

Nanofibers of Silk Fibroin Controlled by the Crystallization of Polyethylene Glycol in Frozen Solution^{*}

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Abstract The conformation of silk fibroin (SF) frozen with polyethylene glycol (PEG) at a molecular weight from 2 kDa to 20 kDa and a mass ratio of PEG:SF from 1:5 to 10:1 was studied by spectral and microscopic methods. It is found that the conformation transition of SF from random coil to β -sheet could be induced by the stress resulting from PEG crystallization at $-20\text{ }^{\circ}\text{C}$, and greatly depended on the cooling rate, PEG:SF mass ratio and PEG molecular weight. These findings provide a new method for the preparation of desired SF nanofibers.

Keywords Silk fibroin; Polyethylene glycol; Freezing; Phase separation; Crystallization

Electronic Supplementary Material Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s10118-017-1974-x>.

INTRODUCTION

Silk fibroin (SF) from *Bombyx mori* (*B. mori*) silkworm is a promising material for biomedical applications due to its outstanding properties^[1, 2]. Recently, more attention has been paid to avoid the agglomeration of SF nanoparticles in biological media^[3, 4], and polyethylene glycol (PEG) conjugation is one of the most commonly used strategies to solve the hurdles in therapeutic delivery^[5]. However, recent studies have found that PEG can induce the conformation transition of SF into β -sheet during the evaporation of water at room temperature^[6]. As a result, lyophilization is one of the best choices for drying these materials, and freezing is a key procedure in the lyophilization^[7]. Previous studies have revealed that the physical environment around proteins changes dramatically during freezing, which induces various stresses (*i.e.*, cold denaturation, freeze-concentration, and ice-induced denaturation) that impact protein stability^[8]. Although SF was reported to be stable during freezing process^[9], the effect of additives, such as PEG, on the conformation of SF during freezing is still unclear, which is crucial for the stability of protein composite materials^[10]. Besides, there was a dispute on the effect of PEGylation on proteins for a long time, which possesses profound significance for the understanding of polymer blends^[11].

In this study, we sought to investigate the conformation transition of SF induced by PEG in frozen solution, and reveal the structures of SF frozen with PEG, and develop an efficient method to control the conformation of SF.

^{*} This work was financially supported by the National Natural Science Foundation of China (No. 21374022).

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Received March 15, 2017; Revised April 28, 2017; Accepted May 8, 2017

doi: 10.1007/s10118-017-1974-x

EXPERIMENTAL

Preparation of Mixtures of SF and PEG

PEG ($M_w = 2, 4, 6, 10$ and 20 kDa) were dissolved in deionized water to prepare 1 wt% PEG solutions, and SF solution was prepared as described in our previous work^[6]. The specific volumes of SF solutions were gently mixed with 1% PEG solutions in centrifuge tubes to get homogeneous solutions with mass ratio of PEG:SF from 1:5 to 10:1. The mixed solutions were frozen and stored at various temperatures for 0 to 8 days. Two freezing temperatures, -20 °C in a freezer and -196 °C in liquid nitrogen, and three storage temperatures, -20 , -80 and -196 °C, were used. After storing for different periods, the frozen solutions were thawed at 25 °C or lyophilized directly for the following analysis.

PEGylation of SF

Monomethoxy-polyethylene glycol-succinimidyl carbonate (mPEG-SC, $M_w = 5$ kDa) of desired concentration was dissolved in a 20 mmol/L sodium phosphate buffer at pH = 8.0, and specific volumes of SF solutions were gently added to the mPEG-SC solution. The PEGylation reaction was carried out at a SF concentration of 10 mg/mL, with a PEG:SF mass ratio of 1:1, and the reaction was carried at 4 °C for 24 h with a stirring speed of 150 r/min. Then, the solution was dialyzed against ultra-pure deionized water using a dialysis tube with a molecular weight cut-off 1.2×10^4 g/mol for 3 days. The final solution was diluted with ultra-pure deionized water to a SF concentration of 1 mg/mL. The PEGylation yield of SF was quantified through a barium chloride PEG assay based on the method of Brown^[12]. Briefly, 160 μ L of the sample was incubated with 40 μ L of barium chloride solution (5 wt% in 1 mol/L HCl). 20 μ L of 0.1 mol/L iodine solution (with 0.3 mol/L KI) was then added to each well and absorbance was measured at 535 nm using a microplate reader (ELx 800, Bio-Tek). PEGylation yield was calculated on a standard curve generated with unconjugated mPEG-SC. The PEGylated SF solution was then frozen at -20 °C for 8 days for the following analysis.

