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Study on the prevention of surface-induced platelet activation by albumin coating

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Abstract—To understand how albumin on the surface inhibits surface-induced platelet activation, we adsorbed albumin on dimethyldichlorosilane-coated glass (DDS-glass) and modified the adsorbed albumin by three different methods. The adsorbed albumin was crosslinked with glutaraldehyde, dried and rehydrated, or digested with trypsin. Surface albumin concentration did not change by crosslinking; however, it decreased by about 15% by a simple dry-and-rehydration process. Trypsin digestion reduced the surface albumin concentration by 50%. Platelets were found to adhere and activate on albumin coated DDS-glass, if the adsorbed albumin was modified. The extent of platelet activation was quantified with two numeric parameters, the spread area and circularity. Fibrinogen adsorption to the dried or digested albumin layer resulted in enhancement of platelet activation, while adsorption of more albumin inhibited platelet activation. The results suggest that albumin can inhibit platelet activation as long as it covers the surface completely and remains flexible on the surface. This study indicates that steric repulsion is one of the mechanisms of surface passivation by albumin.

Key words: surface passivation; protein adsorption; albumin; platelet activation; steric repulsion.

INTRODUCTION

Surface-induced thrombosis remains as one of the major problems in the development of blood-contacting biomaterials [1]. When a foreign surface comes in contact with blood, the initial blood response is adsorption of blood proteins [2, 3]. The adsorption of proteins is followed by platelet adhesion and activation [4]. Surface-induced platelet activation is largely dictated by the type and amount of blood proteins adsorbed at the biomaterial/blood interface.

Adsorption of fibrinogen and other platelet-adhesive proteins is known to accelerate platelet adhesion and activation [5]. Fibrinogen on the surface is thought to activate platelets by interacting with membrane receptors [6]. On the other hand, albumin-adsorbed surfaces are found to minimize surface-induced platelet activation. Both the total number of adherent platelets and the extent of platelet activation are reduced on the albumin-adsorbed surfaces [7, 8]. Lack of albumin receptors on platelet membrane has been attributed to the minimized surface-induced platelet activation by albumin coating (9).

Due to the pronounced albumin effects in preventing platelet adhesion and activation, several approaches for albumin immobilization have been considered. Physical adsorption of albumin on biomaterial surface prior to implantation is the most common approach [10, 11]. Other methods of albumin immobilization include crosslinking with glutaraldehyde [12], crosslinking by gamma-irradiation [13], and photoactivable albumin [14].

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Recently, attention has been directed towards fabrication of biomaterials with enhanced albumin affinity. Eberhart *et al.* [15–17] pioneered an innovative approach for selective adsorption of albumin from blood. They suggested that grafting long carbon chains (C-16 and C-18) on polymer surfaces would provide a regenerating layer of endogenous albumin. Similar materials were later examined using the canine *ex vivo* shunt model [18, 19]. The results showed improved short-term biocompatibility with alkylated surfaces. Although the surface modification increased albumin retention, it still could not achieve total specificity towards albumin. This is important to the long-term usage of biomaterials. Thrombus was present even on alkylated surfaces after long-term continuous exposure to blood [16].

Since the mechanisms governing protein adsorption from a complex protein mixture such as blood are not understood, designing surfaces with specific protein adsorption properties is not yet feasible. Thus, understanding how albumin on the surface reduces adsorption of other proteins and adhesion of platelets is important in the design of biomaterials which would mimic albumin-coated surfaces.

Previously, we have shown that platelets adhere and activate on glass surfaces even in the presence of albumin at a bulk concentration as high as 50 mg/ml [20]. This is mainly due to low albumin affinity to glass surfaces. Platelets can easily displace adsorbed albumin from the glass and interact directly with the glass. Albumin, however, has high affinity to hydrophobic surfaces [21]. For example platelets cannot displace albumin molecules which are adsorbed on hydrophobic surfaces such as dimethyldichlorosilane-treated glass (DDS-glass) [22].

We have examined platelet activation on DDS-glass where albumin was adsorbed and treated by three different modification methods. We have used DDS-glass as a model thrombogenic surface, since our previous study showed that albumin has high affinity to DDS-glass and albumin effects can be readily seen on the surface [22]. Adsorbed albumin was crosslinked with glutaraldehyde, dried and rehydrated, or digested with trypsin. Surface-induced platelet activation on those surfaces was quantitated using two numerical parameters, spread area and circularity.

