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Relationships between Antithrombogenicity and Surface Free Energy of Regenerated Silk Fibroin Films

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Abstract: Silk fibroin (SF) was dissolved in calcium chloride/ethanol/water mixture (1/2/8 in mole ratio) at 70°C for 4 h. The dissolved silk fibroin was regenerated by casting the dialyzed solution into the films. The films were treated with 50% aqueous solution of methanol for different times, and their antithrombogenicity was evaluated by *in vitro* and *in vivo* tests. *In vivo* blood tests were made by a method of peripheral vein indwelling suture. It was found that the silk fibroin had a good antithrombogenicity and an absorbability even though the polymer showed foreign body reaction. Finally, the blood compatibilty of silk fibroin films which were subjected to structural change by the methanol treatment, was examined in connection with their interfacial surface energy, and a correlation between these properties was found to be present.

Keywords: Silk fibroin, Antithrombogenicity, Surface free energy

Introduction

When polymeric materials contact with blood or plasma, rapid protein adsorption takes place. The composition of the adsorbed proteins and the reversibility of the adsorption process mainly depend on the surface characteristics of a polymer[1]. Therefore, the anticoagulant activity or antithrombogenicity of the polymeric materials is mainly affected by surface properties such as surface smoothness, the distribution of anionic functional groups and the density of the electrostatic interaction in the polymer, the balance between hydrophilicity and hydrophobicity on the surface, the microphase-separated heterogeneous domains on the surface, and so on[2,3].

Natural polymers such as polysaccharides and proteins have drawn much attention as biomedical materials due to their biocompatibility and biodegradability to the living body[4]. Among them, some fibrous proteins including silk fibroin (SF) and wool keratin have been noted for their blood-compatible properties[5-8].

Unlike to wool keratin, SF can be solubilized without chemical modification and regenerated into fibers and films [9]. It is also characteristic of SF that the conformational structure as well as surface characteristics of the fibers and films can be varied by chemical treatment. Using the conformational transition of the structure which results in insolubilization of the films, SF has been used as a substrate of enzyme immobilization[10,11].

The aim of this work was to ascertain the effect of conformational change on the antithrombogenicity of SF film and equivocally establish the correlation between the surface free energy and the antithrombogenicity of SF. The change in conformation of regenerated SF films was achieved by methanol treatment. Antithrombogenicity was evaluated by the platelet adhesion and the method of peripheral vein indwelling suture.

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Materials and Methods

Preparation of SF Films

Commercial raw silk fibers (21 den.) of *Bombyx mori* were refined with a 0.3% sodium oleate aqueous solution. The degummed silk fibers were dissolved in the mixed solution of calcium chloride/ethanol/distilled water (1/2/8 in mole ratio) at 70°C for 4 h. The SF solution (10 wt%) was dialysed with cellulose tubular membrane (Sigma Chem. Co., 250-7 μm) in distilled water for 3 days. After removing an undissolved or precipitated part by centrifuging at 12,000 rpm for 20 min, the dialysed SF solution was cast onto acryl plate and dried for one week at room temperature. Since the dried films were soluble in water, the films were fixed between two rectangular acrylic frames and treated with a 50% aqueous solution of methanol from 15 min to 240 min to make the films insoluble. The methanol-treated films were washed with water thoroughly and dried in air. The thickness of the films was 45-60 μm. Sample codes and preparation conditions of SF films are listed in Table 1.

Measurements

The contact angles were measured with contact angle goniometer (Erma, G-I type) at the octane/water/solid interfaces by immersing a film in deionized water for at least

Table 1. Sample code and preparation condition of SF films

Sample code	Solvent treatment condition
SF-15	50% methanol, 15 min
SF-30	50% methanol, 30 min
SF-60	50% methanol, 60 min
SF-120	50% methanol, 120 min
SF-240	50% methanol, 240 min
SF-FA	5% formic acid

24 h to ensure complete hydration. After releasing an octane drop (about 1 μl) beneath the film using a microsyringe, the contact angles on the both sides of each drop were averaged. Ten replicates were carried out for each sample.