Characterizations

Thioflavin T (ThT) fluorescence was recorded by a fluorescence spectrophotometer (QM 40, Photon Technology International Inc.). Circular dichroism (CD) spectra were recorded by Jasco J715 spectropolarimeter. Morphology of SF was studied by Bruker MultiMode 8 atomic force microscopy (AFM) using a silicon probe in tapping mode. Fourier transform infrared (FTIR) spectra were recorded by Nicolet 6700 infrared spectrometer (ThermoFisher, USA) with a diamond attenuated total reflection (ATR) accessory. The amide I band (1580 – 1720 cm^{-1}) was deconvoluted for quantitative analysis using Peakfit 4.12 software^[13]. Mass spectra of mPEG-SC was recorded by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (AB SCIEX 5800) with α -cyano-4-hydroxycinnamic acid (CHCA, $M_w = 189$ Da) as matrix. ^1H -NMR was recorded by a Bruker AVANCE III spectrometer.

RESULTS AND DISCUSSION

Effect of PEG on Conformation of SF in Frozen Solution

ThT is one of the most commonly used fluorescent probes in the evaluation of β -sheet content in proteins, because its fluorescence intensity is remarkably increased when it presents in β -sheet conformer of protein. As can be seen from ThT fluorescent intensity on fibrillation kinetics in Fig. 1(a), SF showed no conformational change after 8 days of storage under -20 °C. However, when SF was frozen with PEG-10kDa, the β -sheet conformer of SF increased gradually. It is notable that such effect depended dominantly on the mass ratio of PEG:SF. These results can also be found in AFM images of SF frozen without or with PEG at different freezing time, as shown in Figs. 2(a) and 2(b). It is found that the morphology of SF remained in a state of amorphous aggregates even after being frozen for 8 days. However, when SF was frozen with PEG, small SF oligomers were formed in 3 days, and gradually elongated into the protofibrils; eventually, SF turned into cross-linked fibrils after being frozen with PEG for 8 days.

To further reveal the effects of mass ratio and molecular weight of PEG on the conformation of SF, SF frozen with PEG at different PEG:SF mass ratios and PEG molecular weight were also studied by ThT

