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Preparation of alginic acid layers on solid substrates for biomedical applications

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Alginic acid was immobilized on γ-aminopropyltriethoxysilane-coated glass as a model substrate since an alginic acid layer was known to prevent cell adhesion. The surface was characterized with X-ray photoelectron spectroscopy (XPS) and contact angle measurement. The coated substrates adsorbed practically no calcium phosphates on their surfaces when soaked in a simulated body fluid (SBF) of Kokubo recipe. Since calcium ions are one of the factors for blood clotting, the present alginic acid coating is one of the candidates to improve blood compatibility of clinical materials.

1. INTRODUCTION

When artificial materials are implanted in living organisms, their surfaces directly come into contact with living tissues or body fluid. Thus, it is important to so optimize the implant surfaces as to promote desired responses to the living organisms. Currently, metals and alloys are mainly used as bone substitutes, artificial tooth roots, artificial heart casing, or artificial heart valves. It is essential to modify the surfaces in wettability, friction, wear, electrochemical passivation, and surface reactivity.

One of the important surface modification methods is to coat the surface with a layer of organic molecules in a form of Langmuir-Blodgett (LB) monolayer and self assembled monolayer (SAM). Recently, the studies of SAM has attracted much attention [1-9]. Alkane thiols can form SAM on gold [1] while silane coupling agents can form it on metals such as silicon [2,3]. SAM-forming techniques can introduce densely packed functional groups on solid substrates [4-8]. Moreover, silane coupling agents are used when organic molecules are covalently immobilized onto the solid substrates [10]. Thus, such SAMs serve molecularly smooth templates for chemical tethering of functional molecules [9].

In this study, we focus our attention to the fabrication of the surfaces with a SAM of a silane coupling agent as the template to chemically immobilize functional molecules so that they can promote desirable responses in organism.

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We have selected alginic acid as the functional molecule to be immobilized on glass substrate, since alginic acid is non-toxic and also an alginic acid layer formed on polymer substrates prevented cell adhesion as Morra *et al.* reported [11]. Surface characterization methods include contact angle measurement and X-ray photoelectron spectroscopy (XPS). Also the ability of calcification of the alginic acid-immobilized surface is examined using a simulated body fluid (SBF), or Kokubo solution, which has the same inorganic ions as the human body plasma in similar concentrations and has been confirmed to well reproduce the in vivo behavior of the materials under in vitro conditions [12].

2. EXPERIMENTAL

Surface modification was performed on 10×10×1 mm glass slide (S-1111; Matsunami Glass Ind., Osaka). Glass substrates were sonicated in ethanol and then in acetone before heating at 500°C for 2 h. The heat-treated specimens were denoted as GS_500. Some of them were silanized with γ-aminopropyltriethoxysilane (γ-APS; Chisso Co., Tokyo) as they were soaked in a 1 vol% toluene solution of γ-APS up to 8 h in air. After rinsed with toluene and ethanol, and finally sonicated in ethanol for 5 min, they were dried in air over 24 h and then kept at 105°C for 10 min. The γ-APS-coupled specimens were denoted as APS_1min or APS_8h depending on the soaking time. Those samples were soaked in aqueous solution of sodium alginate (Nacalai Tesque, Kyoto) containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Wako Chemicals, Osaka) at room temperature for 24 h. The molar ratio between sodium alginate and EDC was 1:1. Then the specimens were rinsed with distilled water, and finally sonicated for 5 min before dried in air over 24 h. Those specimens with immobilized alginic acid were denoted as ALG.

Contact angle of the samples toward distilled water was measured using contact angle measurement equipment, model CA-V (Kyowa Interface Science, Tokyo). For silanized samples, static contact angle was measured. Before and after immobilizing alginic acid onto silanized samples, advancing and receding angles were measured by extending or contracting the water droplet on the specimens.

X-ray photoelectron spectra (XPS) were measured with a Fisons Instruments S-probe ESCA (SSX100S). The instrument was equipped with a monochromatic X-ray source (Al Kα) operating at 10 kV and 210 W. Neutralization of the sample surface was carried out during the measurement by the combined use of a low energy flood gun and an electrically grounded Ni mesh screen placed 1 mm above the sample surface [13,14]. Calibration of the spectra was done by setting the measured binding energy of the C1s peak to 284.1 eV of adventitious carbon accumulated in the analysis chamber of the spectrometer.

SBF was prepared by adding inorganic salts according to a specific recipe and standard procedure [12]. It was kept at 7.4 in pH at 36.5°C. Before soaking in SBF, ALG was soaked in 10 mL of a saturated aqueous solution of $Ca(NO_3)_2$ up to 7 days, expecting that calcium ions were fixed in the alginic acid layer. Since the present study used glass slide as a substrate, the saturated aqueous $Ca(NO_3)_2$ solution was employed to avoid alkali solution. The specimens were rinsed in distilled water for a day. Then, the specimens were soaked in 14 mL of SBF for 14 days, gently rinsed with distilled water, and dried in air. The surface structure was examined with thin film X-ray diffraction (TF-XRD; RAD-IIA, RIGAKU, CuKα, 40 kV, 25 mA) and Fourier transform infrared spectroscopy (FT-IR; FT-IR300,

JASCO, Tokyo).

