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Physical properties and structure of poly(ethylene glycol)–silk fibroin conjugate films

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Poly(ethylene glycol) (PEG)–silk fibroin (SF) conjugate (PEG1–SF) was prepared by the chemical modification of solubilized SF with 2-[*O*-methoxypoly(ethylene glycol)]-4,6-dichloro-*s*-triazine (actPEG1), and the physical properties of the PEG1–SF film were studied. The circular dichroism (c.d.) spectrum of the PEG1–SF film exhibited both negative and positive extrema due to a β-sheet structure. D.s.c. measurements and polarizing microscopic observations at elevating temperature of the PEG1–SF film clarified the thermal behaviour of the PEG1–SF film exhibiting the melting of the PEG chains and the decomposition of SF. In the d.s.c. thermogram of PEG1–SF, the positions of the endothermic peaks due to the melting of the PEG chains and the decomposition of SF scarcely shifted compared to the peak position of actPEG1 and the peak position of the SF having a β-sheet structure, respectively. Judging from these observations, the mutual miscibility between PEG and SF was considered to be poor. The tensile tests of the PEG1–SF and SF films revealed that the PEG-modification of SF improved the elongation at break but reduced the tensile strength. based on these results, a layered structure of the PEG1–SF film was suggested. Copyright © 1996 Elsevier Science Ltd.

(Keywords: silk fibroin–poly(ethylene glycol) conjugates; thermal properties; mechanical properties)

Introduction

Silk fibroin (SF) is a structural protein created by the *Bombyx mori* silkworm and has recently been considered for applications as a biomaterial and a medical material such as an enzyme-immobilization material[1], a cell culture substrate[2], and an oral dosage form[3] because of its unique physicochemical properties. In order to develop new properties of SF and to use SF widely in the biomedical field, we are attempting a chemical modification of the amino acid residues in solubilized SF[4–6].

Poly(ethylene glycol) (PEG) is a nontoxic, nonimmunogenic, and amphipathic polymer and possesses a variety of properties pertinent to biomedical and biotechnical applications[7]. The introduction of PEG into proteins by a chemical modification has been developed[7,8]. In our previous study, we prepared a PEG–SF conjugate (PEG1–SF) by the chemical modification of solubilized SF with cyanuric chloride-activated PEG, namely, 2-[*O*-methoxypoly(ethylene glycol)]-4,6-dichloro-*s*-triazine (actPEG1, MW 5000)[5,8] (*Figure 1*). The ^{1}H n.m.r. spectrum and amino acid analysis of the conjugate suggested that the chlorine atom of the triazine ring of the modifier actPEG1 was substituted by the tyrosine, lysine, and/or histidines residues in SF[5]. Cell attachment and growth on the matrix of the conjugate were also studied using a cell culture method[6]. The conjugate is expected to be used as an adhesion barrier material, because cell attachment and growth on the conjugate were very low in contrast to the SF matrix[6].

In this paper, the physical properties of the PEG1–SF film were studied by c.d. and d.s.c. measurements, tensile tests, and polarizing microscopic observations. From these results, a layered structure of the PEG1–SF film was speculated.

Experimental

Preparation of the PEG1–SF aqueous solution. An aqueous solution of SF was prepared by dissolving degummed SF fibres in 9 M LiBr aqueous solution and dialyzing the dissolved solution against water[4,5]. According to the previous procedure[5], 100 mg of actPEG1 (MW 5000, Sigma Chemical Co.) was added to 6 ml of the 0.32% (w/v) SF aqueous solution containing 0.1 M sodium borate (pH 9.4) at 4°C over 15 min. After the reaction mixture was kept at 4°C for 1 h, the solution was dialyzed against cold 0.1 M phosphate buffer (pH 7.0) and subsequently 5 M urea–0.02 M Tris · HCl buffer (pH 8.0). In order to remove the unreacted modifier, the dialyzed solution was applied to a column of Sephacryl S-300 preequilibrated with 5 M urea–0.02 M Tris · HCl buffer (pH 8.0) and eluted with the same buffer. The eluate of PEG1–SF was collected and dialyzed against water. The solution of PEG1–SF was concentrated to about 1% (w/v) by ultrafiltration using an XM-50 membrane (Amicon Division of W. R. Grace Co.). It was evaluated that PEG1–SF consisted of about 30 wt% SF and 70 wt% PEG by comparing the integral values of the signals in the ^{1}H n.m.r. spectra of PEG1–SF and the mixture of SF and methoxypoly(ethylene glycol)[5].

