. In order to solve these problems, many researchers have blended fibroin with other polymers [6–9] or coated fibroin on other polymers' surface [10, 11], but these methods usually resulted in the decline of biocompatibility. Also, the effect of different conditions such as temperature, drying rate and concentration was studied to try to obtain insoluble fibroin films without methanol [12, 13]. Until now, there is not any report about insoluble fibroin films without methanol treatment. If the insoluble fibroin films could be directly obtained, the excellent biocompatibility of fibroin and the good mechanical properties could be obtained together.

In this article, through blending degummed fibroin fibril or fibroin microspheres with fibroin solution, the insoluble fibroin films were obtained at 40–45 °C without using methanol treatment. The X-ray diffraction (XRD) and the infrared (IR) spectroscopic method were used for the analysis of molecular conformation. The stability of silk fibroin films was determined by the weight loss in water for 24 h at 37 °C and the mechanical properties were also measured in wet state.

Experimental

Materials

Raw silk was degummed with 0.5%(w/w) Na_2CO_3 solution at 100 °C for 1 h and then washed with distilled water. Degummed silk was dissolved in a ternary solvent system of $CaCl_2/H_2O$/EtOH solution (1/8/2 mole ratio) for 30 min at 80 °C and dialyzed to remove salts in a cellulose tube against distilled water for 3 days at room temperature. Through adjusting the weight of fibroin dissolved in ternary solvent, the initial fibroin concentration was about 4%. Then the concentrated solution of silk fibroin was obtained by drying the solution at 45–50 °C with stirring.

The degummed silk (DF) obtained above was cut into small fragment about 0.1 mm and this degummed fibroin was added in the concentrated fibroin solution, then the solution was cast onto polystyrene plates and the solvent evaporated in the air at 40–45 °C.

The ratio of fibroin and DF were modified to obtain insoluble membrane. Table I showed the films' conformation at different conditions and the optimal condition was as follows: the mass percent of DF was 2% and the concentration of fibroin solution kept 15%. The preparation of insoluble fibroin films containing DF was repeated at least three times to ensure the reproducibility.

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TABLE I The effect of different condition to the stability of films in water

	Concentration (%l)	Content of DF(%)	Conformation in water
1	7.5	1	Soluble
2	15	1	Part-soluble
3	7.5	2	Part-soluble
4	15	2	Insoluble, no aggregation of DF
5	15	3	Insoluble but part aggregation of DF

The preparation of fibroin microspheres and films

The degummed fibroin fibrils were cut into 0.1 mm, which made the insoluble films have some protuberances in films' surface. In order to prepare the insoluble films having smooth surface, the insoluble fibroin microspheres were obtained and added into fibroin solution. The degummed silk was added in a ternary solvent system of $CaCl_2/H_2O$/EtOH solution (about 1/10/2 mole ratio) for 30 min at 80 °C, then this mixture was dialyzed to remove salts in a cellulose tube. In the process of dialysis, fibroin precipitation was obtained. After rinsing with distilled water, the precipitation was dried at 80 °C and ground in a mortar to prepare microspheres. The diameters of microspheres were in the range of 70–200 nm. The 0.5 wt% fibroin microspheres was added to the concentrated fibroin solution and the insoluble fibroin films were obtained by drying the solution at 40–45 °C. The experiment was also repeated at least three times.

Measurements

Fourier transform infrared (FTIR) spectra were obtained with a system 2000 spectrometer (Perkin. Co, American). X-ray diffraction curves were recorded with a D-Max-2400 Diffractometer (Rigaku Co), with CuKa Radiation ($\lambda = 1.54$ Å). Scanning electron micrography (SEM) of the fibroin films was carried out using a HITACHI-3500. The tensile strength and elongation at break of the films was measured at after the sample was soaked in water for over 24 h using an Instron 6022 machine. The experimental conditions were 10 mm/min tensile speed and $4 \times 15 \times 0.15$ mm sample dimensions.

