Published in final edited form as:

Langmuir. 2012 February 7; 28(5): 2745–2752. doi:10.1021/la204777x.

Time-dependent Conformational Changes in Adsorbed Albumin and its Effect on Platelet Adhesion

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Abstract

Recent studies have shown that platelets can adhere to adsorbed albumin (Alb) through a receptor-mediated mechanism, but only if the Alb undergoes more than a critical degree of adsorption-induced unfolding. The objectives of this research were to investigate whether Alb that was initially adsorbed in a manner that induced unfolding that was less than this critical level would undergo further unfolding with time; and if so, whether this would induce the onset of platelet adhesion once this critical level was exceeded. To address these questions, CD spectropolarimetry was used to monitor the structure of Alb on OH- and CH₃-functionalized alkanethiol self-assembled monolayer surfaces, with the Alb initially adsorbed under conditions resulting in degrees of unfolding that were below this critical level, and then the adsorbed Alb layers were aged over a six-month period of time in sterile physiological saline at 37°C. Platelet adhesion to Alb was quantified at selected time points via a lactate dehydrogenase (LDH) assay. The results indicate that an adsorbed Alb layer does undergo further structural changes with increasing residence time and supports platelet adhesion once it unfolds beyond the previously determined critical level. These results may be relevant to the clinically observed problem of the onset of late-thrombosis, which occurs on cardiovascular implants such as drug-eluting stents.

Introduction

Proteins tend to undergo structural changes upon contact with solid substrates, which are a determinant of the bioactivity of the tissue-biomaterial interface, and play a critical role in the subsequent cellular responses to the biomaterial^{1–7}. Several studies have shown that structural alterations in adsorbed plasma proteins play a critical role in the ability of non-activated platelets to bind to the adsorbed protein layer^{3, 7, 8} and mediate a thrombotic response. Recent studies by our group showed that the adhesion of non-activated platelets is strongly correlated in a highly linear manner to the degree of adsorption-induced unfolding of both fibrinogen (Fg)⁵ and albumin (Alb)⁶, measured by adsorbed-state circular dichroism spectropolarimetry (CD). In these studies, the degree of unfolding was controlled by varying both surface chemistry and the solution concentration from which the proteins were adsorbed, with a higher solution concentration tending to cause the proteins to undergo a lesser degree of unfolding, as adsorbed proteins had less time to unfold before further unfolding was blocked by neighboring adsorbed proteins.

The results from these studies⁵ revealed that a relatively small base level of platelets adhered to adsorbed Fg even when it underwent no detectable degree of unfolding, with the platelet adhesion response then substantially increasing in direct proportion to the degree of adsorption-induced unfolding in Fg. The platelet response to adsorbed Alb, however, was

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observed to be distinctly different⁶. Platelet adhesion to the adsorbed Alb was found to be near zero until the Alb exceeded a critical degree of unfolding, corresponding to a 34% loss in its α -helical structure, with the platelet adhesion response then increasing with further unfolding of Alb just as strongly as that which was observed for adsorbed Fg. While the discovery that platelets can adhere to adsorbed Alb following a critical degree of unfolding is remarkable, a high degree of Alb unfolding was only found to occur when the Alb was adsorbed from solution concentrations below 10 mg/mL. Given that the plasma concentration of Alb is about 40 mg/mL, these results suggest that when Alb adsorbs from plasma to a biomaterial surface, the Alb will undergo a minimal degree of adsorption-induced unfolding, and thereby not be capable of mediating a platelet adhesion response. However, further scrutiny of this is warranted to assess its potential clinical relevance, particularly since Alb has been conventionally thought to be unable to mediate platelet attachment, even to the point of leading to its use as an intended hemocompatible coating for biomaterial surfaces^{9, 10}.

