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In vitro Platelet Adhesion and Protein Adsorption of Biomedical Implant Modified with Polysaccharide

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Protein adsorption on an alginic acid layer covalently immobilized onto γ-aminopropyl-triethoxysilane (γ-APS)-grafted stainless-steel (SUS316L) was examined. The surface was characterized with contact angle measurement toward water, Fourier transform infrared spectroscopy using a reflection absorption method (FT-IRRAS), and atomic force microscopy (AFM). FT-IRRAS revealed the successful immobilization of γ-APS and alginic acid onto the SUS316L surface. The contact angle measurement and AFM images indicated that the covalently immobilized alginic acid layer was hydrophilic and smooth. Bovine serum albumin (BSA) adsorption on the alginic acid-immobilized SUS316L was examined with FT-IRRAS. BSA adsorbed on the SUS316L substrates with and without γ-APS-immobilization. In contrast, the alginic acid-immobilization prevented BSA adsorption thoroughly. It was suggested that the protein-repelling property of the alginic acid layer should be effective for blood compatibility.
Key words: blood compatibility, alginic acid, stainless steel, platelet adhesion, protein adsorption

1. INTRODUCTION

Artificial materials in contact with human blood should avoid blood clotting on their surfaces. Adverse reactions between artificial materials and blood components are the predominant factors restricting those materials in contact with blood. It is important to optimize the material surface properties so as to promote desired responses to blood. Stainless-steel (SUS316L) and titanium are applied for blood-contacting devices such as stent or artificial heart housing. The poor blood compatibility of those metallic devices has been unsolved yet. One of the strategies to fabricate blood-compatible surface is to coat such materials with anticoagulant agents, and heparin coating is a candidate for attaining a blood compatible surface. However, the blood compatibility of heparin coating is still insufficient because heparin requires the interaction with specific protein, antithrombin, to practice its anticoagulant property [1].

Polysaccharide coating is an alternative method to obtain fouling-resistant surface. Österberg *et al.* [2] and Morra *et al.* [3] reported that polysaccharide coating, for example, dextran or alginic acid, prevented protein adsorption, cell adhesion, and even bacteria adhesion. Since the blood components such as blood cells and proteins are adsorbed on the material surface to diminish functionalities, it is effective to coat the materials with polysaccharide. Recently, we reported that the alginic acid layers immobilized on γ-aminopropyltriethoxy-silane (γ-APS)-grafted SUS316L and titanium reduced *in vitro* platelet adhesion and did not affect the blood-clotting period [4,5]. However, the interaction between alginic acid layers immobilized on the metal surface and blood proteins is still unknown. As to fabrication of blood-compatible surfaces, it is essential to investigate the adsorption characterization of blood proteins because they dominate blood coagulation behavior on the material surfaces.

In the present study, we examined protein adsorption on an alginic acid layer immobilized onto SUS316L. Alginic acid was covalently immobilized onto the SUS316L substrates using carbodiimide, to which γ-APS was grafted to introduced amino groups. The surface was characterized with contact angle measurement toward water, Fourier transform infrared spectroscopy using a reflection absorption method (FT-IRRAS), and atomic force microscopy (AFM). Bovine serum albumin (BSA) adsorption was examined using FT-IRRAS since albumin is a major protein in blood. FT-IRRAS is useful technique for detection of the adsorbed proteins on metal surface [6]. The relationship between platelet adhesion and protein adsorption on the alginic acid layer was discussed.

2. EXPERIMENTAL

2.1 Alginic acid immobilization

Surface modification was performed on 10 x 10 x 2 mm pieces of mirror-polished stainless-steel (SUS316L; JIS G4305-1999). These substrates were washed successively with 0.1 N NaOH, distilled water, and acetone, before dried with nitrogen gas. The cleaned substrates were silanized with γ-APS as they were soaked in a 1 vol% toluene solution of γ-APS. After rinsing with toluene and ethanol, they were heated at 105°C for 10 min. These substrates were denoted as APS. Subsequently, the APS substrates were soaked in an aqueous solution containing sodium alginate and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at room temperature under stirring. After rinsing with distilled water, they were stored in distilled water (MILLIPORE, 18.2 MΩcm) until use. These substrates were denoted as ALG. All substrates were dried with nitrogen gas before use.