Results and discussion

The conformation of fibroin films having DF

FTIR spectra

Sample SF-1 and SF-2 represents the fibroin films dried from 1.5 and 15% concentrations at 40–45 °C, respectively and SF-3 is the insoluble fibroin films containing DF. Fig. 1 showed the FTIR-ATR spectra of films. SF-1 and SF-2 showed absorption bands at 1642 cm^{-1} (amide I), 1515 cm^{-1} (amide II), 1230 cm^{-1} (amide III) and 1053 cm^{-1} in which the bands at 1642 cm^{-1}, 1230 cm^{-1} and 1053 cm^{-1} were assigned to the random α helix/random coil conformation while the band at 1515 cm^{-1} was belonged to the β-sheet conformation [14–16]. So SF-1 and SF-2 both mainly consist of α helix/random coil conformation and some β-sheet

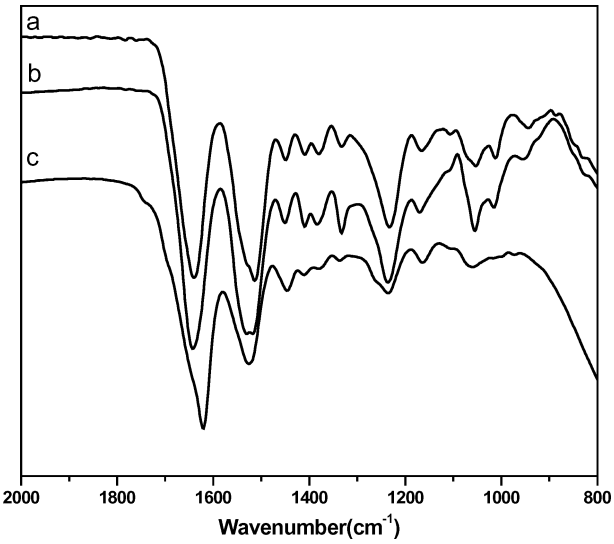

Figure 1 FTIR-ATR spectra of different SF films: (a) SF-1, dried in 1.5% concentration, (b) SF-2, dried in 15% concentration and (c) SF-3, blended with DF.

structure. When DF blended in the concentrated solution (15%) and dried at 40–45 °C, the film (SF-3) showed many new peaks. The amide I bands changed to 1621 cm^{-1} that is characteristic for anti-parallel β-structural frequencies. It could also be found SF-3 showed absorption bands at 1525 and 1068 cm^{-1}, which were belong to β-sheet conformation. While the peak at 1236 cm^{-1} was indicative of a random coil structure, a shoulder at 1265 cm^{-1} was related to a β-structural amide III. Therefore, it suggested that the β-sheet structure increased rapidly when DF (2%) blended with SF.

X-ray diffraction curves

X-ray diffraction curves of three samples were shown in Fig. 2. SF-1 membrane showed a very broad peak at $2\theta = 20$ °C, which was a typical characteristic

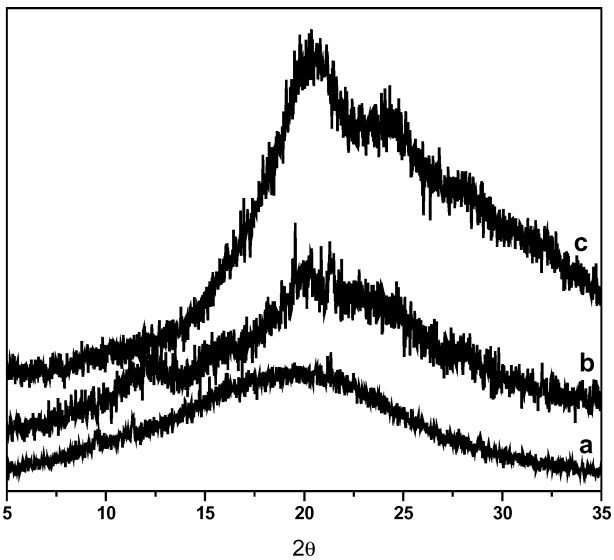

Figure 2 X-ray diffraction patterns of the SF films: (a) SF-1, dried in 1.5% concentration, (b) SF-2 , dried in15% concentration and (c) SF-3, blended with DF.