Alb is the most abundant plasma protein¹¹ and has been shown to be often preferentially adsorbed from plasma on polymer surfaces¹². Blood plasma, of course, contains numerous other types of proteins in addition to Alb, which have also been shown to readily adsorb to surfaces in a competitive manner. For example, apolipoprotein A1 (apo A1), which has often been largely overlooked in blood-material interactions¹³, has been shown to strongly adsorb from plasma on both polymer^{14, 15} and self-assembled monolayer (SAM) surfaces¹⁶, along with other plasma proteins such as albumin, fibrinogen, IgG, and complement proteins factors B, H, and I^{15, 16}. These proteins are generally considered to adsorb in an irreversible manner, apart from being displaced by Vroman effects¹⁷, which have been found to be material-surface dependent. For example, in a study of protein adsorption from dilute plasma on polymers of varying hydrophobicity, Knetsch et al.¹⁸ found that Alb and Fg were the major proteins adsorbed and retained on hydrophobic polymers, while the Alb that was initially adsorbed on the more hydrophilic polymer surfaces was largely displaced over 30–60 min by high density lipoprotein (HDL), for which apo A1 is a major component. Other competitive adsorption studies using purified proteins have shown minimal desorption of adsorbed Alb by more surface-active proteins such as IgG and Fg by Vroman effects^{19, 20} on both hydrophilic and hydrophobic surfaces, with displacement of Alb decreasing with an increase in residence time of Alb on the surface.

From these and numerous other studies over the past several decades, protein adsorption to biomaterial surfaces has been shown to represent very complex and materials-dependent processes that involve many different plasma proteins. The results from many of these experimental studies combined with the fact that Alb is the most abundant plasma protein and has been shown to be one of the first proteins that adsorbs on an implant surface following blood contact²¹, suggests that Alb may be a prominent component of the final, irreversibly adsorbed protein layer on many biomaterial surfaces in vivo. This situation raises the questions of whether irreversibly adsorbed proteins, in particular Alb, which can be expected to largely retain its native state structure following initial adsorption, will eventually undergo structural unfolding as it ages on a biomaterial surface. And, if so, when its degree of unfolding exceeds the previously established critical level⁶, will it then cause non-activated platelets in the blood to begin to adhere and activate on the surface of the material?

If such processes do occur in an adsorbed layer of Alb, as well as in other irreversibly adsorbed proteins, such as Fg, we speculate that they may potentially play a role in the late thrombotic events that have been observed on drug-eluting vascular stents^{22–26}, with these events being directly correlated with prolonged exposure of the stent surface to blood^{1, 2, 27}. This clinical problem, combined with our prior results regarding the relationship between

adsorbed protein structure and platelet adhesion^{5, 6}, raises important questions regarding the influence of residence time on the structure of proteins, such as Alb, that irreversibly adsorb on biomaterial surfaces that are used in blood-contact applications, and their potential association with late thrombotic responses.

Previously reported studies have typically addressed the behavior of adsorbed proteins for only short residence times of up to 2 h²⁸⁻³². In longer-term studies, Lenk and Horbett³³ investigated the post-adsorptive behavior of Fg using infrared spectroscopy, and reported evidence of continued conformational unfolding of adsorbed Fg over a 50 h period, with the elutability of the protein decreasing with continued aging time. In an even longer-term study, Rapoza and Horbett³⁴ examined the elutability of Fg adsorbed from diluted plasma solutions over a 5 day period, and reported that the elutability of Fg using sodium dodecylsulfate (SDS) was lower for adsorption from more dilute plasma, and also significantly lower after 5 days of residence time. These studies suggest that the adhesion strength of adsorbed proteins increases with increased residence time, providing increasing resistance to displacement by other proteins, or competitive agents such as SDS. This behavior can thus be expected to result in the relatively rapid establishment of an effectively irreversible layer of adsorbed protein on a biomaterial surface following exposure to blood.

The irreversible nature of an adsorbed protein layer raises important questions about the fate of these proteins and the possible consequences that the aging of the adsorbed protein layer may have for blood compatibility. To our knowledge, previous studies have not been published that examine this question beyond a period of five days. Based on this recognized gap in the knowledge base, and as a direct extension of our previous studies investigating the correlation between adsorbed Alb conformation and platelet adhesion, the first objective of the studies that we report on in this work was to investigate the extent that time-dependent conformational changes occur in an irreversibly adsorbed, tightly packed layer of Alb that initially is adsorbed in a manner that largely retains its native-state structure. We then sought to determine if this aging-induced unfolding process can cause the adsorbed Alb layer to support platelet adhesion if unfolding occurs beyond the previously determined critical degree of unfolding.