MATERIALS AND METHODS

Preparation of DDS-glass

Glass coverslips (25 × 75 mm, # 1, Bellco, Vineland, NJ) were cleaned with 2% (v/v) Isoclean (Isolab, Akron, OH) solution for 3 h at 60°C. They were then rinsed with distilled water at 60°C and dried. Dimethyldichlorosilane (DDS, Sigma, St Louis, MO)-treated glass (DDS-glass) was prepared by treating clean glass with 5% (v/v) DDS in chloroform for 2 h at room temperature. DDS-glass was washed in chloroform and ethanol in sequence four times and then rinsed with copious amount of deionized distilled water and dried overnight at 60°C.

For measuring the surface protein concentrations using radiolabelled proteins, glass tubings (i.d. 2.5 mm, Kimble, Vineland, NJ) were used instead of coverslips.

Preparation of proteins

Human serum albumin (Fraction V, Sigma) was used as received. Human fibrinogen (Type I, Sigma) was purified by ammonium sulfate precipitation method of Laki (23). Purified fibrinogen was stored in aliquots at −70°C. The concentrations

of both proteins in phosphate buffered saline (PBS, pH 7.4) was monitored by UV absorbance at 280 nm using absorptivities of 5.8×10^2 cm^2/g and 1.506×10^3 cm^2/g for solutions of albumin and fibrinogen, respectively [24, 25].

Protein adsorption

In order to quantify the surface concentrations of albumin and fibrinogen on DDS-glass tubings, the proteins were labeled with ^{125}I (Amersham, Arlington Heights, IL) using Enzymobeads (Bio-Rad, Rockville Center, NY). Labeled albumin or fibrinogen was adsorbed for 1 h at room temperature. Unadsorbed protein was rinsed with PBS and the surface protein concentration was determined by counting the radioactivity using a gamma counter (Gamma 5500B, Beckman, Arlington Heights, IL).

Modifications of adsorbed albumin

The surface albumin was crosslinked with 2% glutaraldehyde solution (E.M. Grade, Polysciences, Warrington, PA) in PBS. After 1 h, excess glutaraldehyde was rinsed and the fixed albumin layer was treated with 0.1 M glycine for 1 h at room temperature. In another study, the surface albumin was dried for 18 h at 60°C. The dried surface was rehydrated with PBS 1 h before addition of platelets. The surface albumin concentrations before and after modification were determined using ^{125}I-labeled albumin.

The adsorbed albumin was also treated with 0.1 mg/ml trypsin (specific activity 10 200 units/mg, Type III, Sigma). Adsorbed albumin was digested with trypsin for 1 h at room temperature. After adsorbed albumin was modified by crosslinking, drying and rehydration, or trypsin digestion, more albumin was adsorbed and the changes in the surface albumin concentration and platelet activation were examined. In some cases, fibrinogen was adsorbed instead of albumin.

Preparation of platelet suspension

Blood was obtained from healthy adult human volunteers and collected in Vacutainers (Becton-Dickinson, Rutherford, NJ) containing sodium citrate buffer. All volunteers were kept free of aspirin and other drugs that can interfere with platelet functions. Platelets were separated from blood proteins using a Sepharose CL-2B column which was pre-equilibrated with PBS without divalent cations [26].

Platelet adhesion and activation

For surface-induced platelet activation studies, an observation chamber was designed as described before [22]. The chamber was assembled using DDS-glass coverslip, spacers, and a microscope slide (25 × 75 mm, Fisher, Chicago, IL). For modification of adsorbed albumin, the bulk albumin concentrations of 1 and 5 mg/ml were used for adsorption. Platelet suspension was added to the albumin-adsorbed surfaces and allowed to spread for 1 h at room temperature. Platelets were then fixed with 2% glutaraldehyde in PBS and stained with 0.1% Coomassie Brilliant Blue (Bio-Rad).

Surface adherent platelets were observed with a video microscope (Diaphot, Nikon, Garden City, NY). The image was transferred to a computer image analysis

system (Imaging Technology, Woburn, MA) through a video processor (Colorado Video, Bouldor, CO). The platelet images were digitized and analyzed with image analysis software obtained from Computer Imaging Applications (Madison, WI). Light micrographs of adherent platelets were taken from the video-monitor screen using a Polaroid instant camera (DS-34, Cambridge, MA).