To estimate the crystallinity of the SF films, which were treated with 50% aqueous solution of methanol for different treating times, IR measurement was performed by Perkin Elmer 1725X FTIR spectrophotometer. The resolution was 4 cm^{-1} and the scan was repeated 100 times. The IR crystallinity index of SF films was calculated by equation (1) proposed by Bhat *et al.*[12] as shown below:

$$\text{IR crystallinity index (CI)} = A_{1265}/A_{1235} \quad (1)$$

where A's are the absorbances at 1265 cm^{-1} and 1235 cm^{-1}, which are assigned to amide III band due to β-sheet and random coil conformation, respectively.

Platelet Adhesion

The platelet adhesion test for the SF films was performed by film depositing method[13]. Samples (2×2 cm) were washed with saline and contact with human anticoagulant citrate dextrose (ACD) blood at 37°C for 10 min. The number of platelets in the sampling bottle was counted by using a haemacytometer (Fortuna) on a phase contrast microscope (model BH-PC, Olympus, ×400). The adhered platelet (%) was measured seven times for each sample and averaged as the following relation.

$$\text{the adhered platelet (\%)} = (1 - N/N_o)\times 100 \quad (2)$$

where N_o and N are the number of platelets before and after film deposition for 10 min, respectively.

Plasma Protein Adsorption

The adsorption of plasma proteins onto the SF film was carried out by the following procedure. Sample film (3×3 cm) was dipped in blood plasma solution and incubated for 60 min in water bath regulated at 37°C. Then sample film was rinsed with distilled water to remove unadsorbed proteins.

In Vivo Tests

The dialysed SF solution was frozen in methanol/dry ice, and amorphous SF flakes were obtained by freeze drying the frozen SF under reduced pressure for 20 h. Then the flakes were dissolved either in distilled water or in 99% formic acid at room temperature and filtered after centrifugation. Subsequently, a polyester suture (International Standard No. 1~0, 10 cm) and polyester meshes (1×3 cm), sterilized with ethanol, were coated twice with the filtered solution (2 wt%). In patricular, the samples prepared from the aqueous solution of fibroin were treated with 50% aqueous solution of methanol at room temperature for different times.

Thrombus Formation Test

The SF-coated polyester suture was inserted through a needle into the periperal vein of a mongrel dog under general anesthesia[14]. After a given period of time the dog was sacrificed by acute exsanguination from the aorta with the administration of heparin. The vessel in which the suture had been inserted was opened in a longitudinal direction and the suture was removed. Then the suture was fixed with a 2.5% glutalaldehyde in phosphate buffered solution for macroscopic examination. The results were evaluated using the following criteria.

(−): no thrombus formation
(+): a partial thrombus formation
(++): thrombus formation along the entire surface
(+++): noticeable thrombus formation along the entire surface to lead to the embolus of a blood vessel

Absorbability Test

The SF-coated polyester suture, for which the thrombus formation test had been completed, was embeded in a molten paraffin and the suture was sectioned using a microtome. After staining with hematoxylin-eosine, the absorbability of the SF was evaluated by microscopical observation according to the following criteria.

(−): no absorption
(+): 25% absorption
(++): 50% absorption
(+++): 75% absorption
(++++): above 95% absorption

Foreign Body Reaction Test

The SF-coated polyester meshes were implanted into the back-muscle of a mongrel dog. After a certain period of time, the meshes were cut off from the muscle with environmental tissues and the extent of the foreign body reaction was evaluated using an optical microscope according to the following criteria.

(−): no foreign body reaction
(±): between (−) and (+)
(+): low foreign body reactions as cell layers which surrounded the sample are below 10, though the cells recognize the sample as the foreign body, and foreign gaint cells or analogous epithelial cells group around the sample
(++): between (+) and (+++)
(+++): high foreign body reaction as a mass of manifold

cells are formed
(++++): significant foreign body reaction enhanced above (+++) as the environmental cells reach necrotic state by a cellular poisoning

Results and Discussion

Surface Free Energy of SF Films with Methanol Treatment

The change in surface free energy of SF films treated with 50% methanolic aqueous solution is shown in Figure 1.

The SF films used were five kinds of samples treated with 50% aqueous solution of methanol for 15 min (SF-15), 30 min (SF-30), 60 min (SF-60), 120 min (SF-120), and 240 min (SF-240). As the treating time increased, the polar component (γ_s^p) of surface tension and total surface tension (γ_s) increased gradually, while the dispersion component (γ_s^d) of surface tension did not change. As reported previously [15], random coil conformation of SF film is transformed into β-sheet one by the methanol treatment, but α-helix portion still partly remains. Such a structural change of SF film due to the methanol treatment results in the increase of total surface tension and polar, especially hydrogen bond components. The IR crystallinity indices of SF films treated with methanolic aqueous solution for 15 min to 240 min are represented in Figure 2.

