ARTICLE

Complement Activation by Polymers Carrying Hydroxyl Groups

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ABSTRACT Hydrogels of polymers carrying surface hydroxyl groups strongly activate the complement system through the alternative pathway, although it has also been reported that solutions of polymers do not. To address these curious, inconsistent results, we examined the effect of polymer states, either immobilized on a surface or soluble in serum, on the complement activation using a surface plasmon resonance apparatus and enzyme-linked immunosorbent assay. We clearly showed that dextran- and poly(vinyl alcohol)-immobilized surfaces strongly activated the complement system but that soluble polymers could not, even when the amounts of the soluble polymers added to serum were 4−2000 times higher than those on the polymer-immobilized surfaces.

KEYWORDS: complement activation • protein adsorption • poly(vinyl alcohol) • dextran

INTRODUCTION

When artificial materials come into contact with blood, the plasma proteins and cells interact with the material surface. Various biological responses are induced, such as adsorption of proteins, activation of the complement and coagulation systems, inflammatory reactions, and cell adhesion. For the development of biomaterials for use in biomedical devices that will be exposed to blood, the complete understanding and control of these interactions are essential.

The complement system, a cascade of enzyme reactions involving approximately 30 fluid-phase and cell-membrane-bound proteins, plays an important role in the body's defense systems against pathogens (1). Activation of the complement system is initiated through three pathways: the classical, lectin, and alternative pathways. The classical pathway is initiated by the formation of immune complexes (2, 3), the lectin pathway is triggered by the binding of mannose-binding lectin to carbohydrate chains on foreign microorganisms (4, 5), and the alternative pathway is activated by the nonspecific binding of complement protein C3 to nucleophilic groups on foreign substrates (6, 7).

Complement activation by polymeric materials has been extensively studied in relation to hemodialysis membranes. For example, dialysis membranes made of cellulose or its derivatives activate the complement system (8, 9). Cross-linked dextran (Sephadex) also activates the complement system (10). Other studies indicate that polymeric membranes carrying surface hydroxyl groups, or nucleophilic groups, strongly activate the complement system through the alternative pathway (11−15). In contrast to these observations, Videm and Mollens reported that solutions of dextran, which have a medicinal application as a blood substitute and a plasma expander, did not strongly activate the complement system at the dextran concentrations used in clinical settings (16). These studies were conducted by different research groups under different experimental conditions, making direct comparisons difficult. These seemingly contradictory results led us to question whether the state of the polymer, either immobilized on a surface or free in solution, affects the complement activation.

In the present study, we used dextran and poly(vinyl alcohol) (PVA) as model polymers carrying hydroxyl groups. We examined the deposition of serum proteins and complement fragment C3b onto polymer-immobilized surfaces by using a surface plasmon resonance (SPR) apparatus. Using enzyme-linked immunosorbent assay (ELISA), we also compared the release of complement fragments C3a and SC5b-9 after exposure of serum samples to either a polymer-immobilized surface or a solution of polymer dissolved in a neutral buffer.

MATERIALS AND METHODS

Preparation of the Serum and Buffer. Blood samples, collected from eight healthy volunteer donors who had a meal at least 4 h prior to donation, were pooled and mixed together. To separate serum, aliquots (10 mL) of the blood were divided equally into sterile glass tubes, left on a clean bench at ambient temperature for 30 min for blood coagulation to occur, and then centrifuged at 2500 rpm at 4 °C for 30 min. The serum supernatants were then pooled in a bottle, mixed well, divided equally into vials in an ice bath, and stored at −80 °C until use.

Veronal buffer (VB), composed of 5 mM sodium barbital (Nacalai Tesque, Inc., Kyoto, Japan), 142 mM NaCl, 3.7 mM HCl, 1.5 mM $CaCl_2$, and 10 mM $MgCl_2$ (pH 7.4), was prepared according to the protocol for CH50 measurement (17). For investigations involving the inhibition of complement activation,