The extent of surface-induced platelet activation was quantified using two numerical parameters, spread area and circularity of platelets. Circularity (C) is defined by the following equation:

$$C = P^2/4\pi A$$

where P and A are the perimeter and spread area of platelets, respectively [22]. The number of platelets examined ranged from 30 to 100 depending on the type of surface. The data represents average ±SEM for at least four independent experiments.

Scanning electron microscopy

Platelets in suspension were allowed to adhere and activate on DDS-glass and albumin-adsorbed DDS-glass for 1 h at room temperature. Adherent platelets were fixed with 2% glutaraldehyde in PBS and dehydrated in serial dilution of ethanol (25–100%). Dehydrated platelet samples were dried with a critical point drier (CPD 020, Balzers, Arlington Heights, IL) using CO_2 as a transitional fluid. Surfaces were then sputter-coated with gold-palladium and examined with JSM-840 scanning electron microscope (JEOL, Peabody, MA) using 10 kV of accelerating voltage.

RESULTS

Quantitation of platelet activation

As platelets activate on the surface, they change their shape from discoid to circular form. Thus, as platelets activate the spread area increases. For fully activated platelets, the spread area is usually greater than 40 μm^2. The circularity, however, can increase or decrease depending on the shape of the platelet. The circularity is largely dependent on the number and length of pseudopods present on the partially activated platelets. Circularity has a minimum value of 1.0 for perfectly circular platelet and the value becomes larger as the platelet shape becomes more complex. If platelets are fully spread, all the pseudopods are retracted and the circularity usually becomes less than 2.0 [22].

Figure 1 shows scanning electron micrographs of platelets spread on DDS-glass (A) and contact adherent platelets on albumin-adsorbed DDS-glass (B). On control DDS-glass, platelets adhered and activated completely. Platelets had spread to a circular shape and the granules were released from the center of the spread platelets (Fig. 1-A). The average spread area of platelets on control DDS-glass was about 40.3 μm^2 and the circularity value was 1.78. Platelets on albumin-adsorbed surface, however, had a small spread area of 19.6 μm^2 and high circularity of 3.75 which reflects the presence of several pseudopods. As shown in Fig. 1-B, only a small number of platelets were contact adherent with extended pseudopods.