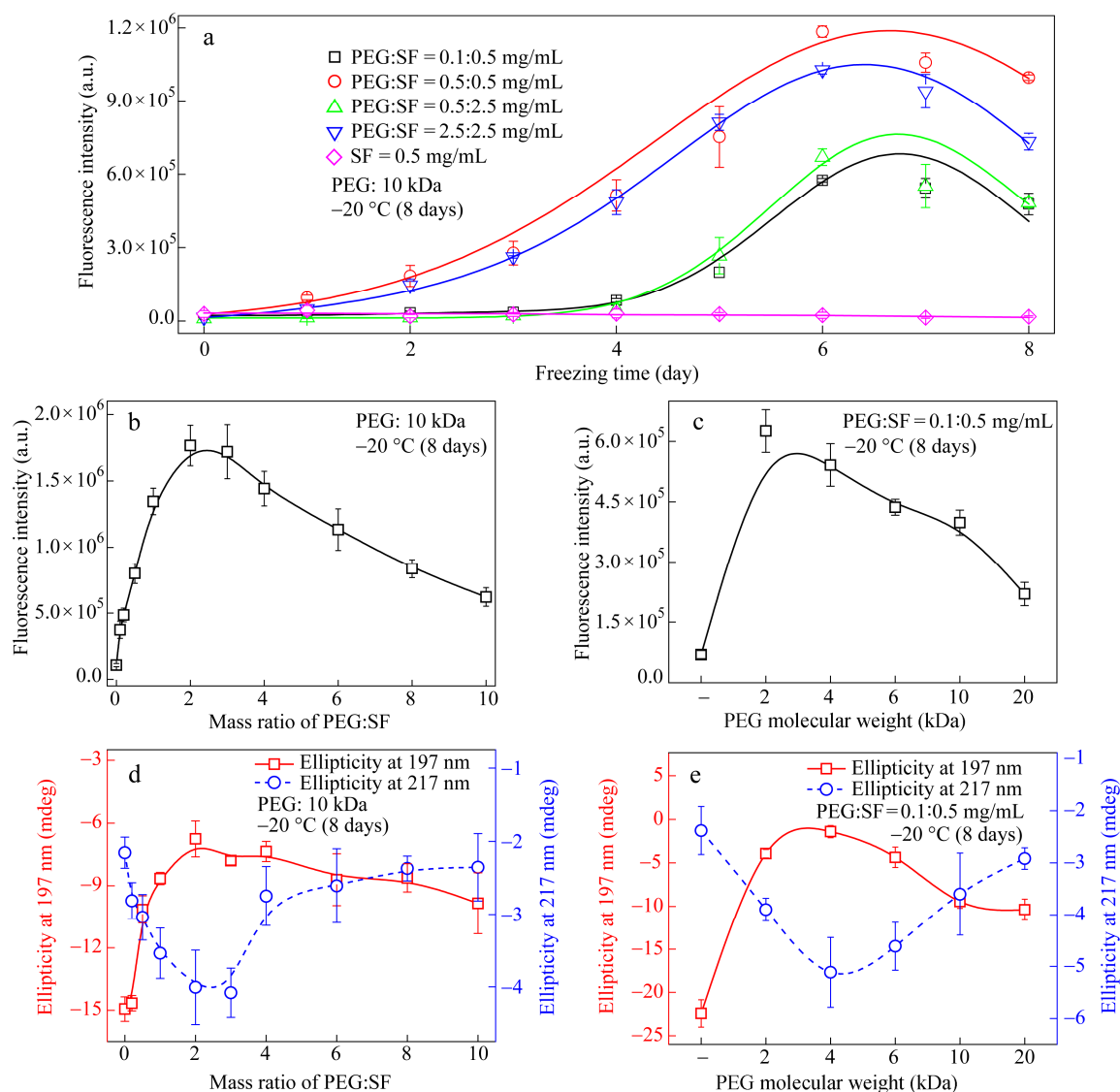


Fig. 1 Conformational characteristics of SF frozen with PEG: (a) Fibrillation kinetics of SF frozen with or without PEG-10kDa; ThT fluorescence intensity of SF (0.5 mg/mL) after being frozen for 8 days with (b) different PEG:SF mass ratios and (c) different PEG molecular weights, respectively; Dependence of ellipticity at 197 and 217 nm on (d) PEG:SF mass ratio and (e) PEG molecular weight, respectively

fluorescence assay and CD measurements. Figure S1 (in supporting information, SI) shows the CD spectra of SF frozen with PEG, and Fig. 1(d) shows the effect of PEG:SF mass ratio on the ellipticity at 197 and 217 nm in CD spectrum, which is assigned to the random coil and β -sheet conformer, respectively. As can be seen in Figs. 1(b) and 1(d), both results from ThT fluorescence assay and CD spectrum show that the amount of β -sheet conformer of SF increased in the beginning, and then decreased as the PEG:SF mass ratio increased from 3 to 10. In addition, the amount of the formed β -sheet conformers decreased as the PEG molecular weight increased at a given content, as shown in Figs. 1(c) and 1(e). Results from AFM in Fig. 2 show that the amount of fibrils increased as the PEG:SF mass ratio increased from 0.2 to 2 (Fig. 2b-8 days, Figs. 2c and 2d), but decreased as the ratio further increased from 2 to 10 (Figs. 2e and 2f). Moreover, the morphologies of fibrils induced by PEG were dependent on molecular weight of PEG (Fig. 2b-8 days and Figs. 2g-2j). Large molecular weight PEG induced more tangled fibrils than the smaller one. These results indicate that PEG can induce the conformation