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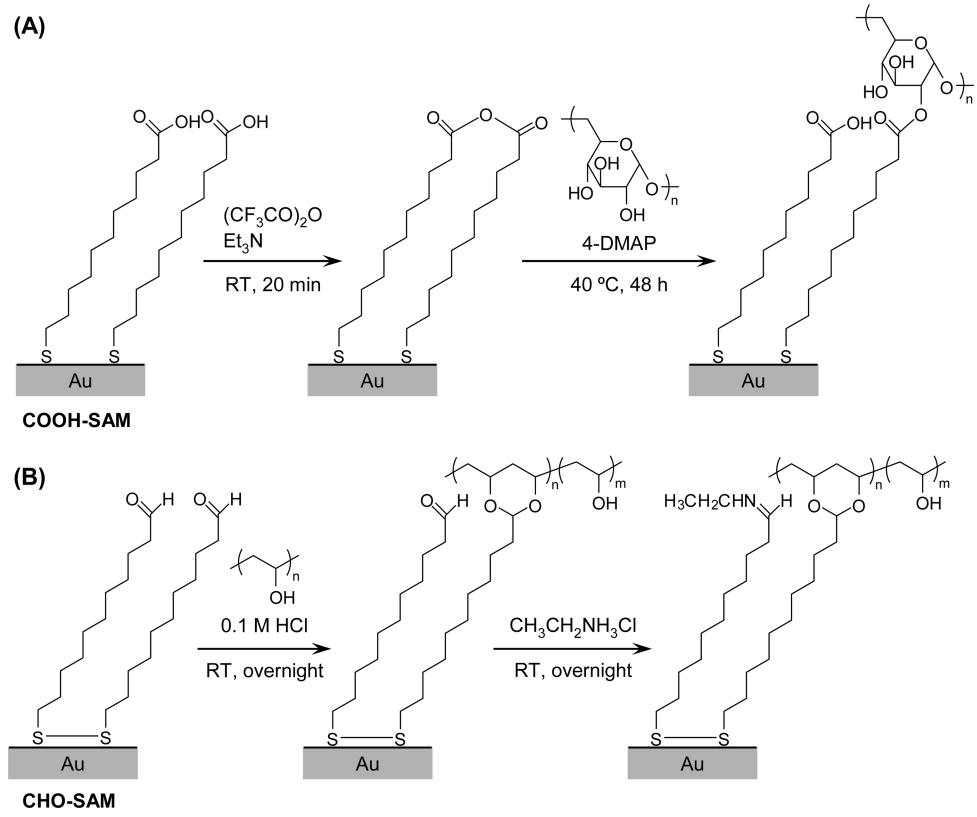
Scheme 1. Immobilization of Dextran (A) and PVA (B) to SAMs on Gold



ethylenediaminetetraacetic acid (EDTA; Dojindo Laboratories, Kumamoto, Japan) was added to VB (without $CaCl_2$ and $MgCl_2$) for a final concentration of 10 mM (EDTA/VB). To block the classical pathway, ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Sigma-Aldrich, St. Louis, MO) and $MgCl_2$ were added to VB (without $CaCl_2$ and $MgCl_2$) at final concentrations of 10 and 2.5 mM, respectively (EGTA-Mg^{2+}).

Preparation of the Dextran-Immobilized Surface. Glass plates (BK7, refractive index = 1.515, size = 25 × 25 × 1 mm, Arteglass Associates Co., Kyoto, Japan) were immersed for 5 min into a piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide) at room temperature, washed three times with deionized water, sequentially rinsed three times with deionized water and 2-propanol, and finally stored in 2-propanol until use. These glass plates were dried under a stream of dried nitrogen gas and then mounted on the rotating stage of a thermal evaporation coating apparatus (V-KS200, Osaka Vacuum, Ltd., Osaka, Japan). A chromium layer was deposited at 1 nm of thickness, and then a gold layer was deposited at 49 nm of thickness onto the glass plates.

The gold-coated glass plate was immersed in a 1 mM solution of 11-mercaptoundecanoic acid (Sigma-Aldrich, St. Louis, MO) in ethanol at room temperature for 24 h to form a self-assembled monolayer (SAM) carrying carboxylic acid functional groups. The plate was washed with ethanol three times and dried in a stream of nitrogen gas. The plate was then immersed in a mixture of 0.1 M trifluoroacetic anhydride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.2 M triethylamine (Nacalai Tesque, Inc., Kyoto, Japan) in anhydrous *N*,*N*-dimethylformamide for 25 min to form interchain anhydrides (18) (Scheme 1A). The plate was washed with dichloromethane, dried in a stream of nitrogen, and then immersed in a 10% dextran (MW = 200 000−300 000, MP Biomedicals, Solon, OH) solution in dimethyl sulfoxide supplemented with 2 mg/mL of 4-(dimethylamino)pyridine (Nacalai Tesque) as a catalyst (19). After incubation at 40 °C for 48 h, the plate was washed with Milli-Q water and stored in Milli-Q water until use.

Preparation of the PVA-Immobilized Surface. The gold-coated glass plate was immersed in a 0.5 mM solution of 11-undecanal disulfide (ProChimia Surfaces Sp. Z O.O., Sopot, Poland) in ethanol at room temperature for 24 h to form aldehyde-terminated SAM (Scheme 1B). The plate was washed with ethanol three times and dried in a stream of nitrogen gas. The plate was then immersed in a 1% PVA (DP = 1700, DS = 98%, Unitika Ltd., Osaka, Japan) aqueous solution containing 0.1 M HCl and incubated at room temperature for 24 h. The plate was washed with Milli-Q water and dried in a stream of nitrogen gas. To block unreacted aldehyde groups, the plate was immersed in a 1 M aqueous solution of ethylamine hydrochloride. The plate was washed with Milli-Q water and stored in Milli-Q water until use.

Surface Characterization of Dextran- and PVA-Immobilized Surfaces. X-ray photoelectron spectroscopy (XPS) was employed to obtain atomic compositions of surfaces using an ESCA-850 V instrument (Shimadzu Co., Kyoto, Japan). Magnesium was used as a target of the electrons generated by the filament with an electric current of 30 mA at 8 kV. The takeoff angle of the sample surface was 90°, and the operating pressure was less than 1×10^{-5} Pa. All spectra were corrected by referring to the peak of Au 4f to 83.8 eV.