2.2 Surface characterization

The contact angle of the samples toward distilled

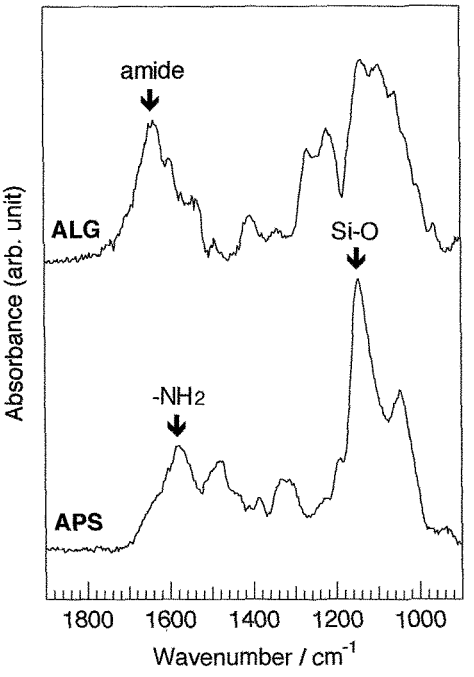

Fig. 1 FT-IRRAS spectra for APS and ALG

Table I Contact angle (°) toward water on the surface of SUS316L, APS and ALG

sample	contact angle (°)
SUS316L	83 ± 2
APS	49 ± 1
ALG	28 ± 2

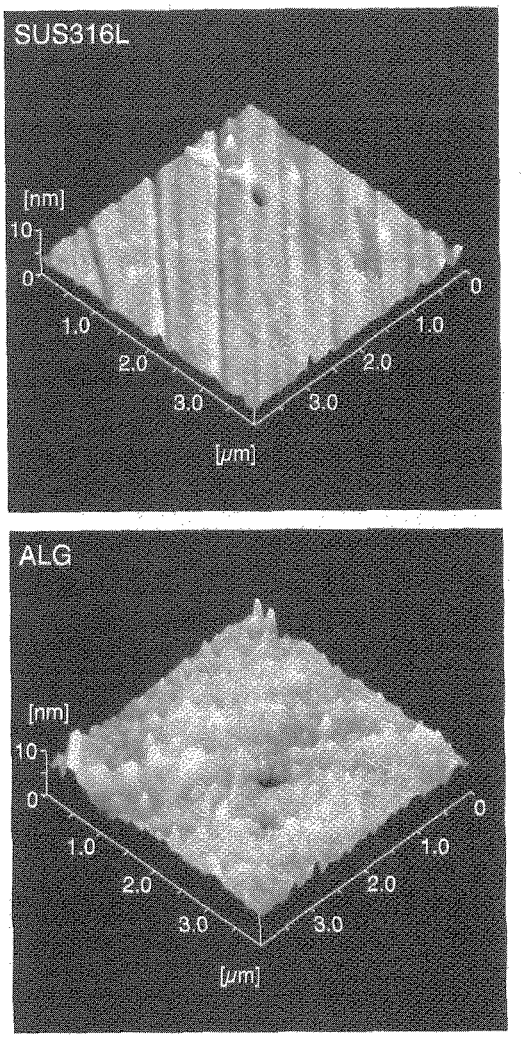

Fig. 2 AFM images of SUS316L and ALG surface

water was measured using contact angle measurement equipment, model CA-V (Kyowa Interface Science, Japan). FT-IRRAS spectra were measured using a FT-IR spectrometer, model Nexus 470 (Thermo Nicolet, USA) equipped with a Smart SAGA system (Thermo Nicolet, USA). The signals from 256 scans at a resolution of 4 cm^{-1} were collected. The spectrum for the as-cleaned SUS316L substrate was taken as the background. The AFM images were obtained using a Nanopics 2100 (Seiko Instruments, Japan) that was operated with a damping mode in air at ambient temperature. Surface roughness was calculated from the images using the instrument software.