As the treating time increases, the IR cystallinity index of SF films increases rapidly in the beginning and slowly after 60 min. This increment in the IR crystallinity was closely correlated with conformational change of SF films, random coil to β-sheet, involved in methanol treatment.

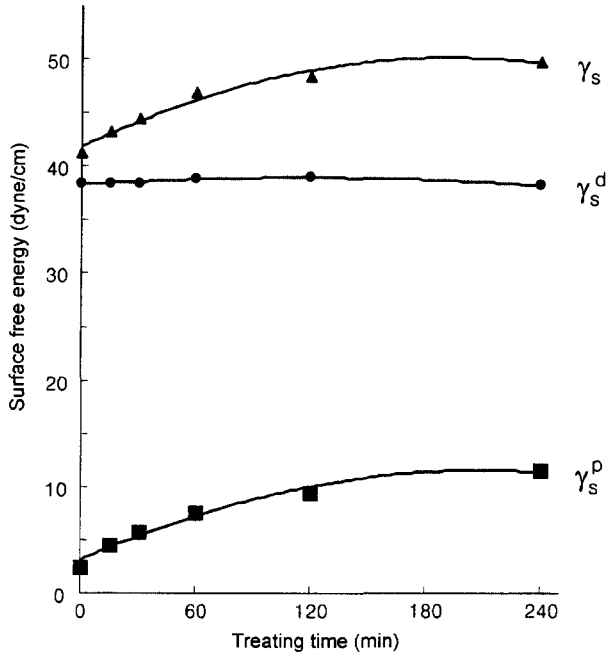

Figure 1. Effect of treating time on surface free energy of SF films treated with 50% aqueous solution of methanol.

Platelet Adhesion Behavior of SF Films

When a biomaterial contacts with blood, the thrombus is

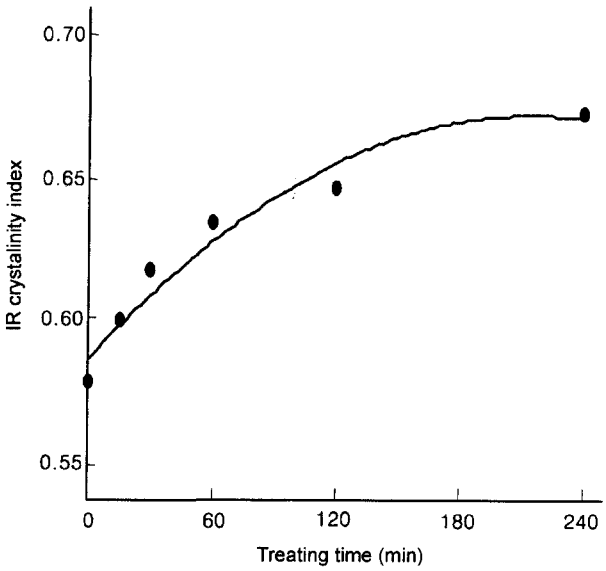

Figure 2. Effect of treating time on IR crystallinity index of SF films treated with 50% aqueous solution of methanol.