IR adsorption spectra of sample surfaces were collected by the reflection−adsorption method (FTIR-RAS) using a Spectrum One spectrometer (Perkin-Elmer Inc., Boston, MA) equipped with a reflector (Harrick Scientific Co., Ossining, NY) and a mercury−cadmium telluride detector cooled by liquid nitrogen. Glass plates with a gold layer of 199 nm thickness were used for FTIR-RAS analysis. Spectra were acquired (128 scans at 4 cm^{-1} resolution from 750 to 4000 cm^{-1}) using the p-polarized IR laser beam at an incident angle of 75° in the chamber purged with dry nitrogen gas.

Static water contact angles on surfaces were determined by the sessile drop method using a contact-angle meter (CA-X, Kyowa Interface Science Co. Ltd., Saitama, Japan) at room temperature. A 10 μL water droplet was placed on a substrate,

and the contact angle was determined three times. This procedure was repeated five times at different places on the same surface.

SPR. The SPR apparatus employed in this study (11) was a homemade apparatus prepared as described by Knoll (20). The BK7 glass plate with the gold layer (49 nm in thickness) was coupled to a hemicylindrical prism with an immersion oil (n = 1.515, Cargille Laboratories, Cedar Grove, NJ). The sample surface was irradiated with a p-polarized He−Ne laser light (λ = 632.8 nm) through the prism. The intensity of the reflected light was monitored as a function of the incident angle. The incident angle, at which the reflectivity reached a minimum, was described as the SPR angle.

The thickness of an immobilized polymer layer in air was also estimated by SPR. The glass plate with the polymer layer was set on the SPR instrument, and the reflectivity was measured in air as a function of the incident angle of the laser light. The thickness of the polymer layer was determined by the SPR angle shift using Fresnel's law for the multilayer system BK7/Cr/Au/SAM/polymer/air (20, 21). The refractive indices for SAM, dextran, and PVA were 1.45, 1.54 (value for cellulose instead of dextran) (22), and 1.52 (22), respectively.

Protein Adsorption from Human Serum. Protein adsorption from undiluted human serum was examined by SPR. A flow chamber with a sample plate was placed on a prism of the SPR apparatus, and VB was circulated at a flow rate of 3.0 mL/min in the flow chamber assembly. The reflectance was monitored during the flow of the liquid samples at an incident angle of 0.5° less than the SPR angle. Undiluted human serum was then introduced into the flow chamber assembly for 90 min. To wash out serum from the sample surface, VB was introduced and circulated for an additional 20 min. All experiments were performed at 37 °C. The introduction of undiluted human serum resulted in a large increase in the reflectance (bulk effect) due to its high refractive index. To correct for changes in the reflectance, standard solutions with different refractive indices (Mill-Q water, VB, and 2 M NaCl) were employed.

To identify proteins deposited on the surface, solutions of specific antibodies were flowed through the apparatus after exposure of the sample surface to human serum. A 1% solution of antiserum diluted in VB was flowed for 90 min onto the surface of the protein layer formed on the surface, and then VB was introduced for 20 min to wash out the serum solution. Antibodies employed were polyclonal rabbit antihuman C3b antiserum (RAHu/C3b; Nordic Immunology, Tilburg, The Netherlands), polyclonal sheep antihuman C1q (PC020; The Binding Site Ltd., Birmingham, U.K.), and polyclonal goat antihuman IgG (109-005-088; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The thickness of the protein layer was calculated from the shift in the SPR angle (ΔSPR) using Fresnel fits for the system BK7/Cr/Au/SAM/protein/water, where the refractive index of SAM and protein was assumed to be 1.45. The amounts of proteins adsorbed onto the surfaces were estimated from the thickness, presuming that the density of the protein layer was 1 as follows (11):

$$\text{amount of adsorbed protein (ng/cm}^2) = 500\Delta\text{SPR (deg)} \quad (1)$$

For studies requiring conditions that block complement activation, serum was supplemented with EDTA at a final concentration of 10 mM. To examine the effects of inhibition of the classical and lectin pathways, serum was supplemented with EGTA and $MgCl_2$ (final concentrations of 10 and 2.5 mM, respectively).

Quantification of Generated Complement Fragments. To prepare a cell, a silicone spacer (inner diameter = 20 mm; thickness = 1 mm) was placed between the two test surfaces. Serum (approximately 340 μL) was injected into the circular space in the cell and incubated for 90 min at 37 °C. Serum was then collected, and EDTA was added at a final concentration of 10 mM to halt the complement activation. The concentration of the complement fragments (C3a and SC5b-9) in the fluid phase was determined by commercially available ELISA kits (C3a, OptEIA Human C3a ELISA, BD Biosciences Pharmingen, San Diego, CA; SC5b-9, SC5b-9 plus ELISA, Quidel Corp., San Diego, CA).