TABLE II The weight loss in water of different fibroin films at 37 °C

Fibroin concentration (%)	Content of DF or microspheres (%)	Weight loss (%)
1.5	0	100
15	0	35 ± 3.5
15	2 DF	2±1
15	0.5 microspheres	4.5±0.5

diffraction pattern of amorphous silk fibroin while SF-2 films exhibited the peaks at 12 °C and 28.5 °C, which were assigned to the α crystal (silk I) as well as the peaks at 20 °C and 24 °C, which belonged to β crystal (silkII). The results suggested that SF-2 had both α crystal and β crystal structures [7–9]. After DF (2%, w/w) blended in 15% fibroin solution and dried at 40–45 °C, the peaks at 20.9 °C and 24.5 °C could be found, which were attributed to β crystal structure. At the same time, the peaks at 12 °C and 28.5 °C, the typical peaks of α crystal has disappeared, which meant the α crystal changed into β-sheet conformation. It confirmed the results of FTIR and suggested that DF could induce the β-sheet conformation transition in concentrated fibroin solution.

The weight loss in water

The stability in water was important for the use of biomaterials, so we use the weigh loss in water to measure the stability of fibroin films (Table II). Every sample was tested three times and the average value was obtained. Sample SF-1 was soluble in water and the weight loss of SF-2 was 35%. But the weight loss of SF-3 was only 2% for 24 h and nearly kept constant when it was immersed in water for 10 days. The results suggested that the films blended with DF could keep stable in water. Because the α-helix/random coil conformation was considered as soluble while the β-sheet conformation was insoluble, the stability of fibroin films in water meant the increase of β-sheet conformation and DF could induce the transformation from α-helix/random coil conformation to β-sheet crystal.

Mechanical properties

Mechanical properties are of primary importance for determining the performance of materials expected to undergo various types of stresses in use. In our study, the stress-strain curves were obtained in the wet state, which was more important in practical applications of biomaterials than that in dry state. Table III showed the mechanical properties of different fibroin films. Every sample was tested three times and the average value was obtained. Haeyong Kweon *et al.* [6] reported that the tensile strength and the elongation at break were 4.5 MPa and 10% when the fibroin films treated with methanol. In our study, the tensile strength and the elongation at break of pure fibroin films dried at 40–45 °C were 0.94 MPa and 21.9%. When DF was added, the tensile strength and elongation changed to 6.47 MPa and 16%, respectively. That meant the mechanical properties were improved remarkably and were superior to fibroin films treated with methanol owning to the increase of β-sheet crystal.

TABLE III Mechanical properties of fibroin films in wet state dried at 40–45 °C

	Tensile strength (MP)	The elongation at break (%)
Pure fibroin films	0.94 ± 0.5	21.9 ± 4
Fibroin films containing microspheres	6.47 ± 1.3	16 ± 2

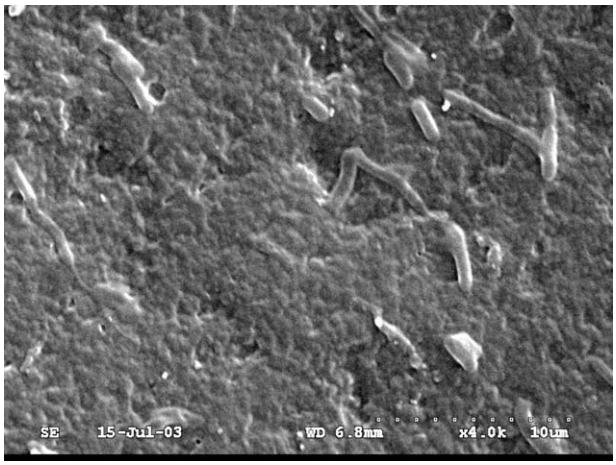

Figure 3 The SEM of fibroin films containing degummed fibroin.

The properties of fibroin films containing microspheres

The surface morphology of films

The fibroin films containing DF had so many protuberances in films' surface that this surface was not suitable for many applications (Fig. 3). In order to obtain the relative smooth surface, the insoluble fibroin microspheres were produced and added to the fibroin solution to prepare the insoluble fibroin films dried at 40–45 °C (sample SF-4). The surface morphology of film and microspheres was shown in Fig. 4. The fibroin microsphere's sizes ranged from 70 to 200 nm. The fibroin films containing microspheres had very smooth surface.