2.3 Protein adsorption

In the protein adsorption experiment, bovine serum albumin (BSA, Sigma) was used. The substrates were soaked in the phosphate buffer solution (PBS, pH7.2) for at least 2 h and rinsed with distilled water, for which the background FT-IRRAS spectra were measured. The substrates were soaked in 1 mL of PBS containing various concentration of BSA at 36.5°C for 1 h. Then, the loosely adsorbed proteins were removed with PBS and distilled water. After drying with nitrogen gas, FT-IRRAS spectra for the substrates after soaking in BSA solution were collected by averaging the signal of 256 scans at a resolution of 4 cm^{-1}.

3. RESULTS AND DISCUSSION

Fig. 1 shows the FT-IRRAS spectra for APS and ALG. In the APS spectrum, the absorption peaks of amino groups and Si-O bonds were detected at 1580cm^{-1} and 1145 cm^{-1}, respectively. In the ALG spectrum, the amide bond was detected at 1635 cm^{-1}. This amide bond was derived from condensation reaction between the amino groups of γ-APS and the carboxyl groups of alginic acid, indicating that the alginic acid was immobilized on SUS316L via γ-APS in covalent bonding. Table 1 shows the values of contact angle for SUS316L, APS and ALG. SUS316L surface was hydrophobic, while γ-APS and alginic acid-immobilization changed the wettability. The ALG surface was more hydrophilic than the SUS316L and APS surfaces. An alginic acid molecule has hydrophilic groups such as hydroxyl and carboxyl groups. Thus, the contact angle data confirmed the immobilization of alginic acid on SUS316L.

Fig. 2 shows the AFM images of the SUS316L and ALG surfaces. One may find some grooves on the

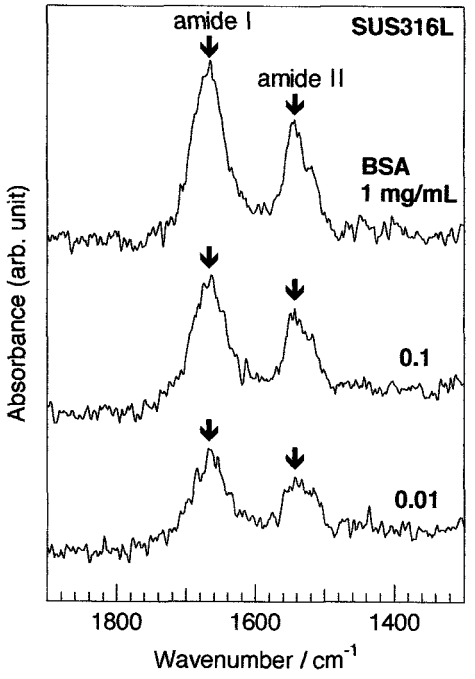

Fig. 3 FT-IRRAS spectra for BSA adsorbed on SUS316L in 0.01, 0.1 and 1 mg/mL

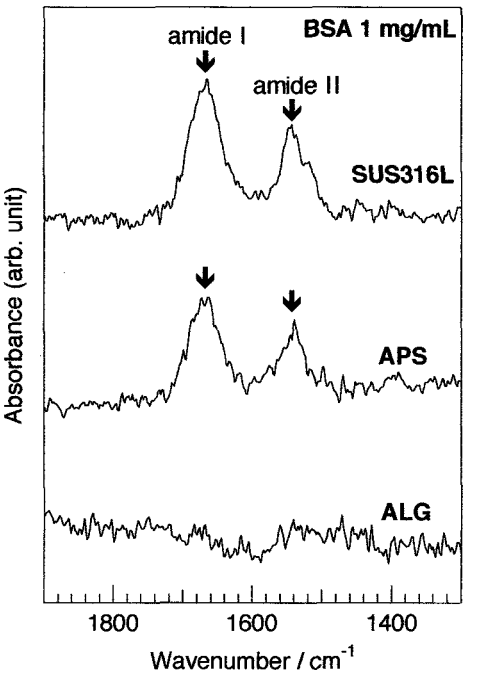

Fig. 4 FT-IRRAS spectra for BSA adsorbed on SUS316L, APS and ALG in a concentration of 1 mg/mL

SUS316L surface due to polishing procedure. On the ALG surface, no grooves were observable but the surface was slightly rougher than the bare SUS316L. However, the roughness of both surface was less than 1 nm. Thus, it was feasible that the evaluation of protein adsorption on the ALG was performed using FT-IRRAS technique although the surface morphology of the ALG was different from that of bare SUS316L.