Quantification of Generated Complement Fragments in a Polymer Solution. Aliquots of dextran or PVA solutions in VB were added to human serum. The final concentration of dextran or PVA in the assay was varied (dextran, 0.002−1%; PVA, 0.001−0.1%), while the final concentration of serum was held constant at 95%. Serum samples supplemented with EDTA and zymosan (10 mg/mL) were used as negative and positive controls, respectively. The procedure for quantification of complement fragments was the same as that described above.

Statistical Analysis. Significant differences between two groups were examined using the Student's t test. A $p < 0.05$ was considered as statistically significant. All statistical calculations were performed by the software *JMP*, version 7.0.1 (SAS Institute, Cary, NC).

RESULTS

Surface Characterization. Using FTIR-RAS, XPS, and water contact-angle measurements, we characterized the dextran and PVA immobilized onto SAMs on gold (Scheme 1).

Dextran was immobilized through ester bonds onto a COOH-SAM (Scheme 1A). The FTIR-RAS spectrum for COOH-SAM showed asymmetric and symmetric C−H stretching bands of methylene groups at 2922 and 2854 cm^{-1} and two C═O stretching bands at 1739 and 1718 cm^{-1}, which were caused by the free and hydrogen-bonded carboxylic acids, respectively (18) (Figure 1A). After the treatment of COOH-SAM with trifluoroacetic anhydride, two C═O stretching bands appeared at 1819 and 1733 cm^{-1}, which were assigned to in-phase and out-of-phase stretching bands of the two coupled carbonyl groups, respectively (18). The spectrum of the dextran-immobilized surface showed new bands at 3100−3600 cm^{-1}, assignable to an O−H stretching band, and at 1148 and 1051 cm^{-1}, assignable to C−O−C stretching (23).

PVA was immobilized onto a SAM functionalized with aldehydes through acetal bonds (Scheme 1B). The FTIR-RAS spectrum of CHO-SAM showed two C−H stretching bands at 2928 and 2852 cm^{-1}, which were assigned to the asymmetric and asymmetric C−H stretching of methylene groups, respectively (Figure 1B). The band observed at 1729 cm^{-1} was assigned to the C═O stretching band of the carbonyl group (24, 25). The spectrum of the PVA-immobilized surface shows new bands at 3100−3600 cm^{-1}, assigned as O−H stretching bands, at 1145 and 1098 cm^{-1} assigned respectively as the C−O stretching in the crystalline region and the C−O stretching in the amorphous region, and at 845 cm^{-1}, corresponding to CH_2 rocking (26). For the PVA-immobilized surface after blocking with ethylamine hydrochloride, the intensity of the C═O stretching band at 1729 cm^{-1} decreased (Figure 1B,c).

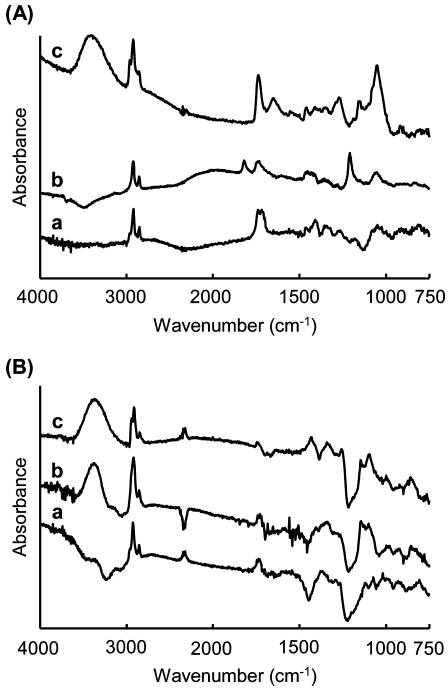


FIGURE 1. FTIR-RAS spectra of modified gold surfaces: (A-a) COOH-SAM; (A-b) interchain anhydride-SAM; (A-c) dextran-immobilized surface; (B-a) CHO-SAM; (B-b) PVA-immobilized surface; (B-c) PVA-immobilized surface followed by blocking with ethylamine hydrochloride.

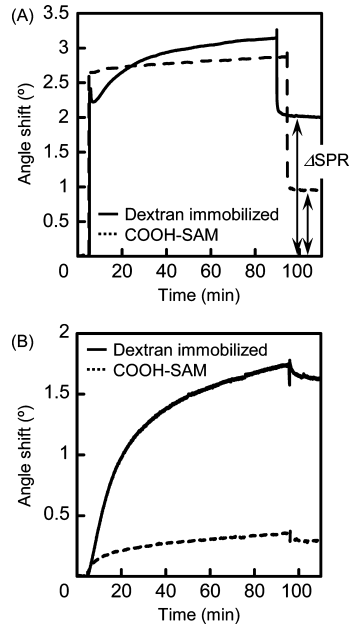


FIGURE 2. SPR sensorgrams during exposure of COOH-SAM and dextran-immobilized surfaces to normal human serum (A) and subsequent exposure to anti-C3b antiserum (B). ΔSPR indicates an increase in the SPR angle after exposure to serum and subsequent rinsing with a buffer and corresponds to the amount of adsorbed serum proteins according to eq 1.