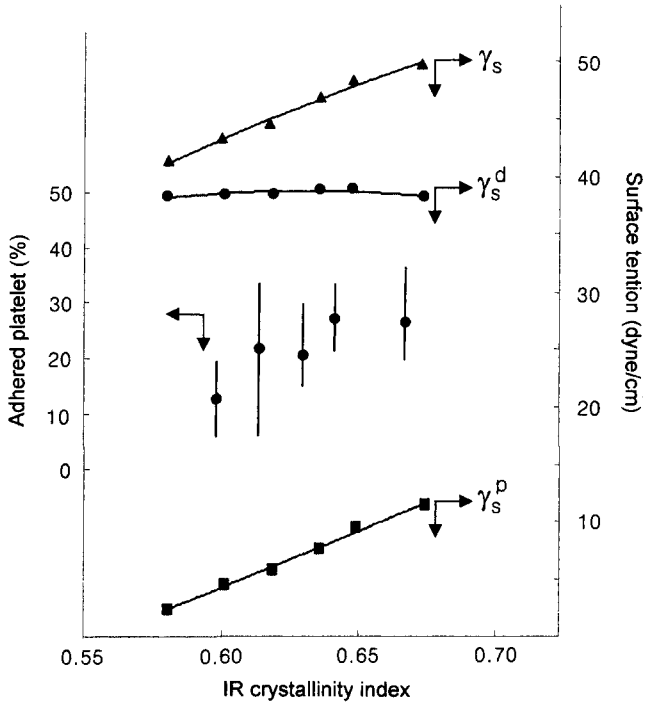

Figure 3. Relation between IR crystallinity index and adhered platelet (%) and between IR crystallinity index and surface tension of SF films.

formed by many factors, but the most important event with the biomaterial is the platelet adhesion behavior. In Figure 3, the adhered platelet (%) of SF films was evaluated in connection with the surface free energy components and the IR crystallinity index of SF films.

As seen in Figure 3, SF-15 sample with low IR crystallinity index (0.60) exhibited the lowest value of adhered platelet (12%). The adhered platelet (%) of SF films gradually increased with the increase of methanol treated time and thus IR crystallinity index. It is obvious that the surface free energy of SF films, especially the polar component, has an influence on its platelet adhesion.

Antithrombogenicity of SF Films (*in Vivo* Test)

In vivo results on the thrombus formation, absorbability and foreign body reaction of regenerated SF samples are shown in Table 2.

According to the criteria of the thrombus formation employed in this work, the regenerated SF samples examined here exhibited an excellent blood compatibility except for SF-FA sample. In Figure 3, SF-15 sample with low IR crystallinity index (0.60) was found to suppress platelet adhesion and, therefore, antithrombogenic. Considering that SF-FA sample had higher IR crystallinity index (0.72) and this sample was found to be thrombogenic, the above results suggest that the conformation of SF would strongly influence its antithrombogenicity. Thus, it becomes obvious that the surface energy of SF film has an influence on its antithrombogenicity.

On the other hand, SF showed about 75% absorbability after implantation for 2 weeks, irrespective of the treatment conditions. However, the results of foreign body reaction indicated that the SF-FA acted on the organic cells as a foreign body.

Griffith Surface Energy Analysis on Antithrombogenicity of SF Films

Kaeble and Moacanin[16] analysed the relationship of interfacial tension between adsorbed plasma protein layer (phase 1), blood plasma (phase 2) and material surface (phase 3) on the basis of the Griffith fracture mechanics for the blood compatibility. Equation (3) represents the relation between surface energies of the three phases and critical crack propagation stress.

$$\sigma_C = \left(\frac{2E\gamma_G}{\pi C}\right)^{1/2} = \left(\frac{2E}{\pi C}\right)^{1/2}(R^2 - R_0^2)^{1/2} \geq 0 \quad (3)$$

where

$\gamma_G = R^2 - R_0^2$
$R_0^2 = 0.25\ \{(\alpha_1 - \alpha_3)^2 + (\beta_1 - \beta_3)^2\}$
$R^2 = (\alpha_2 - H)^2 + (\beta_2 - K)^2$
α_1, β_1 = square roots of the respective dispersion and polar components of surface free energy of adsorbed plasma protein layer (phase 1)
α_2, β_2 = square roots of the respective dispersion and polar components of surface free energy of blood plasma (phase 2)
α_3, β_3 = square roots of the respective dispersion and polar components of surface free energy of biomaterial (phase 3)
$H = 0.5(\alpha_1 + \alpha_3)$ (an averaged value from the dispersion components of surface free energies of phase 1 and phase 3)
$K = 0.5(\beta_1 + \beta_3)$ (an averaged value from the polar components of surface free energies of phase 1 and phase 3)
σ_C = critical crack propagation stress
γ_G = Griffith surface energy for fracture
E = Young's modulus
C = crack length

On the other hand, when blood contacts with biomaterial, the initial event is the adsorption of blood plasma protein. In this process plasma proteins are adsorbed onto the biomaterial surface until a surface equilibrium concentration is reached and this adsorbed protein layer changes the surface energy of biomaterial to cause a low interfacial tension against blood plasma. Therfore, a central issue of blood compatibility can be related to the resistance to detachment of the plasma protein layers which cover and biologically modify the biomaterial surface. The modified Griffith relation of equation (3) provide a quantitative means for evaluating the Griffith energy γ_G required to detach the adsorbed protein layers (phase 1) from the biomaterial (phase 3) in the presence of blood plasma (phase 2).