3. RESULTS AND DISCUSSION

After the heat treatment at 500°C for 2 h, the contact angle toward distilled water on the surface of the glass substrate was less than 6°. This indicated that the heat treatment cleaned the glass surfaces enough to evaluate the change in contact angle after silanization. Fig. 1 presents the contact angles for the glass substrate silanized with γ-APS as a function of period of soaking in the γ-APS: toluene solution. The contact angle increased very quickly within the first 1 minute from ~6° to about 50° and then gradually increased to about 58° at 1 h. It was also found that soaking for 1 h or longer did not change the contact angle. Those results indicated the formation of thin film of γ-APS on the glass substrate. Thus, 1 h of silane-coupling was sufficient (sample APS_1h) for immobilization of alginic acid.

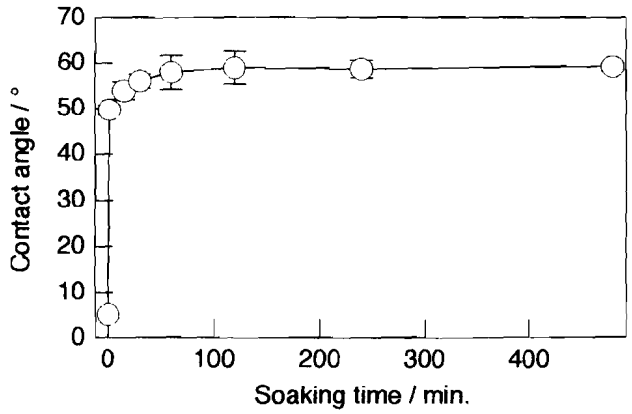

Fig. 1 Contact angle (°) toward distilled water on the surface of the glass substrates as a function of the soaking time in 1 vol% γ-APS: toluene solution.

Table 1 shows the advancing and receding contact angles for the glass substrates after immobilization of alginic acid. Immobilization of alginic acid decreased advancing and receding contact angles. One can attribute the decrease of receding contact angle to the affinity of ALG surfaces toward water. That is, the decrease of receding contact angles are due to the hydrophilic groups of alginic acid such as hydroxyl and carboxyl groups.

Table 1 Advancing and receding contact angles (°) toward distilled water on the surface of the glass substrates. APS_1h: surface silanized with grafting γ-aminopropyltriethoxysilane, ALG: alginic acid-immobilized surface.

Sample	Advancing ang.	Receding ang.
APS_1h	62.7±1.5	40.3±4.1
ALG	54.2±2.6	16.8±3.4

Table 2 Surface composition (% atom) derived from XPS analysis. GS_500: heated glass surface (before the chemical modifications), APS_1h: surface silanized with grafting γ-aminopropyltriethoxysilane, ALG: alginic acid-immobilized surface.

Sample	C	O	N	Si	Na	K	Ca
GS_500	11	56	0	21	7	4	1
APS_1h	29	44	4	18	2	2	1
ALG	50	32	4	12	1	0	1

Table 2 shows the surface composition data of GS_500, APS_1h, and ALG from the XPS analysis. The sodium, potassium, and calcium signals were derived from the glass component oxides. Carbon was detected on all the samples, while nitrogen was detected on the surface of APS_1h and ALG. The silanization obviously contributed to immobilizing amino groups on the specimen surface. The immobilization of alginic acid to the silanized surface (APS_1h) increased in the carbon to nitrogen ratio.

Fig. 2 shows C1s photoelectron spectra of GS_500, APS_1h, and ALG. They were deconvoluted to a few component peaks 1 through 4. Table 3 lists their values of binding energy (BE), full widths at half maximum (FWHM), and the relative area of the deconvolution components. ALG exhibited an additional one, peak 4, with a greater binding energy, 288.8 eV, assignable to the carbon atoms in the carboxyl groups of alginic acid. One might expect that an amide bond would be derived by a reaction between an amino group of γ-APS and a carboxyl group of alginic acid. The C1s peak at binding energy 287.5 eV was assigned to a carbon atom in the amide bond. However, it was too difficult to deconvolute the signal of an amide bond from the N1s adjacent peak (data was not shown here).

Hanakawa *et al.* reported that alginic acid fibers treated with the saturated $Ca(OH)_2$ aqueous solution formed apatite on their surface in SBF