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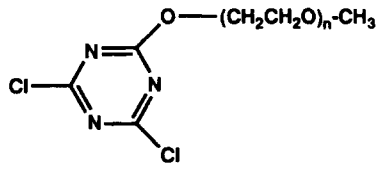

actPEG1

Figure 1 Chemical structure of actPEG1

Preparation of the PEG1–SF and SF films. The films of PEG1-SF and SF were obtained by casting each solution containing 0.5–1% (w/v) SF protein onto thin polyethylene films as casting substrates and drying at ambient relative humidity at room temperature[4,5]. While the SF film was transparent, the PEG1–SF film was slightly turbid. These films were used for d.s.c. measurements, tensile tests, and polarizing microscopic observations. As a control specimen, the SF having a β-sheet structure was prepared by immersing the SF film in a 50% (v/v) methanol–water mixture for 4 h at room temperature and drying the immersed SF film at ambient relative humidity at room temperature[5].

Measurements. The film samples for c.d. spectra of PEG1–SF and SF were prepared by casting each solution containing 0.2% (w/v) SF protein on a quartz disc and drying at ambient relative humidity at room temperature[4]. C.d. spectra were observed with a JASCO-J600 automatic recording spectropolarimeter at room temperature.

Differential scanning calorimetry (d.s.c.) measurements were performed using a Thermoflex DSC-10A (Rigaku Denki Co.) at a heating rate of 10°C min^{-1}. D.s.c. range and sample weight were 2.5 mcal s^{-1} and about 2 mg, respectively. The open aluminium cell was swept with nitrogen gas during the course of the analysis. The samples were dried by keeping them in a desiccator containing P_2O_5 before use.

The PEG1–SF and SF films were tensile tested to determine their tensile strength and elongation at break using a Tensilon UTM-II tensile tester (Toyo Baldwin Co.). All tests were carried out at a test extension speed of 20 mm min^{-1} and at 20°C and 65% relative humidity. Strips (15 × 3 mm) cut from the films were used for the test.

Polarizing microscopic studies were performed using an Olympus BHS-751P polarizing optical microscope with a hot stage (Mettler FP82HT).

Results and discussion

Figure 2 shows the c.d. spectra of the PEG1–SF and SF films. The c.d. spectrum of the SF film showed two broad negative bands at about 205 and 220 nm assigned to a random coil conformation containing small amounts of β-sheet and helix structures[9]. On the contrary, the spectrum of the PEG1–SF film exhibited a negative extremum at 218 nm and a positive extremum at 198 nm, which were characteristic of a β-sheet structure[9]. In our previous study, the i.r. spectrum of the PEG1–SF film exhibited absorption bands attributed to a β-sheet structure[5]. In the i.r. spectrum of the intact SF film, absorption bands attributed to a random coil

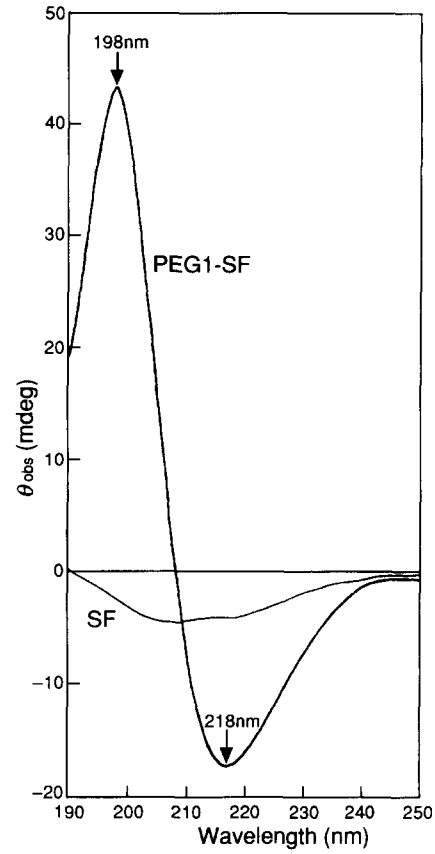

Figure 2 C.d. spectra of the SF and PEG1-SF films

conformation were observed[5]. Thus, the results of the c.d. spectra were consistent with those of the i.r. spectra.