Materials and Methods

Gold substrates

Quartz slides ($0.375'' \times 1.625'' \times 0.0625''$, Chemglass) were used for CD experiments, while 18 mm square cover glasses (VWR Scientific, Catalog No. 48368-040) were used as substrates for the platelet adhesion experiments. These substrates were cleaned as described earlier^{5, 6, 35}. Briefly, the substrates were incubated in a piranha solution (7:3 v/v $\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$) at 50°C for 30 min, followed by an RCA basic wash (1:1:5 v/v $\text{NH}_4\text{OH}:\text{H}_2\text{O}_2:\text{H}_2\text{O}$), and this cleaning procedure was repeated twice. The cleaned substrates were then rinsed copiously with 100% ethanol (Pharmco-Aaper; Catalog No. 111000200) and nanopure water, and finally dried using a stream of nitrogen gas.

The cleaned substrates were coated with a chromium adhesion layer followed by a gold layer via thermal vapor deposition. A 50 Å chromium adhesion layer and 1,000 Å of gold were deposited on the cover glasses for the platelet adhesion studies, while the quartz slides for CD were coated with 30 Å of chromium and 100 Å of gold. The thicknesses of the gold and chromium layers were also verified using a DekTak profilometer and a GES5 ellipsometer (Sopra, Inc., Palo Alto, CA).

Formation of self-assembled monolayers (SAMs) of alkanethiols

1-Dodecanethiol ($\text{SH}-(\text{CH}_2)_{11}\text{CH}_3$; Aldrich; CH_3) and 11-Mercapto-1-undecanol ($\text{SH}-(\text{CH}_2)_{11}\text{OH}$; Aldrich; OH) in 100% ethanol were used as the alkanethiols for creating the self-assembled monolayer (SAM) surfaces, as described previously³⁵.

The gold-coated substrates were dipped in a modified piranha wash (4:1 v/v $\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2$), followed by an RCA basic wash, for 1 minute each and then rinsed copiously with 100% ethanol, to clean them. The cleaned gold substrates were then incubated in 1.0 mM alkanethiol solutions for 24 h, as per the established protocols described previously^{36, 37}.

Prior to the protein adsorption step, the SAM surfaces were cleaned to remove any traces of hydrophobic contaminants on their surface³⁸. The CH_3 SAMs were sonicated in ethanol, hexane and ethanol, and then rinsed with nanopure water. The OH SAMs were sonicated in ethanol, and then incubated in a 25 mM potassium phosphate buffer containing 0.005 volume % Triton-X-100 (Sigma; Catalog No. T-9284) in order to block off hydrophobic defect sites (e.g. grain boundaries), and then rinsed thoroughly with acetone, ethanol and nanopure water to remove loosely-bound Triton.

Buffers

25 mM potassium phosphate buffer (pH 7.4), prepared by combining appropriate amounts of the mono- and dibasic salts (Sigma-Aldrich) to maintain the pH at 7.4, was used for all adsorption experiments. This buffer is recommended for CD experiments to determine the secondary structure of proteins^{21, 39, 40} as it permits measurement of CD spectra with minimal noise below 200 nm, especially the positive CD peaks at 193 and 195 nm, which are critical for the accurate determination of the α -helix and β -sheet content of the proteins, respectively.

The platelet suspension buffer (PSB, pH 7.4) contained 137 mM NaCl, 2.7 mM KCl, 5.5 mM dextrose, 0.4 mM sodium phosphate monobasic, 10 mM HEPES, and 0.1 U/mL apyrase⁴¹. The presence of apyrase in PSB prevents the washed platelets from becoming unresponsive towards ADP-stimulation, and enables them to retain their physiological properties, and respond to platelet agonists in a similar fashion as that observed for platelets in citrated platelet-rich plasma (PRP)⁴². 2.5 mM CaCl_2 and 1.0 mM MgCl_2 were added to the PSB to give a platelet suspension buffer with metal ions (PSB+MI).