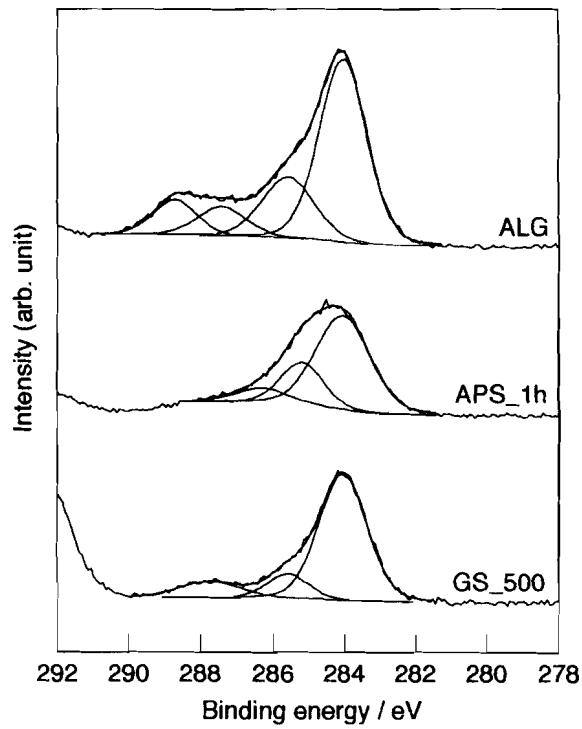

Fig. 2 C1s XPS spectra for the chemically modified glass substrates. GS_500: heated glass surface (before the chemical modifications), APS_1h: surface silanized with grafting γ-aminopropyltriethoxysilane, ALG: alginic acid-immobilized surface.

Table 3 Analysis of the C1s XPS spectra. Binding energy (BE), full widths at half maximum (FWHM), and relative area of the deconvoluted components of the C1s peaks. GS_500: heated glass surface (before the chemical modifications), APS_1h: surface silanized with grafting γ-aminopropyltriethoxysilane, ALG: alginic acid-immobilized surface.

	Peak1			Peak2			Peak3			Peak4		
	BE	FWHM	Area	BE	FWHM	Area	BE	FWHM	Area	BE	FWHM	Area
Sample	(eV)	(eV)	(%)	(eV)	(eV)	(%)	(eV)	(eV)	(%)	(eV)	(eV)	(%)
GS_500	284.1	1.6	75	285.6	1.4	13	287.8	2.0	12			
APS_1h	284.1	1.8	66	285.2	1.4	24	286.3	1.8	10			
ALG	284.1	1.6	58	285.6	1.7	22	287.5	1.6	10	288.8	1.4	10

within 7 days [15]. Thus, in the present study ALG was also soaked in SBF for 14 days and the calcification ability was examined. Fig. 3 shows TF-XRD patterns and FT-IR reflection spectra of the surface of the specimen, with or without incubation in saturated $Ca(NO_3)_2$ solution, after soaking in SBF for 14 days. Regardless to incubation, no appreciable changes were detected in the XRD patterns nor in the FT-IR spectra after soaking in SBF for 14 days. It was reasonable that the FT-IR spectra showed no signals of γ-APS and alginic acid, because the layers were of order of nanometers in thickness. These results indicated that there are no apatite or calcium phosphate or calcium carbonate depositions on the ALG surface.

It is necessary to prevent formation of thrombi and calcification on the surface of the materials when the materials contact with blood. This study indicated that alginic acid layer formed on ceramic substrate prevented calcification on its surface in SBF. Moreover, after Morra *et al.* [11] an alginic acid layer prevented from cell adhesion. Therefore, the present surface modification process is applicable to the materials that are required to be not-blood clotting.

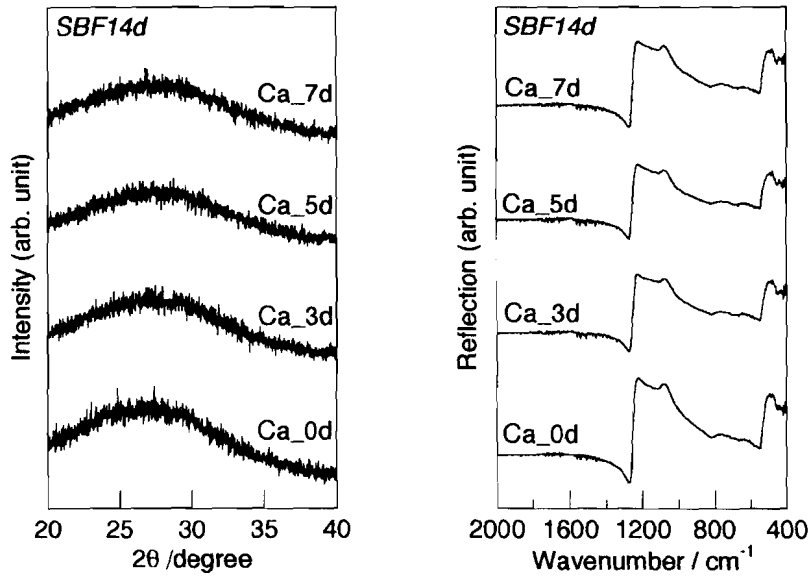

Fig. 3 TF-XRD patterns and FT-IR spectra for ALG treated with or without saturated $Ca(NO_3)_2$ solution up to 7 days and subsequently soaked in SBF for 14 days.

4. SUMMARY

Alginic acid has been successfully immobilized onto the glass substrate to which are introduced amino groups by grafting γ-aminopropyltriethoxysilane. Those glass substrates did not form apatite although treated with the saturated $Ca(NO_3)_2$ aqueous solution up to 7 days. Thus, it is concluded that the present modification process is applicable to the materials that should not result in blood clotting when in contact with blood.

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