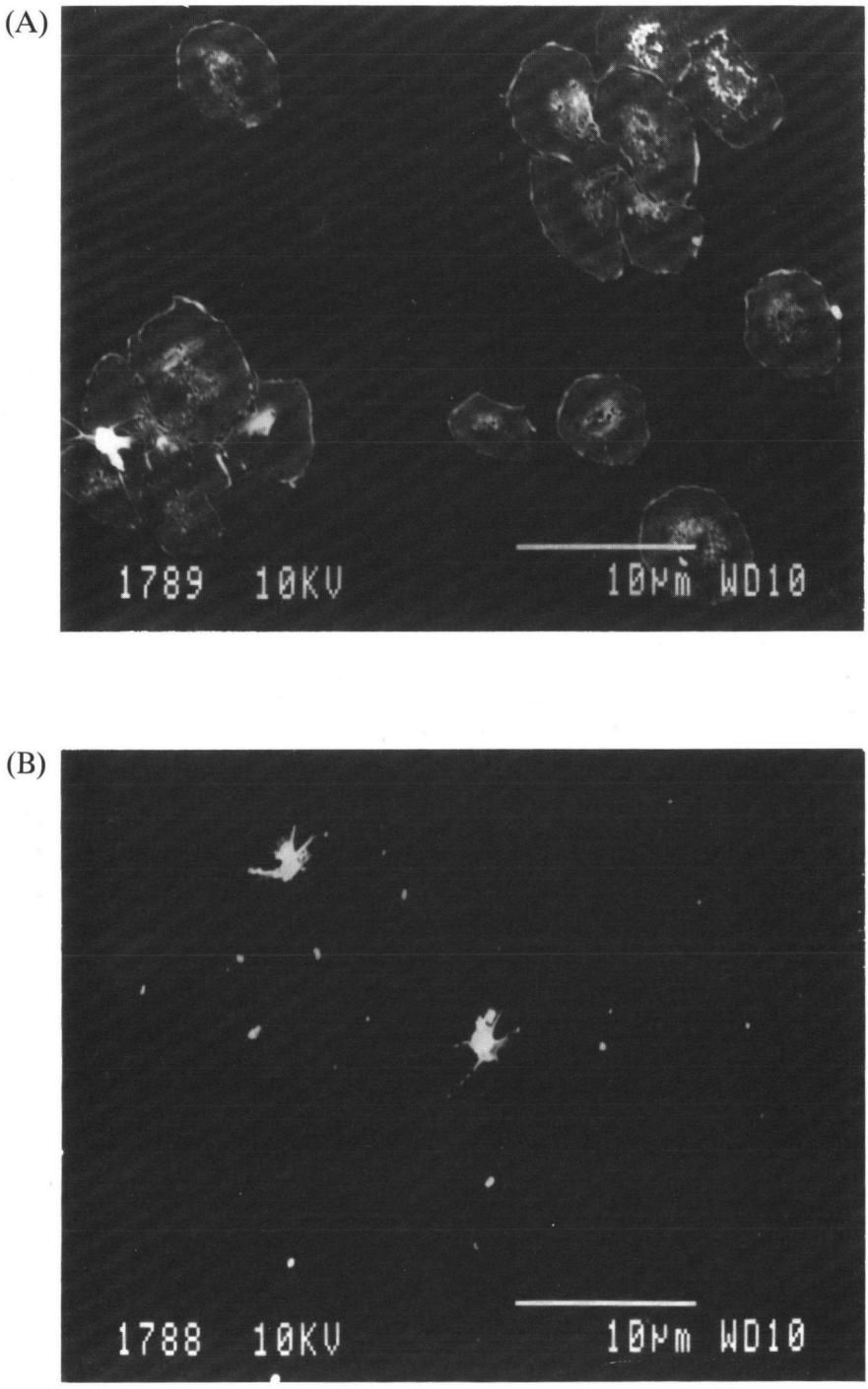

Figure 1. Scanning electron micrographs of platelets activated on DDS-glass (A) and contact adherent platelets on albumin-adsorbed DDS-glass (B). Albumin at a bulk concentration of 1 mg/ml was adsorbed for 1 h at room temperature.

Effects of glutaraldehyde crosslinking of adsorbed albumin on platelet activation

Table 1 shows the data on platelet activation on albumin adsorbed DDS-glass. The surface albumin concentrations were 0.29 and 0.31 $\mu g/cm^2$ when the albumin concentration in the adsorption solutions were 1 mg/ml and 5 mg/ml, respectively. The surface albumin concentrations did not change by crosslinking with glutaraldehyde. Platelets, however, adhered and activated on albumin-adsorbed surfaces after crosslinking. Spread area of platelets increased from 19.6 to 36.3 and 11.8 to 36.6 μm^2 for 1 and 5 mg/ml albumin, respectively. The circularity values also decreased from 3.75 to 2.16 and 3.43 to 2.03, respectively. Apparently, platelets could adhere and activate on the albumin-coated surface, if the albumin was crosslinked with glutaraldehyde.

Addition of fibrinogen to the crosslinked albumin did not significantly change the area and circularity values. Fibrinogen is adsorbed on crosslinked albumin layer at the concentration of 0.02 $\mu g/cm^2$. The effect of fibrinogen on platelet activation was not quite pronounced. This may be due to the low surface fibrinogen concentration. If fibrinogen had effectively adsorbed to the crosslinked albumin surface, the platelet spread area would be higher than 40 μm^2 and the circularity much less than 2.0 [22].

Addition of unlabeled albumin did not remove the crosslinked albumin layer from the surface as expected (*e* in Table 1). The platelet activation profile also remains similar to the one on the crosslinked albuminum layer (compare *c* and *e* in Table 1). The spread area remained at 26.7 and 34.5 μm^2 for 1 and 5 mg/ml albumin, respectively. The circularity values were also similar to the ones obtained on crosslinked albumin layer. The surface albumin concentration remained at 0.28 $\mu g/cm^2$ even after addition of more ^{125}I-labeled albumin to the crosslinked albumin layer.