transition of SF from random coil to β -sheet, and small molecular weight PEG is more efficient for the transition than large one. As a result, PEG-2kDa was employed for further study of the conformation transition of SF affected by cooling rate and stored temperatures.

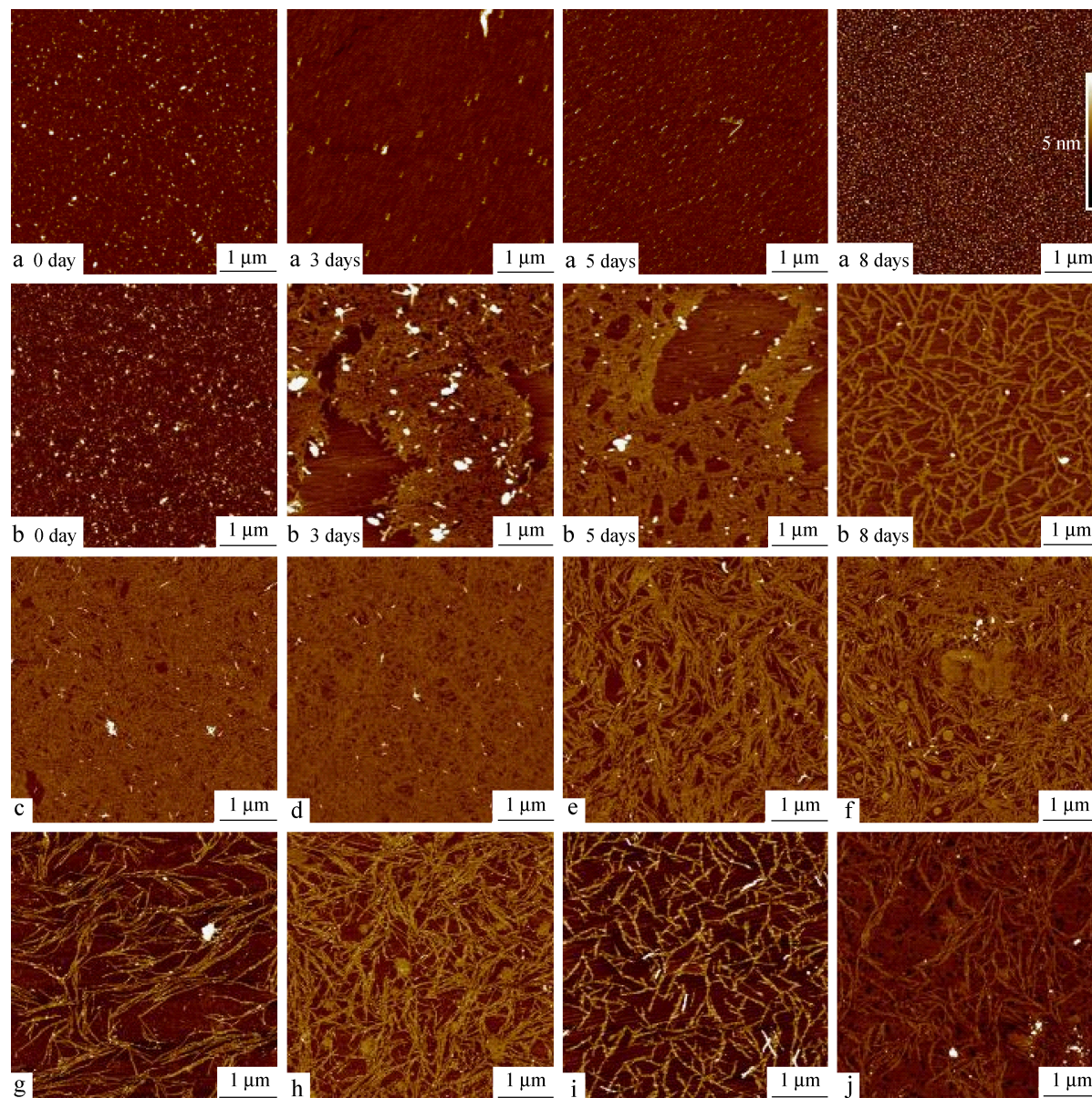


Fig. 2 AFM images of SF frozen (a) without and (b) with PEG-10kDa (PEG:SF = 0.2) at different freezing time, respectively; Morphologies of SF frozen with PEG-10kDa at PEG:SF mass ratio of (c) 1, (d) 2, (e) 5 and (f) 10, respectively; Morphologies of SF frozen with different molecular weight of PEG at mass ratio of PEG:SF = 0.2: (g) 2 kDa, (h) 4 kDa, (i) 6 kDa and (j) 20 kDa

Effect of Frozen and Stored Temperatures on Conformation of SF

As shown in Fig. 3(a), ThT fluorescence of SF frozen with PEG-2kDa at -20°C shows the same tendency (black column) as the results with PEG-10kDa (Fig. 1a), while the tendency for those frozen rapidly in liquid nitrogen and then stored at -20°C (red column) is different, where the fluorescence intensity did not decrease at high PEG:SF mass ratio. In addition, the samples frozen rapidly in liquid nitrogen and then stored under -80°C

(blue column) and $-196\text{ }^{\circ}\text{C}$ (green column), respectively, showed no conformational change after 8-day storage. As shown in Fig. 3(b), SF fibrils formed with PEG-2kDa at mass ratio of 1:1, by frozen rapidly and stored at $-20\text{ }^{\circ}\text{C}$ are short and unified in nanoscale about 120–150 nm.