The d.s.c. thermograms of the samples are shown in *Figure 3*. As one can see in the thermogram of PEG1–SF (*Figure 3d*), two endothermic peaks at 58°C and 281°C were observed. The modifier actPEG1 showed an endothermic peak at 59°C (*Figure 3c*). The melting point of actPEG1, as measured using a melting-point apparatus, was 56–58°C. The melting point of pure PEG (MW 4000–6000) was reported to be around 59°C[10]. Thus, the d.s.c. peak of actPEG1 at 59°C is considered to be due to the melting of the PEG chains. The peak of PEG1–SF at 58°C is also considered to be due to the melting of the PEG chains.

In the d.s.c. thermogram of the SF having a random coil conformation (*Figure 3a*), an exothermic peak attributed to the conformational transition from a random coil conformation to a β-sheet structure was observed at about 228°C together with an endothermic peak at 278°C due to the thermal decomposition of SF[11,12]. In contrast, the d.s.c. thermogram of the SF having a β-sheet structure exhibited only an endothermic peak at 284°C due to the thermal decomposition[11,12] (*Figure 3b*). Accordingly, the peak of PEG1–SF at 281°C is suspected to correspond to the decomposition of SF. The thermal behaviour of PEG1–SF without an exothermic peak also suggests that the conformation of PEG1–SF is a β-sheet structure. In the d.s.c. thermogram of PEG1–SF, the positions of the endothermic peaks due to the melting of the PEG chains and the decomposition of SF scarcely shifted compared to the peak position of actPEG1 and the peak position of the SF having a β-sheet structure, respectively. Judging

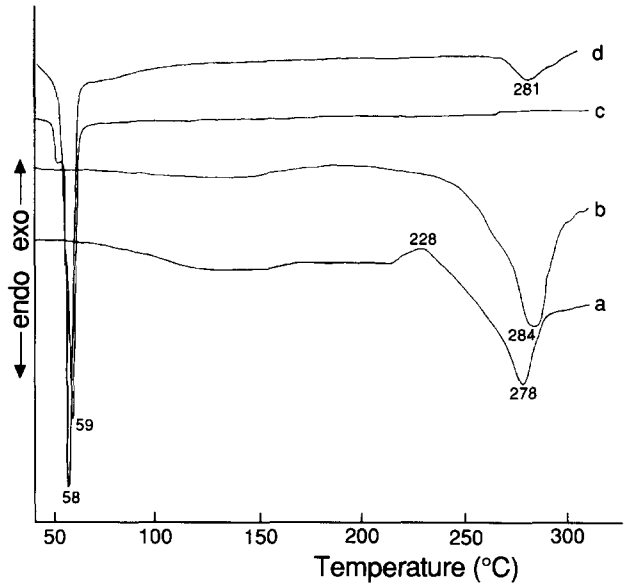

Figure 3 D.s.c. thermograms of SF (a), the SF having a β-sheet structure (b), actPEG1 (c), and PEG1-SF (d)

from these observations, the mutual miscibility between PEG and SF was considered to be poor.

The PEG1–SF film was examined using a polarizing optical microscope, as shown in *Figure 4*. At room