FTIR spectra of films containing microspheres

Fig. 5 showed the FTIR-ATR spectra of films (SF-4) and the FTIR spectra of microspheres. Characteristic peaks are at 1514, 1620 and 3285 cm^{-1} for the fibroin films (Fig. 5(a)), while these peaks shifted to 1531, 1656, 3292 and 3471 cm^{-1} for the microspheres (Fig. 5(b)). The results indicated that the fibroin films containing fibroin microspheres were mainly composed of β-sheet conformation. Because the microspheres were prepared through a ternary solvent system of $CaCl_2/H_2O/EtOH$ solution (about 1/10/2 mole ratio) to breach the β-sheet conformation, the microsphere appears many random conformation bands. However, the peaks at 3292 cm^{-1} and 1265 cm^{-1} and the insolubility in water suggested that the microspheres still have many β-sheet conformation which can induce the conformational transformation of fibroin films as crystal nucleation center.

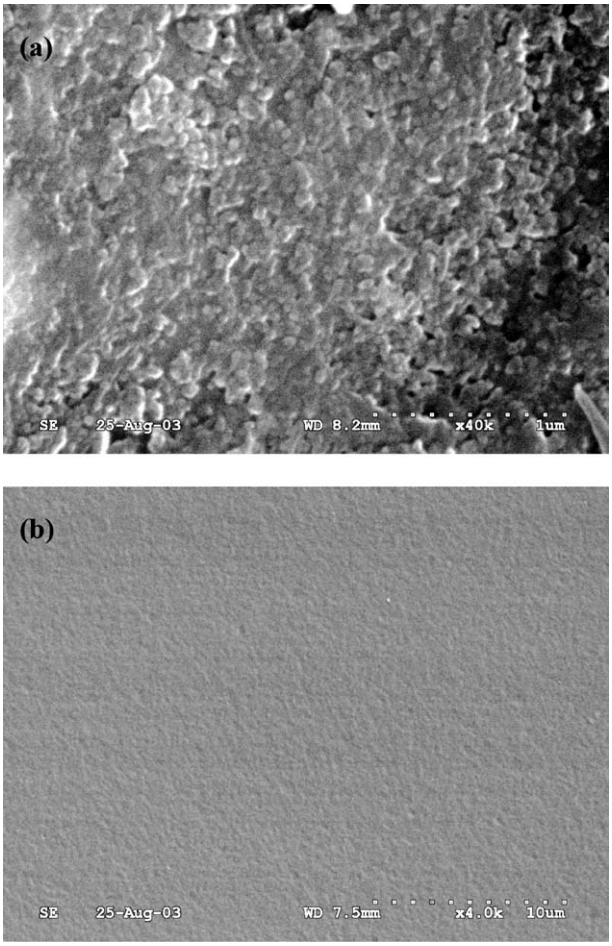

Figure 4 The SEM of fibroin: (a) insoluble fibroin microspheres and (b) fibroin films containing microspheres.

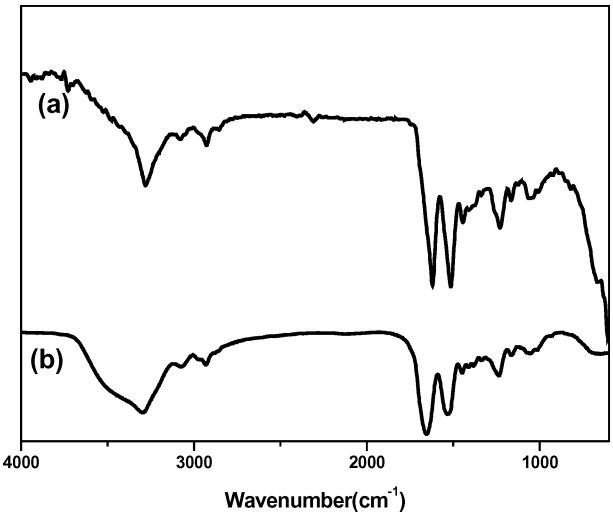

Figure 5 FTIR spectra of fibroin: (a) fibroin films containing microspheres and (b) insoluble fibroin microspheres.