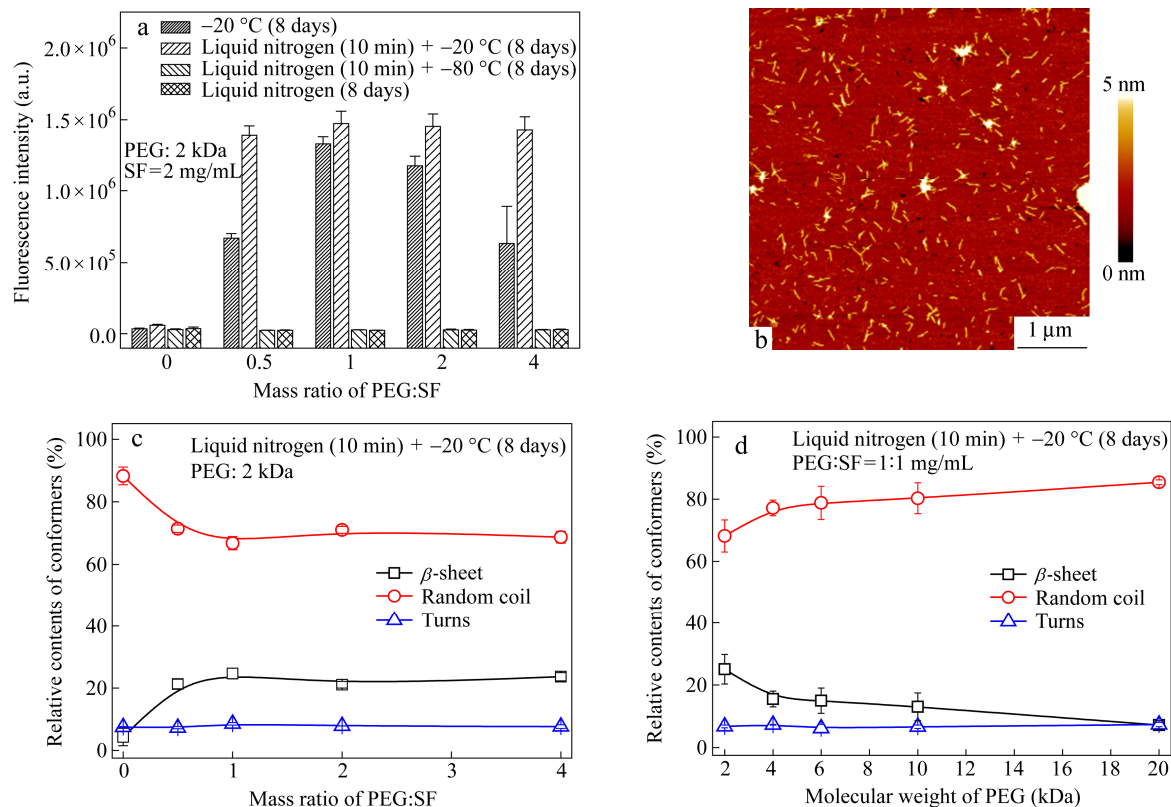


Fig. 3 (a) ThT fluorescence intensity of SF with PEG-2kDa on different PEG:SF mass ratios at different frozen and stored temperatures; (b) AFM image of SF with PEG-2kDa at mass ratio of 1:1, frozen rapidly in liquid nitrogen and then stored at $-20\text{ }^{\circ}\text{C}$ for 8 days; The relative contents of β -sheet, random coil and turn conformers versus (c) PEG-2kDa mass ratio and (d) PEG molecular weight, respectively, studied by ATR-FTIR

To exclude the effect of thaw on conformation transition, the frozen solutions were lyophilized immediately without the thawing process, and the conformation of SF was studied by ATR-FTIR (shown in Fig. S2, SI). As shown in Figs. 3(c) and 3(d), when the samples were frozen rapidly in liquid nitrogen and then stored at $-20\text{ }^{\circ}\text{C}$ for 8 days, the conformation of SF frozen with PEG-2kDa gradually changed from random coil to β -sheet, and the change got lowered as PEG molecular weight increased. The results indicated that the conformation transition of SF induced by PEG occurred during the freezing process instead of the thawing process.

Stability of PEGylated SF in the Frozen Solution