Effects of drying and rehydration of adsorbed albumin on platelet activation

The surface albumin concentration decreased by about 15% after drying and rehydration as shown in Table 2. This decrease was due to the loss of albumin from the surface rather than the loss of free ^{125}I. Then radioactivity in the rehydrating solution disappeared when trichloroacetic acid was added to precipitate albumin from solution. Platelets activated completely on albumin-adsorbed surface after drying and rehydration. The spread area of platelets increased to 36.3 and 39.1 μm^2 for bulk albumin concentration of 1 and 5 mg/ml, respectively. The circularity values decreased to 1.78 and 1.81, respectively. This clearly indicates that platelets were fully activated on albumin-adsorbed surfaces after drying (compare *a* and *e* in Table 2).

Addition of fibrinogen to the dried albumin layer resulted in adsorption of fibrinogen. The surface fibrinogen concentrations were 0.04 and 0.05 $\mu g/cm^2$ for surfaces adsorbed with albumin at the bulk concentrations of 1 and 5 mg/ml, respectively. Platelets were found to spread even more on the fibrinogen adsorbed surface than on the albumin surface after drying and rehydration. The area of spread platelets increased to 41.5 and 42.1 μm^2 after addition of fibrinogen to the once-dried albumin layer. The circularity also decreased to 1.60 and 1.40 for 1 and 5 mg/ml albumin, respectively. These values indicate complete activation of platelets on the surface [22].

Addition of more unlabeled albumin to the dried albumin layer does not change the surface albumin concentration significantly. This suggests that the additional

Table 1.
Effect of glutaraldehyde crosslinking of adsorbed albumin on platelet activation

Bulk albumin Conc. used for adsorption	Surface protein concentration ($\mu g/cm^2$)				Platelet activation			
	Albumin		Fibrinogen		Spread area (μm^2)		Circularity	
	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml
DDS-glass[a]	0.00				40.3 ± 2.3		1.79 ± 0.12	
Albumin[b]	0.29 ± 0.02	0.31 ± 0.01			19.6 ± 1.3	11.8 ± 0.7	3.75 ± 0.12	3.43 ± 0.41
Alb-Fix[c]	0.28 ± 0.01	0.30 ± 0.02			36.3 ± 1.9	36.6 ± 1.7	2.16 ± 0.11	2.03 ± 0.07
Alb-Fix-Fib[d]	0.26 ± 0.01	0.29 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	36.9 ± 2.7	38.7 ± 1.5	2.01 ± 0.40	1.82 ± 0.08
Alb-Fix-Alb[e]	0.28 ± 0.02	0.26 ± 0.01			26.7 ± 1.7	34.5 ± 1.8	2.15 ± 0.91	1.82 ± 0.07

[a] Control DDS-glass without any preadsorbed albumin.
[b] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass for 1 h at room temperature.
[c] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass and crosslinked with 2% (w/v) glutaraldehyde in PBS for 1 h at room temperature.
[d] Fibrinogen at a bulk concentration of 0.1 mg/ml was adsorbed for 1 h at room temperature on the crosslinked albumin layer.
[e] Unlabeled albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed for 1 h at room temperature on the crosslinked albumin layer. The surface concentration of unlabeled albumin was not accounted for in this measurement.

Table 2.
Effect of drying and rehydration of adsorbed albumin on platelet activation

Bulk albumin Conc. used for adsorption	Surface protein concentration ($\mu g/cm^2$)				Platelet activation			
	Albumin		Fibrinogen		Spread area (μm^2)		Circularity	
	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml
DDS-glass[a]	0.00				40.3 ± 2.3		1.79 ± 0.12	
Albumin[b]	0.29 ± 0.02	0.31 ± 0.01			19.6 ± 1.3	11.8 ± 0.7	3.75 ± 0.12	3.43 ± 0.41
Alb-Dry[c]	0.24 ± 0.01	0.27 ± 0.01			36.3 ± 2.9	39.1 ± 2.4	1.78 ± 0.10	1.81 ± 0.07
Alb-Dry-Fib[d]	0.25 ± 0.03	0.26 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	41.5 ± 3.3	42.1 ± 4.3	1.60 ± 0.10	1.40 ± 0.05
Alb-Dry-Alb[e]	0.26 ± 0.03	0.27 ± 0.01			26.1 ± 2.1	10.6 ± 0.9	5.18 ± 1.24	4.12 ± 0.46