Table 3 shows γ_G and the other parameters calculated on

Table 2. *In vivo* results of SF

Sample	Thrombus formation		Absorbability	Foreign body reaction	
	Implantation period (day)		Implantation period (day)	Implantation period (day)	
	1	14	14	14	28
SF-FA[a)]	–	++	+++	++	+++
SF-15[b)]	–	–	+++		
SF-240[b)]	–	–	+++		

[a)] as coated.
[b)] treated with 50% aqueous methanol solution after coating: the numbers denote treatment time (min).

Table 3. Calculated Griffith surface energies for SF films required to detach the absorbed protein layer (1) from implant surface (3) in blood plasma (2)

Implant	SF-15	SF-30	SF-60	SF-240
α_3	6.48	6.26	6.43	6.43
β_3	1.95	2.00	2.05	2.26
α_1	5.72	5.81	5.78	5.81
β_1	3.73	3.75	3.81	3.90
α_2	4.67	4.67	4.67	4.67
β_2	7.14	7.14	7.14	7.14
H	6.10	6.04	6.11	6.12
K	2.84	2.88	2.93	3.08
R_0^2	0.94	0.82	0.88	0.77
R^2	20.53	20.02	19.60	18.59
γ_G	19.59	19.20	18.72	17.82

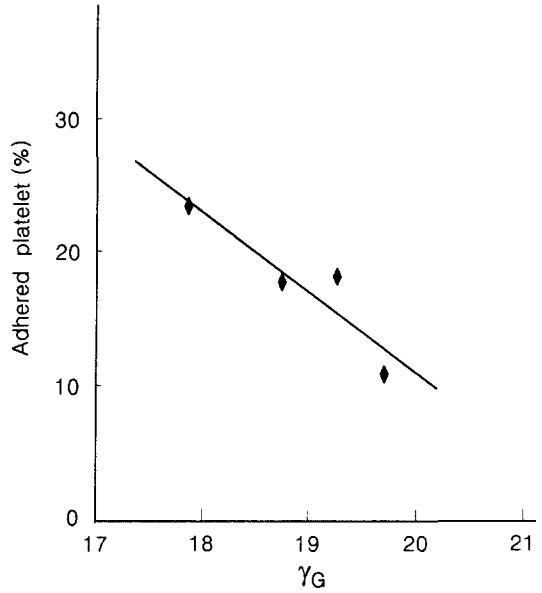

Figure 4. Adhered platelet (%) as a function of Griffith surface energy for SF films.

the 50% methanol treating SF films.

In Table 3, α_1 and β_1 values of blood plasma protein layer adsorbed in equilibrium on the SF film were obtained from contact angle measurement. The other values in Table 3 were obtained by using equation (3) for each SF sample.

Kaelble and Moacanin reported that the Griffith surface energy γ_G (and related critical mechanical stress σ_C) decreasd as the polar component of surface free energy β_3 of material surface increased. The results from this study also showed that γ_G decreased gradually, from 19.59 to 17.82, with increment of β_3. The adhered platelet (%) was plotted against γ_G in Figure 4. Figure 4 showed that in the lower value of γ_G, the platelet adhesion was suppressed.

The nature of polymer surface controls the initial protein adsorption, and the adsorbed protein layer determines the platelet adhesion and subsequently the extent of platelet adhesion is known to be important in the following blood coagulation process. Therefore, the estimation of the modified Griffith surface energy assoiciated with protein adsorption could offer an indicator in blood compatibility of the material.

Conclusions

The regenerated SF films were treated with 50% aqueous solution of methanol for different times to make them water-insoluble, and their antithrombogenicity was evaluated by platelet adhesion and *in vivo* tests.

The platelet adhesion (%) of silk fibroin films increased due to the increment of polar component of surface tension or IR crystallinity index, as the methanol treated time lengthened. From the surface energy analyses using the modified Griffith surface energy γ_G, the blood compatibility of the silk fibroin films was considered to be closely related to their surface free energy. It was concluded from the results of *in vitro* and *in vivo* tests that the silk fibroin could be utilized as a biomaterial having antithrombogenicity and absorbability.

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