mPEG-SC is one of the most commonly used derivatives for the PEGylation of protein^[14, 15]. mPEG-SC reacts preferentially with lysine residues to form a carbamate linkage, but also possibly reacts with histidine and tyrosine residues^[16]. Previous studies have shown that there are many tyrosine residues (5 mol%, about 277 residues) in SF available for the reaction with mPEG-SC. Lysine (0.3 mol%, 12 residues) and histidine (0.1 mol%, 5 residues) residues in SF can also participate in this reaction with very low quantities^[17]. Figure S3(A) (in SI) shows the mass spectra of mPEG-SC; the molecular weight of mPEG-SC is about 4951 Da. As shown in Figs. S3(B)–S3(D) (in SI), the binding of mPEG-SC to SF was confirmed by ^1H -NMR, and the PEGylation yield was about $(90 \pm 3)\%$ quantified through the barium chloride PEG assay.

As shown in Fig. 4(a), the CD spectrum of SF had a negative band around 208–222 nm and a negative band

near 195 nm, which indicated that SF before PEGylation mainly consisted of α -helix and random coil. However, the CD spectrum of SF-PEG conjugate showed a standard feature of random coil, which indicated that PEGylation kept the SF in random coil conformer. However, after being frozen at -20°C for 8 days, the conformation of SF-PEG conjugate changed from random coil to β -sheet. Besides, as shown in Fig 4(b), the amount of β -sheet conformer of SF detected by ThT fluorescent showed no obvious change after PEGylation, but increased dramatically after being frozen at -20°C for 8 days.