X-ray diffraction curves

In order to confirm the effects of microspheres, the XRD curves of the films were shown in Fig. 6. The fibroin microspheres had two diffraction peaks at 20.4 and 24 °C, which confirmed the existence of β-sheet crystal. Compared with the results of FTIR, the microspheres may have irregular structure in its periphery and have β-sheet crystal in its center. When the films containing microspheres were obtained, the characteristic

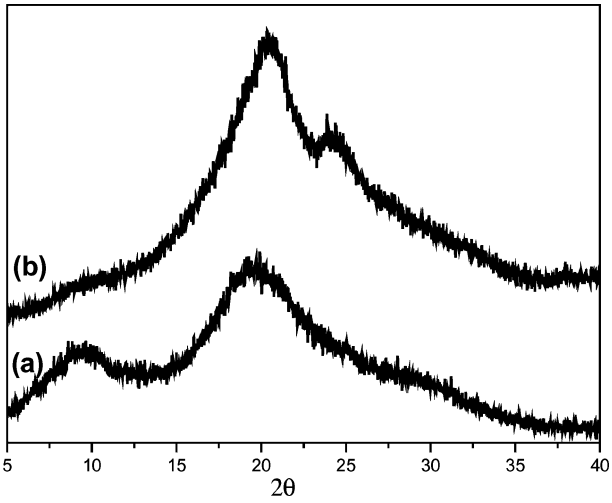

Figure 6 XRD curves of fibroin: (a) fibroin films containing microspheres and (b) insoluble microspheres.

peaks appeared at 9.0, 20.7 and 24 °C, all corresponding to β-sheet crystal. The results also suggested that fibroin microspheres induced the transformation of fibroin films from irregular structure to β-sheet conformation.

The weight loss in water

The weight loss in water of SF-4 at 37 °C was 4.5% when fibroin films immersed in water for 24 h, then, the weight loss nearly came to nought in the later 10 days. This means that the films containing microspheres were stable in water and could be used in many biomedical applications.

Discussion

When DF or fibroin microspheres were added in the concentrated fibroin solution, the β-sheet conformation increased and the insoluble films were obtained at 40–45 °C. Hyoung-Joon Jin and David L. Kaplan [17] found that fibroin aggregated into larger 'globules' and gel-like states as the concentration of silk fibroin increased and it was easy to be transferred to β-sheet crystal. In our study, the concentration was similar to that of the above report, it could also be formed the gel-like state in the concentrated solution. Because DF and microspheres has many oriented β-sheet crystal and can be used as crystal nucleation center, the addition of DF and fibroin microspheres induced the transformation from gel-like states to β-sheet crystal. On the other hand, according to the results of FTIR and XRD, the β-sheet crystal increased and the α helix/random coil conformation decreased when DF and fibroin microspheres were added. This confirmed that DF and microspheres really facilitated the transformation of fibroin from α helix/random coil to β-sheet crystal.

In order to study the effect of DF and microspheres in detail, the crystallinity of fibroin films was calculated according to the ratio of 1265 and 1235 cm^{-1} peaks from FTIR spectrum [18]. The results were shown in Table IV. When fibroin solution was concentrated

TABLE IV Crystallinity of the regenerated SF films

Fibroin films	Crystallinity (%) (calculated from FTIR spectrum)
SF-1, 1.5% concentration	0
SF-2, 15% concentration	20.9
SF-3, containing DF	38.2
SF-4, containing microspheres	46.6

from 1.5% (SF-1) to 15% (SF-2), the crystallinity of fibroin films increased from nought to 20.9%. The result suggests that the formation of gel-like states in concentrated solution facilitates the β-sheet crystal. When DF and microspheres were added to the concentrated fibroin solution, the crystallinity of fibroin films increased to 38.24% (SF-3) and 46.57% (SF-4), respectively. The results confirmed that DF and microspheres induced the formation of β-sheet crystal. More importantly, the effect of microspheres is more effective than that of DF though the DF ratio added in fibroin solution is fourfold of microsphers. Considering the size of microspheres was at 70–200 nm while DF was about 0.1 mm, the number of microspheres is much more than that of DF. This confirmed that DF and microspheres could be used as the nucleation center of β-sheet crystal.