[a] Control DDS-glass without any preadsorbed albumin.
[b] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass for 1 h at room temperature.
[c] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass and dried at 60°C for 18 h and rehydrated in PBS for 1 h before exposure of platelets.
[d] Fibrinogen at a bulk concentration of 0.1 mg/ml was adsorbed for 1 h at room temperature on the dried albumin layer.
[e] Unlabeled albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed for 1 h at room temperature on the dried albumin layer.

albumin did not displace absorbed albumin molecules from DDS-glass. Platelet activation profile, however, is different after addition of more albumin compared to the dried albumin layer. The spread area of the platelets decreased to 26.1 and 10.6 μm^2 for surfaces adsorbed with 1 and 5 mg/ml albumin, respectively. The circularity values also increased to 5.18 and 4.12 due to the presence of several long pseudopods. Addition of more ^{125}I-labeled albumin shows that the surface albumin concentration was restored to 0.29 $\mu g/cm^2$.

Effects of tryptic digestion of adsorbed albumin on platelet activation

The adsorption of trypsin on DDS-glass did not have any effect on platelet activation as shown in Table 3. The extent of platelet activation after adsorption of 0.1 mg/ml trypsin on DDS-glass was similar to that on control DDS-glass. The surface albumin concentration after tryptic digestion was found to decrease by almost 50% from 0.29 to 0.15 and 0.31 to 0.17 $\mu g/cm^2$ for 1 and 5 mg/ml bulk albumin concentrations used for adsorption, respectively.

Platelet activation on trypsin-digested albumin surface was also increased compared to native albumin. The spread area increased to 26.1 and 27.3 μm^2 for bulk albumin concentrations of 1 and 5 mg/ml, respectively. The circularity values also decreased to 2.68 and 2.40, respectively. Platelets adhered and activated more on the trypsin-digested albumin layer than on the native albumin layer. The extent of activation, however, was not as complete as that on control DDS-glass. This suggests that the exposure of bare surface sites by tryptic digestion is not as extensive as by drying.

Addition of fibrinogen to the digested layer of albumin reduced the albumin surface concentration even further (*e* in Table 3). The surface fibrinogen concentrations on the trypsin-digested albumin layer were 0.05 and 0.06 $\mu g/cm^2$ for 1 and 5 mg/ml albumin, respectively. Fibrinogen appears to displace digested albumin molecules from DDS-glass. This indicates that the surface affinity of albumin is reduced by digestion with trypsin.

Platelet activation was also increased by addition of fibrinogen to the trypsin-digested albumin layer. The spread area changed slightly to 28.5 and 31.3 μm^2 for 1 and 5 mg/ml albumin, respectively. It is interesting to notice here that the spread areas are not as high as those observed on the dried albumin layer (compare *d* in Table 2 and *e* in Table 3). The circularity values, however, decreased significantly to 1.50 and 1.40, respectively. Platelets retracted all the pseudopods if fibrinogen was adsorbed on the trypsin-digested albumin layer.

Addition of more unlabeled albumin to the trypsin-digested albumin layer did not change the surface concentration significantly. This suggests that albumin does not have the same effect of displacement as seen with fibrinogen. This is probably because albumin does not have the same affinity to hydrophobic surfaces as fibrinogen does. The addition of more albumin, however, resulted in prevention of platelet activation. The spread area of platelets decreased to 14.5 and 16.3 μm^2 for 1 and 5 mg/ml albumin, respectively. The circularity values also increased to 5.84 and 3.87, respectively, due to several pseudopods extended from the platelets. These values correspond with platelets that are only contact adherent on the surface. The total albumin concentration also increased to 0.30 $\mu g/cm^2$ upon addition of ^{125}I-labeled albumin to the trypsin-digested layer.