Dextran- and PVA-immobilized surfaces were also characterized by XPS, water contact-angle, and SPR measurements (Table 1). The oxygen concentration increased upon immobilization of either dextran or PVA. For CHO-SAM and PVA-immobilized surfaces, N 1s signals were observed when the free aldehyde groups were blocked by ethylamine through Schiff base formation. The water contact angle decreased after immobilization of either dextran or PVA. The relatively high water contact angles for CHO-SAM and PVA-immobilized surfaces may be attributed to the introduction of hydrophobic ethylene chains after reaction with ethylamine. The thicknesses of the dextran and PVA layers in the dry state determined by SPR measurements were 2.4 and 1.7 nm, respectively.

Taken together, these results indicated that dextran and PVA layers were successfully immobilized on SAMs as depicted in Scheme 1.

Complement Activation on Dextran- or PVA-Immobilized Surfaces Observed by SPR. Complement activation on dextran- or PVA-immobilized surfaces was examined using an SPR apparatus (Figures 2 and 3). When COOH-SAM or dextran-immobilized surfaces were sequentially exposed to VB and undiluted human serum, rapid increases of the SPR angle were observed. These increments are due to the large changes in the refractive indices when going from VB to human serum. The SPR angle then slowly increased for COOH-SAM. For the case of the dextran-immobilized surface, the angle gradually increased an additional 0.9° after several minutes of induction time. When serum was removed by flushing VB through the system, the SPR angle rapidly decreased because of the change in the refractive index of the solution. The SPR angle shift (ΔSPR) after the introduction of VB into the chamber (Figure 2A) is attributed to protein adsorption (see eq 1) and indicated that a larger amount of serum proteins had adsorbed onto the dextran-immobilized surface as compared to COOH-SAM in undiluted serum. After the surfaces were washed with VB, an antihuman C3b antiserum solution was applied to the surfaces carrying adsorbed serum proteins to evaluate the amount of C3b or C3bBb that was produced by complement activation (Figure 2B). The SPR angle shifts caused by binding of the anti-C3b antibody on COOH-SAM and dextran-immobilized surfaces were 0.29° and 1.63°, respectively. The large amount of the anti-C3b antibody

Table 1. Characteristics of Polymer-Immobilized Surfaces[a]

		atomic composition (%)				
	contact angle (deg)	C	O	N	S	thickness (nm)
COOH-SAM	16.8 ± 4.1	82.2 ± 1.0	13.5 ± 0.9	0	4.3 ± 0.4	
dextran-immobilized	5.3 ± 0.1	65.6 ± 1.5	32.4 ± 1.5	0	2.0 ± 0.1	2.4 ± 0.1
CHO-SAM	70.5 ± 1.1	77.6 ± 2.7	9.9 ± 1.7	4.0 ± 1.7	8.5 ± 1.4	
PVA-immobilized	36.8 ± 1.4	71.2 ± 1.0	23.9 ± 1.8	2.1 ± 0.8	2.8 ± 0.9	1.7 ± 0.0

[a] Data shown are means ± SD ($n = 3$).

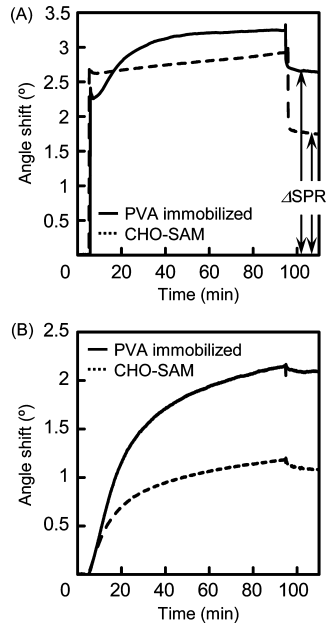


FIGURE 3. SPR sensorgrams during exposure of CHO-SAM after blocking with ethylamine hydrochloride and PVA-immobilized surfaces to normal human serum (A) and subsequent exposure to anti-C3b antiserum (B). ΔSPR indicates an increase in the SPR angle after exposure to serum and subsequent rinsing with a buffer and corresponds to the amount of adsorbed serum proteins according to eq 1.