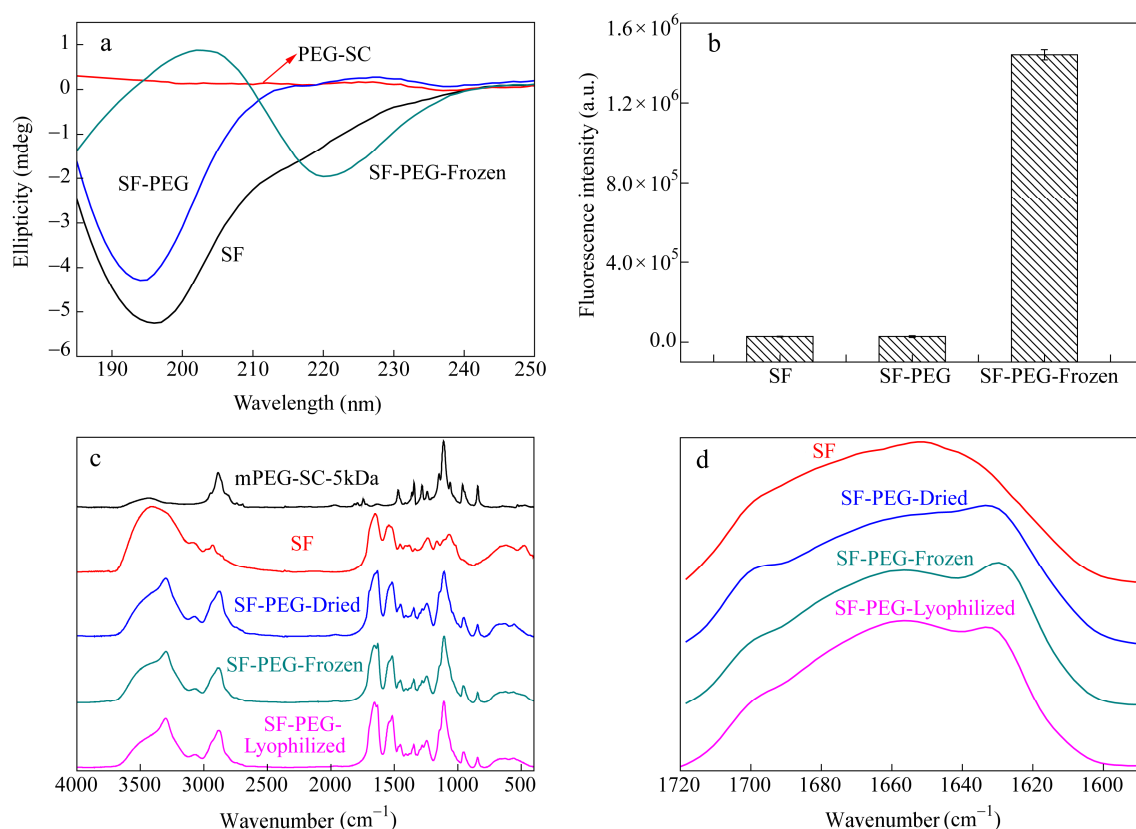


Fig. 4 (a) Circular dichroism spectra of PEG-SC, SF, SF-PEG conjugate and SF-PEG conjugate after being frozen at -20°C for 8 days; (b) ThT fluorescence intensity of SF, SF-PEG conjugate and SF-PEG conjugate after being frozen at -20°C for 8 days; FTIR spectra of (c) full range and (d) amide I band of mPEG-SC-5kDa, SF and SF-PEG conjugates with different treatments

SF-PEG-Dried represents the SF-PEG conjugates dried by evaporation at room temperature; SF-PEG-Frozen represents the SF-PEG conjugates lyophilized after 8-day storage at -20°C ; SF-PEG-Lyophilized represents the SF-PEG conjugates lyophilized after being frozen in liquid nitrogen for 10 min.