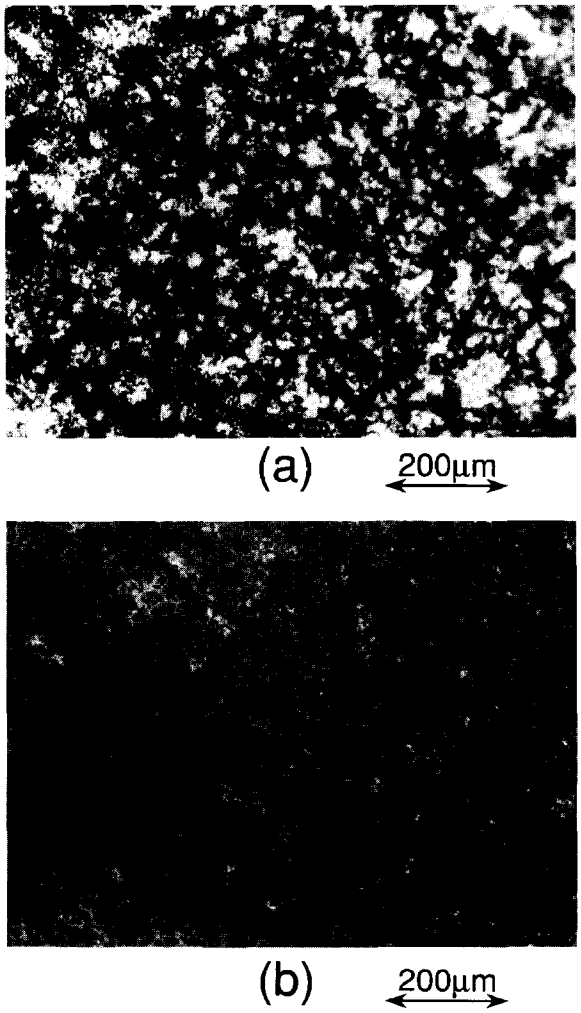

Figure 4 Polarizing optical micrographs of PEG1-SF at 20°C (a) and 100°C (b)

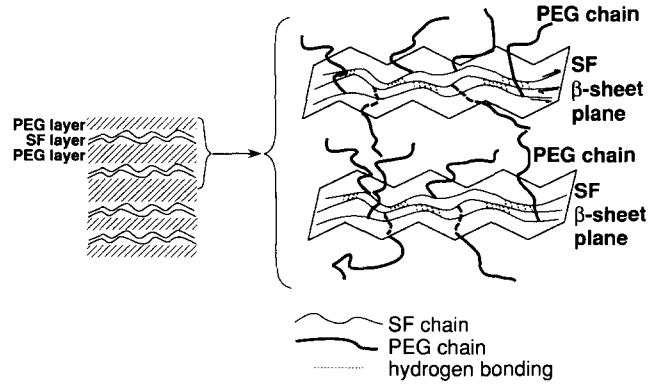

Figure 5 Schematic representation of the PEG1-SF film

temperature, shiny anisotropic images were observed (*Figure 4a*) and suggested that PEG1–SF was in a crystalline state. At 57–58°C, the shiny images disappeared. This means that the melting of the crystalline PEG chains occurred. After the melting of the crystalline PEG chains, the melt of PEG and the crystalline SF coexisted at temperatures up to about 260°C (*Figure 4b*). The above observations support the belief that the endothermic peak at 58°C in the d.s.c. thermogram of PEG1–SF was due to the melting of the PEG chains. The crystalline SF gradually disappeared at 260–290°C, and an isotropic pattern was observed at 290°C. Therefore, this observation also supports the belief that the endothermic peak of PEG1–SF at 281°C was attributed to the thermal decomposition of SF.

The tensile strength and elongation at break of the PEG1–SF film were evaluated to be 0.08 kg mm^{-2} and 5.6%, respectively, while the tensile strength and elongation at break of the SF film were 1.67 kg mm^{-2} and 1.9%, respectively. These results revealed that the PEG-modification of SF improved the elongation at break but reduced the tensile strength. We reported that the X-ray diffraction pattern of the PEG1–SF film suggested a decrease in the degree of the crystallinity of the SF chains in PEG1–SF[5]. Thus, it is considered that the decrease in the tensile strength of the PEG1–SF film results from the decrease in the degree of the crystallinity of SF by the introduced PEG chains. The ductile behaviour of the PEG1–SF film was mainly governed by the ductile PEG matrix.

The results of c.d. and d.s.c. measurements indicated that the introduction of the PEG chains into SF induced the formation of a β-sheet structure. The d.s.c. thermogram of PEG1–SF suggested that the mutual miscibility between the PEG chains and the SF chains was poor, and therefore the PEG chains may form a PEG-rich phase separated from the SF domains. Thus, it is estimated that the introduced PEG chains extend perpendicular to the β-sheet plane of SF without hindering the formation of a β-sheet and form the PEG-rich phases. The tensile properties of PEG1–SF can be explained by supposing that the sliding between SF sheet planes took place due to the ductile PEG-rich phases. Based on the above results, we speculate the layered structure of the PEG1–SF film shown in *Figure 5*. The PEG1–SF units stack on top of one another.

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