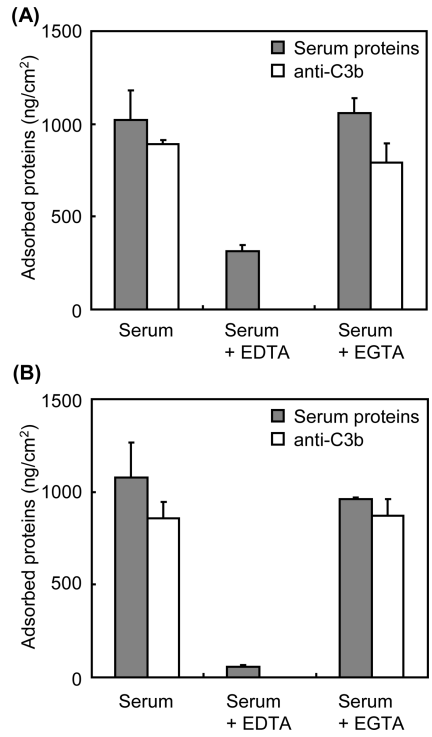


FIGURE 4. Amounts of serum proteins adsorbed on dextran- (A) or PVA-immobilized (B) surfaces and subsequent anti-C3b antibodies bound to those surfaces with a serum protein layer. Data shown are means ± SD ($n = 3$). Serum, normal human serum (NHS); serum + EDTA, NHS + 10 mM EDTA; and serum + EGTA, NHS + 10 mM EGTA + 2.5 mM $MgCl_2$.

bound on the dextran-immobilized surface suggested that a major component in the layer of serum protein on the surface was C3b or C3bBb.

Complement activation on the PVA-immobilized surface and its basal substrate (CHO-SAM) was also examined by SPR (Figure 3). Aldehyde groups on both surfaces were blocked with ethylamine. For ethylamine-blocked CHO-SAM, the SPR angle rapidly increased just after exposure to human serum and then was followed by a slow increase over 90 min of incubation (Figure 3A). The initial increase of the SPR angle was due to the changes of the refractive indices from VB to serum (Figure 3A). For the case of the PVA-immobilized surface, the SPR angle greatly increased to 3.2° after several minutes of induction time. The amount of adsorbed proteins remaining on the PVA-immobilized surface after washing with VB exceeded that for CHO-SAM. The SPR angle shifts caused by binding of the anti-C3b antibody on the surfaces carrying adsorbed serum proteins were 1.09° and 2.09°, respectively (Figure 3B), and suggested that a large amount of complement component, C3b or C3bBb, was immobilized onto the PVA-immobilized surface.

EDTA is known to inhibit all three pathways of complement activation, while EGTA-Mg^{2+} can inhibit only the classical and lectin pathways. To distinguish which of the complement pathways are activated on dextran- and PVA-immobilized surfaces, protein adsorption and subsequent binding of the anti-C3b antibody were examined in serum supplemented with EDTA and with EGTA-Mg^{2+} (Figure 4). When serum supplemented with 10 mM EDTA was employed, the amounts of adsorbed proteins greatly decreased on all of the dextran- and PVA-immobilized surfaces. In particular, protein adsorption on the PVA-immobilized surface was less than ~60 ng/cm^2, indicating that the PVA surface was practically resistant to protein adsorption when the complement activation was inhibited. Binding of the anti-C3b antibody was not observed on either surface in the presence of EDTA. Thus, the deposition of C3b or C3bBb on dextran- and PVA-immobilized surfaces shown in Figures 2 and 3 was due to complement activation by any of the three pathways. However, in the presence of 10 mM EGTA and 2 mM Mg^{2+} in serum, the large amounts of adsorbed protein and bound anti-C3b antibody observed on dextran- and PVA-immobilized surfaces did not differ significantly from the serum controls in the absence of chelating agents. Taken together, these results suggest that exposure of serum to dextran- and PVA-immobilized surfaces activated the complement system through the alternative pathway.

Generation of Complement Fragments. We compared the releases of complement fragments C3a and SC5b-9 by the ELISA method after serum samples were exposed to surfaces carrying either immobilized dextran or PVA or when mixed with solutions of dextran or PVA.

The amount of SC5b-9 was markedly increased when serum was exposed to surfaces carrying either dextran or PVA (Figure 5). Thus, this strong activation of the complement system by dextran- or PVA-immobilized surfaces was consistent with the results of the SPR measurements summarized in Figure 4. The amounts of dextran and PVA on the surfaces, which were estimated from their thicknesses (Table 1), were 1.5 and 1.1 μg, respectively, presuming that the densities of the polymer layers were 1. In the experi-

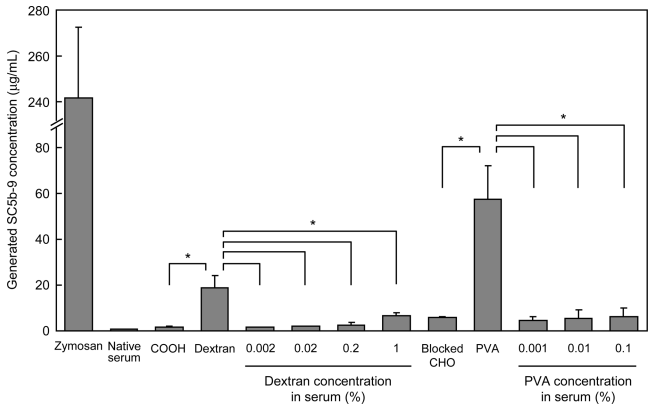


FIGURE 5. Concentrations of SC5b-9 generated in serum exposed to four different kinds of surfaces and serum mixed with soluble dextran or PVA. Data shown are means ± SD ($n = 3$). An asterisk denotes a significant difference ($p < 0.05$).