Table 3.
Effect of digestion of adsorbed albumin with trypsin on platelet activation

Bulk albumin Conc. used for adsorption	Surface protein concentration ($\mu g/cm^2$)				Platelet activation			
	Albumin		Fibrinogen		Spread area (μm^2)		Circularity	
	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml	5 mg/ml
DDS-glass[a]	0.00				40.3 ± 2.3		1.79 ± 0.12	
Trypsin[b]	0.00				42.1 ± 3.5		1.78 ± 0.09	
Albumin[c]	0.29 ± 0.02	0.31 ± 0.01			19.6 ± 1.3	11.8 ± 0.7	3.75 ± 0.12	3.43 ± 0.41
Alb-Trypsin[d]	0.15 ± 0.01	0.17 ± 0.04			26.1 ± 1.2	27.3 ± 0.8	2.68 ± 0.09	2.40 ± 0.28
Alb-Trypsin-Fib[e]	0.09 ± 0.01	0.10 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	28.5 ± 3.9	31.3 ± 1.6	1.50 ± 0.10	1.40 ± 0.30
Alb-Trypsin-Alb[f]	0.14 ± 0.01	0.16 ± 0.01			14.5 ± 0.9	16.3 ± 1.7	5.84 ± 0.55	3.87 ± 0.60

[a] Control DDS-glass without any preadsorbed albumin.
[b] Trypsin at a bulk concentration of 0.1 mg/ml was adsorbed on DDS-glass for 1 h at room temperature.
[c] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass for 1 h at room temperature.
[d] Albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed on DDS-glass and digested with 0.1 mg/ml trypsin for 1 h at room temperature.
[e] Fibrinogen at a bulk concentration of 0.1 mg/ml was adsorbed for 1 h at room temperature or the trypsin-digested albumin layer.
[f] Unlabeled albumin at a bulk concentration of 1 or 5 mg/ml was adsorbed for 1 h at room temperature on the trypsin-digested albumin layer.

DISCUSSION

Albumin is the most abundant protein in human blood. The concentration of albumin in healthy adults varies from 35 to 50 mg/ml [27]. Albumin, a non-glycoprotein, has some unique properties that distinguish it from other globular proteins in the blood. It is an acidic protein with high aqueous solubility and stability. The stability against heat denaturation and low pH conditions is due to 17 disulfide bonds in albumin molecule [28]. High aqueous solubility is attributed to the polar surface of the molecule. There are 585 amino acid residues in human serum albumin, 181 of which have either acidic or basic side-chains. At pH 7, albumin molecule has a net charge of −15 [29].

Surface-induced conformational change in albumin molecule upon adsorption has been studied with Fourier transform infra-red spectroscopy with attenuated total internal reflection (FTIR/ATR) and total internal reflection fluorescence (TIRF) [30–32]. FTIR/ATR studies have shown that upon adsorption, albumin structure changes to increase the content of β-sheets [30]. TIRF analysis has shown that the surface interaction of albumin leads to two distinct states of adsorption, a tightly held layer and a loosely held layer [31]. Tilton *et al.* [32] measured the lateral diffusion of albumin upon adsorption to hydrophilic and hydrophobic surfaces by TIRF with photobleaching recovery. They concluded that the adsorbed albumin is able to migrate laterally over several micrometers by diffusion. The extent of lateral mobility was greater on hydrophobic surfaces than on hydrophilic surfaces.

The treatment of the absorbed albumin with glutaraldehyde is expected to inhibit lateral movement as well as the motional freedom of the protein segments. Glutaraldehyde, a bifunctional reagent, has been used for crosslinking proteins in electron microscopy, leather tanning, and X-ray crystallography [33, 34]. Glutaraldehyde reacts predominantly with amine groups on the protein to form inter- and intramolecular crosslinking. The ε-amino group of L-lysine residues are considered to be the main targets of glutaraldehyde [35]. Human serum albumin molecule has 59 residues of L-lysine [29].

Quicho and Richards studied carboxypeptidase-A liquid crystals after crosslinking with glutaraldehyde [36, 37]. Crosslinked crystals were insoluble in aqueous buffers, more stable than native enzyme, and had improved mechanical strength. X-ray crystallographic studies with crosslinked crystals displayed a significantly less smearing in electron density maps compared to control enzyme crystals [37]. The authors attributed this to the loss in motional freedom of surface amino acid side-chains by crosslinking. The three dimensional structure of the enzyme and the rate of substrate catalysis, however, remained the same as control.

Platelets were able to adhere and activate completely on crosslinked albumin layer. Crosslinking with glutaraldehyde is expected to reduce the motional freedom of adsorbed surface accessible segments of the adsorbed albumin molecule. Therefore, the decreased flexibility of the adsorbed albumin by crosslinking probably results in loss of surface passivating effects. Addition of more proteins to the crosslinked albumin layer does not have any effect on the surface albumin concentration. Apparently, the adsorbed albumin after crosslinking is still able to interact strongly with DDS-glass and remains on the surface. The data in Table 1 suggest that fibrinogen or additional albumin added to the crosslinked albumin layer does not adsorb significantly on the crosslinked albumin layer.