Conclusions

By dissolving silk fibroin with triad solvent $CaCl_2/H_2O/EtOH$ solution, concentrating solution to 15%, and adding DF or fibroin microspheres to concentrated solutions, we obtained the insoluble films when the said solution was dried at 40–45 °C. FTIR and XRD spectra suggested the β-sheet conformation increased rapidly when DF or microspheres were added in fibroin solution and used as crystal nucleation center. Although the fibroin films blending with DF had many protuberances, the films containing fibroin microspheres had the smooth surface and could be used effectively in biotechnological materials and biomedical application. The tensile strength and elongation of the films were 6.47 Mpa and 16%, respectively. Because the preparation of insoluble films was very temperate and no harmful solvent such as methanol was used, the method would be very useful for biomedical applications.

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References

1. G. H. ALTMAN, R. HORAN, H. H. LU, J. MOREAU, I. MARTIN, J. C. RICHMIND and D. L. KAPLAN, *Biomateri.* **23** (2002) 4131.
2. M. DEMURA and T. ASAKURA, *J. Memb. Sci.* **59** (1991) 39.
3. S. SOFIA, M. B. MCCARTHY, G. GRONOWICZ and D. L. KAPLAN, *J. Biomed. Mater. Res.* **54** (2001) 139.
4. T. MASUHIRO, G. YOKO, N. MASAOBU, M. NORIHIKO, K. NOBUTAMI and F. GIULIANO, *J. Polymer Sci. Part B: Polymer Phys.* **32** (1994) 961.
5. M. NORIHIKO, T. MASUHIRO and N. MASANOBU, *Poymer* **31** (1990) 265.
6. HAEYONG KWEON, HYUN CHUI HA, IN CHUL UM and YOUNG HWAN PARK, *J. Appl. Polym. Sci.* **80** (2001) 928.
7. H. Y. KWEON, S. H. PARK, J. H. YEO, Y. W. LEE and C. S. CHO, *ibid.* **80** (2001) 1848.
8. YUYU SUN, ZHENGZHONG SHAO, MINGHUA MA, PING HU, YUSHUN LIU and TONG YIN YU, *ibid.* **65** (1997) 959.
9. YASUTOMO NOISHIKI, YOSHIHARU NISHIYAMA, MASAHISA WADA, SHIGENORI KUGA and JUN MAGOSHI, *ibid.* **86** (2002) 3425.
10. ANNA CHIARINI, PAOLA PETRINI, SABRINA BOZZINI, ILARIA DAL PRA and UBALDO ARMATO, *Biomaterials* **24** 789.
11. KAIYONG CAI, KANGDE YAO, SONGBAI LIN, ZHIMING YANG, XIUQIONG LI, HUIQI XIE, TINGWU QING and LAIBAO GAO, *ibid.* **23** (2002) 1153.
12. O. N. TRETINNIKOV and Y. TAMADA, *Langmuir* **17** (2001) 7406.
13. J. NAM and Y. H. PARK, *J. Appl. Polymer Sci.* **81** (2001) 3008.
14. Y. TSUBOI, T. IKEJIRI, S. SHIGA, K. YAMADA and A. ITAYA, *Appl. Phys. A*, **73** (2001) 637.
15. A. B. MATHUR, A. TONELLI, T. RATHKE and S. HUDSON, *Biopoly* **42** (1997) 61.
16. H. YOSHIMIZU and T. ASAKURA, *J. Appl. Polym. Sci.* **40** (1990) 1745.
17. H. J. JIN and D. L. KAPLAN, *Nature* **424** (2003) 1057.
18. IN CHUL UM, HAEYONG KWEON, YOUNG HWAN PARK and SAM HUDSON, *Intern. J. Biological Macromolecules* **29** (2001) 91.

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