Fig. 3 shows the FT-IRRAS spectra for BSA adsorbed on SUS316L in various concentrations. The absorption peaks were detected around 1660 cm^{-1} and 1530 cm^{-1}, which were assigned to the amide I and amide II bands of BSA, respectively. The amide I absorption peaks were gradually large depending on BSA concentrations. Fig. 4 shows the FT-IRRAS spectra for BSA adsorbed on SUS316L, APS and ALG. On the SUS316L and APS surface, the amide I and amide II absorption peaks were detected. On the other hand, neither amide I nor amide II bands were detected on the ALG surface. FT-IRRAS is a useful technique for evaluation of the adsorbed proteins on metal surfaces. However, it should be taken into consideration that the intensity of the absorption peaks depends on the orientation of the molecules adsorbed on a metal surface. Imamura *et al.* applied this technique to derive the amount of β-lactogloburin on stainless-steel using the intensities of amide I bands [6]. They mentioned that the orientation was independent of the amount of adsorbed proteins because the FT-IRRAS profile was independent of the amount of the protein adsorbed. Therefore, in the preliminary experiment, we confirmed that the shapes of spectra were similar to each other by collecting the spectra for the dried droplets of the protein solutions with different concentrations. Thus, we discussed the intensity of the amide I bands as the standard of the amount of adsorbed protein. Fig. 5

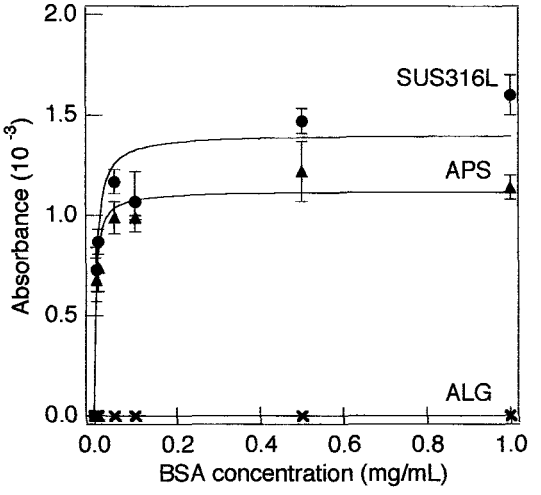

Fig. 5 Relationship between BSA concentration and FT-IRRAS absorbance due to amide I band

shows the relationship between the intensity of amide I band and BSA concentration. The saturated amount of the adsorbed BSA decreased in the order, SUS316L > APS > ALG, while the amount on ALG was practically zero. This order was well explained by the change in contact angle. That is, the BSA adsorption proceeds due to hydrophobic interaction. However, for conclusion, more experiments are necessary.

In our previous study, we found that an alginic acid layer immobilized on SUS316L and titanium reduced *in vitro* platelet adhesion and did not affect blood-clotting times [4,5]. Platelet adhesion leading to thrombus

formation on the material surface typically follows the adsorption of blood proteins. Therefore, attaining blood compatibility is to prevent blood protein adsorption. From the present results of the suppressed BSA adsorption due to an alginic acid layer immobilization, it is concluded that the protein-resistant property directly corresponds to better blood compatibility of the alginic acid layer on SUS316L.

4. CONCLUSIONS

An alginic acid layer was successfully immobilized onto γ-APS-grafted stainless-steel (SUS316L). The surface was characterized with contact angle measurement toward water, FT-IRRAS and AFM. BSA adsorption on the surfaces was examined using FT-IRRAS. BSA adsorbed on SUS316L and γ-APS-grafted surface, while the alginic acid-immobilized surface prevented BSA adsorption. From our previous result that showed the good blood compatibility of alginic acid layers on SUS316L compared with bare one, less protein adsorption leads to blood-compatible surface.

5. ACKNOWLEDGEMENT

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