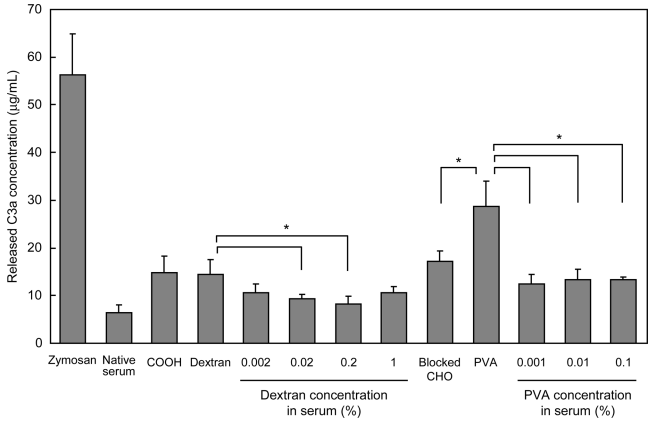


FIGURE 6. Concentrations of C3a generated in serum exposed to four different kinds of surfaces and serum mixed with soluble dextran or PVA. Data shown are means ± SD ($n = 3$). An asterisk denotes a significant difference ($p < 0.05$).

mental setting, 1.5 μg of dextran or 1.1 μg of PVA were exposed to 340 μL of serum. When the effects of soluble dextran and PVA on complement activation were examined, the polymer concentrations in serum ranged from 0.02 to 10 mg/mL for dextran and from 0.01 to 1 mg/mL for PVA. Even though the amount of generated SC5b-9 increased as the concentration of added polymer increased in the serum samples, these values were much lower than those observed for immobilized surfaces. Even in the presence of 2200 times more soluble dextran and 300 times more soluble PVA in the serum, the amount of released SC5b-9 did not achieve the levels observed with the immobilized polymer surfaces. Therefore, dextran and PVA dissolved in serum cannot effectively activate the complement system.

The amounts of C3a released were also determined (Figure 6). The effects of the polymer state on C3a release were not so clear but followed the same general tendency as those observed in SC5b-9 release. When dextran or PVA solutions were added to serum, the levels of generated C3a were lower than those for dextran- and PVA-immobilized surfaces.

DISCUSSION

Dextran and PVA are water-soluble, nonionic polymers carrying hydroxyl groups. Because these polymers do not strongly interact with proteins (27−30), surface modification with these polymers should provide nonfouling surfaces and may be applied to medical devices and biosensors. However, the protein adsorption behavior from serum differs from that of a solution of a single protein. We previously examined protein adsorption on SAMs of alkanethiols carrying hydroxyl groups (11, 12) and hydroxyl-terminated oligo(ethylene glycol) (15). Although those surfaces showed low protein adsorption from solutions containing a single protein, large amounts of proteins adsorbed on those surfaces and the complement system were strongly activated when the surfaces were exposed to normal human serum. Such diverse behaviors are expected to occur on dextran- and PVA-immobilized surfaces.

Cross-linked dextran (Sephadex) (10), dextran-grafted nanoparticles (31−33), and PVA-coated polyethylene tubes (34) strongly activate the complement system, but soluble dextrans do not, even at the concentrations found in plasma substitutes (16). The present study was designed to investigate this discrepancy.

Our SPR measurements clearly demonstrated that large amounts of serum proteins were deposited on dextran- and PVA-immobilized surfaces (Figures 2 and 3). Binding of the antibody against C3b identified the deposited proteins as C3b or C3bBb. Both nonspecific protein adsorption and C3b deposition on these surfaces were greatly reduced in the presence of 10 mM EDTA, which blocks all three complement system activation pathways (Figure 4). These observations indicated that the complement system was strongly activated by these surfaces.

The basal surfaces used for polymer immobilization (COOH-SAM for dextran and CHO-SAM for PVA) also activated the complement system, but to a lesser extent (Figures 2 and 3). Complement activation on material surfaces is closely related to nonspecific adsorption of serum proteins (35). On COOH- and CHO-SAMs, the amounts of bound anti-C3b were small compared with those of adsorbed serum proteins, suggesting that serum proteins other than C3b adsorbed on the surfaces. When dextran or PVA was immobilized, nonspecific protein adsorption was greatly reduced by the shielding effect of the polymers. The contribution of the basal surfaces to the complement activation is minimal, and our data indicate that the complement system was mainly activated by dextran or PVA on the surface of the substrate.