Proteins denature rapidly when exposed to air [38]. Drying and rehydration of adsorbed albumin caused the reduction of the surface albumin concentration by approximately 15%. The decrease in surface albumin concentration resulted in the exposure of bare surface sites. Platelets were fully activated on the surface and this agrees with our previous study which showed that platelets could activate fully as long as a small fraction of the surface (2–15%) was covered with fibrinogen [39]. DDS-glass itself is highly thrombogenic even without the adsorbed fibrinogen [22, 39].

Proteolytic enzymes have been used extensively to investigate protein structures [40]. Trypsin is a serine protease that cleaves specifically at the carboxy end of either L-lysine or L-arginine residues in proteins [41]. The molecular structure of albumin has sufficient flexibility for accommodation to different environmental circumstances. This flexibility is due to the loop-link-loop structure of albumin molecule [28]. One approach of investigating the flexibility of albumin in the adsorbed state is by trypsin digestion. Each human serum albumin molecule has 83 possible (59 L-lysine and 24 L-argine) sites of cleavage by trypsin [29].

The reduction in the surface albumin concentration may be primarily due to the partial cleavage of the albumin segments exposed to trypsin. The remaining portions of partially digested albumin molecules are expected to cover most of the surface. This is why platelets were not able to fully spread despite the 50% decrease in the surface albumin concentration (compare *c* in Table 2 and *d* in Table 3).

For hydrophilic polymers to be effective in steric repulsion, they must meet the following requirements. First, the polymer molecules should have sufficient affinity to the surface so that they can anchor tightly to the surface. Second, part of the polymer should also extend into the bulk solution. Thus, copolymers containing both hydrophilic and hydrophobic segments are more effective than homopolymers [42]. Finally, the polymer molecules should cover most of the surface. If a significant portion of the surface is exposed, steric repulsion is not effective in preventing adsorption of other polymer molecules or proteins. In colloidal chemistry, incomplete surface coverage is known to facilitate coagulation of dispersed particles rather than to prevent it [43].

All the above three requirements can be met by albumin which is adsorbed on hydrophobic DDS-glass. Albumin adsorption to hydrophobic surfaces is usually irreversible [9]. Therefore, albumin interactions with hydrophobic DDS-glass is effective in anchoring the molecule to the surface. Inter- and intramolecular cross-linking with glutaraldehyde reduces the flexibility of the albumin segments, which reduces the passivating effects of albumin. Drying of adsorbed albumin leads to decrease in surface coverage. The vacant sites created on the surface can effectively adsorb additional proteins such as fibrinogen, albumin, and platelet membrane proteins. Therefore, incomplete surface coverage by albumin after drying is probably responsible for loss of surface passivation. Tryptic digestion of the absorbed albumin removes significant fraction of albumin molecules and thus results in the reduction in the thickness of the flexible segments. Decrease flexibility of surface segments and partial exposure of bare surface sites after digestion are probably responsible for loss of surface passivating effects.

The relationship between colloidal stabilization by steric repulsion and prevention of platelet adhesion by the albumin layer was first proposed by Morrissey [44]. It should be noted that albumin can be used for the stabilization of colloidal gold

particles [45] and negatively charged polystyrene latices [46, 47]. Van der Scheer *et al.* (46) concluded that the optimum stability of polystyrene latices was obtained only when albumin molecules completely covered the surface of the latices. Further experimental evidence of colloidal stabilization with adsorbed albumin on polystyrene latices has been obtained by Tamai *et al.* [47]. In high ionic strength media, adsorbed albumin was effective in preventing coagulation of latices by steric repulsion.

Adsorbed albumin can be displaced by other thrombogenic proteins such as fibrinogen in the blood. Therefore, for long-term use of biomaterials simple adsorption of albumin as a method of creating passive surfaces is not adequate. To guarantee the continued presence of albumin on the surface, covalent grafting of albumin to the surface is preferred. We are currently pursuing several approaches that allow covalent grafting of albumin to various biomaterials including inert polymers such as polyethylene and polypropylene.

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