To further study the stability of PEGylated SF during drying process, SF-PEG conjugates were dried by lyophilization or evaporation at room temperature, and the conformation of SF was studied by FTIR. As shown in Figs. 4(c) and 4(d), the conformation of SF-PEG conjugates dried by evaporation changed from random coil (around 1650 cm^{-1}) to β -sheet (1630 cm^{-1}), which was in accordance with our earlier study with the PEG/SF blend system^[6]. In addition, such conformation transition could also be found in the SF-PEG conjugates dried by lyophilization, and the 8-day storage at -20°C formed more β -sheet conformers. As the AFM images shown in Fig. 5, PEGylated SF solution dried directly resulted in lots of parallel nanofibers (about 300–800 nm in length), and the 8-day storage at -20°C increased the length of nanofibers (about 500–800 nm in length). The results above indicated that PEGylation could keep SF in random coil conformers in solution state, but induce the conformation transition of SF from random coil to β -sheet during the drying and frozen process. These results

were in accordance with that obtained from the PEG/SF blend system.

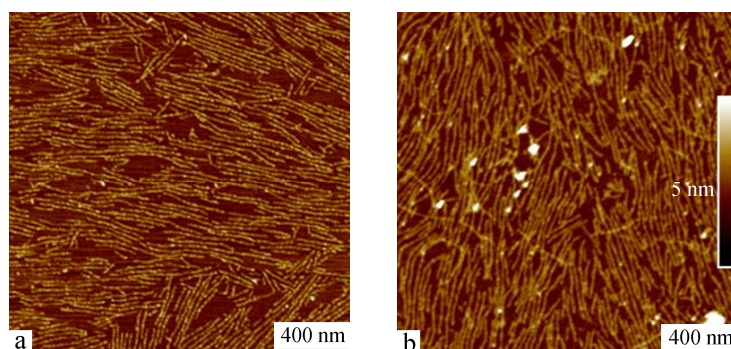


Fig. 5 AFM images of SF-PEG conjugates (a) without or (b) with the frozen (8-day storage at $-20\text{ }^{\circ}\text{C}$) process

Discussion

Results reported here indicate that PEG can induce the conformation transition of SF from random coil to β -sheet and nanofiber formation in $-20\text{ }^{\circ}\text{C}$ frozen solution. This effect greatly depended on the conditions of sample preparation, such as cooling rate, PEG:SF mass ratio and PEG molecular weight. Previous research has found that SF is incompatible with PEG^[6, 18], but PEG fails to induce the conformation transition of SF even at a high concentration (50% V/V)^[19], which indicates that water sorption by PEG is not the critical factor for the conformation transition of SF. Therefore, we suggest that the conformation transition of SF can be induced by the stress resulted from PEG crystallization.

When the solution was frozen, phase separation into PEG-rich and protein-rich phases occurred. When stored at temperature higher than the glass transition temperature (T_g) of PEG ($-45\text{ }^{\circ}\text{C}$), the PEG molecular chains were movable and re-arranged themselves into crystalline state, leading to the stress which induced the conformational transition of SF in PEG-rich phase, and the large molecular weight PEG induced more tangled SF nanofibers for its large gyration radius. When stored below T_g of PEG, SF showed no conformational change for the PEG molecules were rigid in glassy state. It is also easy to understand that PEG with small molecular weight has more obvious impact on the conformation transition of SF. Since the phase separation is more favorable for large PEG molecules, there are few SF presented in the PEG-rich phase. In addition, phase separation occurs easier at a slow cooling rate, therefore the influence on the conformation transition of SF was weaker when samples with high PEG:SF mass ratio were frozen at $-20\text{ }^{\circ}\text{C}$ than that at $-186\text{ }^{\circ}\text{C}$. Interestingly, we found that the unified nanofibers of SF could be manufactured only by simply controlling the cooling rate, mass ratio and molecular weight of PEG as showed in Fig. 3(b).

CONCLUSIONS

In this work, we found that freezing either SF and PEG mixture solution or PEGylated SF solution can cause the conformation transition of SF and nanofiber formation, which suggests that the transition is induced by the stress resulted from PEG crystallization. The formation of SF nanofibers can be well controlled by cooling rate, PEG:SF mass ratio and PEG molecular weight. This work may provide guidance for manufacturing SF nanofibers for biomaterial usage.

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