Three pathways can activate the complement system: the classical, the lectin, and the alternative pathways. Complement activation via the classical pathway is triggered by nonspecifically adsorbed proteins such as immunoglobulins when artificial materials are brought into contact with serum. Adsorption of immunoglobulins on a material surface leads to rapid complement activation through the classical pathway (36). In our experiment, binding of anti-IgG was not observed on dextran- and PVA-immobilized surfaces after a short incubation (5 min) of human serum, indicating that IgG did not adsorb onto these surfaces (data not shown). The lectin pathway is initiated by binding of the complex of

mannose-binding lectin and the mannose-binding lectin-associated serine proteases to exposed mannose groups on the cell surface (4, 5). Specific antibodies against dextran in normal serum initiated the complement activation (37). Both the classical and lectin pathways can be inhibited by the addition of EGTA-Mg^{2+} in serum. For dextran- and PVA-immobilized surfaces, blocking by the addition of EGTA-Mg^{2+} resulted in only small decreases in the amounts of adsorbed proteins and bound anti-C3b compared to control serum samples lacking EGTA-Mg^{2+} (Figure 4). These results suggest that complement activation on dextran- and PVA-immobilized surfaces is mainly mediated by the alternative pathway and is initiated by immobilization of C3b onto hydroxyl groups of dextran and PVA.

ELISA measurement showed that complement fragment SC5b-9 generated in serum markedly increases when dextran- and PVA-immobilized surfaces contact to serum (Figure 5). In contrast, the C3a concentration exhibited a small increase for the dextran-immobilized surface compared with a dextran solution (Figure 6). It was reported that generated C3a adsorbs on the material surface (38). Because immobilized dextran does not fully inhibit nonspecific adsorption of serum proteins (Figure 4A, serum + EDTA), adsorption of C3a on the dextran-immobilized surface is considered to occur, which results in a decrease in the apparent concentration of C3a released in serum. Nevertheless, C3a release exhibited the same tendency as SC5b-9 release.

Dextran- and PVA-immobilized surfaces strongly activated the complement system, but soluble polymers in serum could not. Thus, the state of the polymer greatly modulates the complement activation behavior. For dextran-coated nanoparticles, it was reported that complement activation depends on dextran conformation (31, 33). However, the cause for the different behaviors in polymers immobilized on substrates and soluble polymers is not clear yet. At this point, we speculate following two mechanisms which induces the differences. Complement activation through the alternative pathway is initiated in the fluid phase with spontaneous and continuous generation of C3b (7). C3b alters its conformation, resulting in exposure of a highly reactive thioester group on the surface of a C3b molecule (39−41). The thioester group becomes accessible for nucleophilic attack but is readily hydrolyzed by the surrounding water (half-life = ∼60 μs) (42). When the short-lived C3b is formed near surface carrying nucleophilic groups, it easily reacts with nucleophilic groups and is immobilized on the surface followed by the formation of a complex with factor B to give C3 convertase, or C3bBb. C3bBb effectively converts C3 to C3b near the surface; the C3b molecules near the surface shortly immobilized on the surface and stabilized. Thus, the surface-bound C3b amplifies the complement activation. On the other hand, when a polymer solution is added to serum, spontaneously formed C3b is also stabilized by reacting with a nucleophilic group of the polymer in the serum and then C3bBb is formed. C3bBb effectively converts C3 to C3b, but C3b hardly finds a nucleophilic group and thus reacts with it much more slowly than that formed near the surface carrying nucleophilic groups, because a local concentration of nucleophilic groups near the formed C3b is much lower in the serum than on the surface.

Binding of pro-enzyme factor B to surface-bound C3b and subsequent cleavage of factor B by factor D yield the C3bBb complex (C3 convertase), which converts C3 into C3a and C3b. In addition, it results in the formation of $C3b_2Bb$ (C5 convertase), which cleaves C5 to ultimately lead to the terminal complement complex C5b-9. While surface-bound C3b amplifies the complement activation, factors H and I inactivate C3b. These amplification/inactivation reactions are considered to be determined by the accessibility of each factor to susceptible sites of surface-bound C3b. The crystal structure of C3bBb (43) and the three-dimensional structure of C3bBb determined by electron microscopy (44) revealed that factor B binds to C3b apart from the thioester domain, which contains a thioester bond for covalent binding to surfaces. The crystal structure of the complex of C3b and the factor H fragment (∼31 kDa) also revealed that the factor H fragment binds a large portion of C3b including the thioester domain (45). For C3b bound to dextran and PVA on surfaces, activator (factor B) is expected to be able to interact with their binding sites, but full length factor H (155 kDa) and I (88 kDa) hardly access their binding site in C3b because of steric hindrance as a result of the binding sites of factors H and I being surrounded by immobilized polymers and a planar substrate. On the other hand, for C3b bound to a dextran and PVA soluble chain in serum, regulators (factors H and I) may have access to their binding site in C3b because C3b carries a relatively small soluble dextran or PVA chain.

To elucidate these speculations, we have undertaken an investigation of the effect of the water content of the PVA and dextran layers on the complement activation and we intend to examine the effect of polymer sizes and geometries, that is, soluble polymer chains, nanogels, and flat layers of dextran and PVA, on the complement activation. These results will be reported in due course.

CONCLUSION

We clearly showed that dextran- and PVA-immobilized surfaces strongly activated the complement system, but soluble dextran or PVA in serum could not, even when the amounts of the soluble polymers added to serum were 4−2000 times greater than those on the polymer-immobilized surfaces. These results suggest that the physical states of the polymers, that is, immobilized on the surface or soluble in serum, greatly modulate complement activation.

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