Protein adsorption

The Alb stock solution was prepared by dissolving human Alb (Sigma, Catalog No. A9511) in 25 mM phosphate buffer solution (pH 7.4), and protein adsorption was carried out as described previously^{6, 35}, at a bulk solution concentration of 10.0 mg/mL, so as to obtain an irreversibly adsorbed, tightly packed layer(s) of adsorbed Alb.

The cleaned SAM surfaces were immersed in 25 mM potassium phosphate buffer (pH 7.4), and then a suitable amount of Alb stock solution was added to give the desired bulk protein solution concentration. Special care was taken to ensure that the tip of the pipette was held below the air-water interface to avoid denaturation of the protein at this interface. The protein adsorption step lasted for 2 h, after which the protein solutions over the SAM surfaces were gently flooded over with phosphate buffer for 5 min with pure buffer to wash away the bulk protein solution as well as any loosely adherent protein. The SAM surfaces could then be removed from the pure buffer solution for further analysis without dragging them through the denatured protein film that can be expected to be present at the liquid-air interface if the protein solution had not been replaced with pure buffer via the infinite dilution step prior to removal of these surfaces.

CD studies to quantify the adsorption-induced conformational changes and total surface coverage of Alb on SAM surfaces

A Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD) was used to determine the native and adsorbed secondary structures of Alb, as well as the surface coverage of adsorbed Alb, as described earlier^{6, 35}. The native solution structure of Alb was determined using a high-transparency quartz cuvette (Starna Cells, Inc., Atascadero, CA), while the adsorbed structure of Alb on the SAM surfaces was determined using a special custom-made cuvette³⁵, which our group designed to maximize the signal-to-noise ratio. The ellipticity of the samples (θ , in mdeg) was converted to molar ellipticity (designated as $[\theta]$, with standard units of deg cm²/dmol) using the following equation^{40, 43}:

$$[\theta] = (\theta / M_0) / (10,000 \cdot C_{\text{soln}} \cdot L), \quad (1)$$

where θ is the ellipticity in mdeg, L is the path length of the cuvette in cm, C_{soln} is the solution concentration of the protein in g/mL, and M_0 is the mean residue molecular weight of 118 g/mol.

Since proteins exhibit an absorbance peak at 195 nm⁴⁴, a calibration curve plotting the height of this peak (A_{195}) as a function of C_{soln} for various known concentrations of Alb was created, as described earlier³⁵. The slope of this plot is “ $\epsilon_{\text{protein}} \cdot L$ ” from Beer’s Law, which can be written as:

$$A_{195} = \epsilon_{\text{protein}} \cdot C_{\text{soln}} \cdot L \quad (2)$$

where $\epsilon_{\text{protein}}$ is the extinction coefficient of the protein in mL g⁻¹ cm⁻¹ (or cm²/g) and L is the path length of the cuvette.

The term “ $C_{\text{soln}} L$ ” in eq. 2 has units of g/cm², which is equivalent to the amount of protein per unit area (Q_{ads}). Assuming that the absorbance is dependent on the total amount of protein present per unit area through which the light beam passes, irrespective of whether the protein is in the solution or the adsorbed state, the calibration curve of A_{195} vs. C_{soln} can also be used for calculating the surface coverage of adsorbed Alb on the SAMs (i.e., Q_{ads}). The validity of this method for measuring the amount of adsorbed protein has been confirmed by independent measurement of Q_{ads} from the thickness of the adsorbed protein film obtained by ellipsometry³⁵ using de Feijter’s formula⁴⁵.

Hence, in the calculation for the molar ellipticity of the adsorbed Alb layer on the SAMs, the term “ $C_{\text{soln}} L$ ” in eq. 1 can be replaced by the term Q_{ads} to give the following equation:

$$[\theta] = (\theta / M_0) / (10,000 \cdot Q_{\text{ads}}), \quad (3)$$

The CD spectra (molar ellipticity vs. wavelength) thus obtained were deconvoluted using the SP-22X algorithm and analyzed using the CONTIN/LL software packages to quantify the percentage of α -helix and β -sheet content of the native/adsorbed Alb⁴⁶.

Platelet adhesion

The platelet adhesion experiments were carried out using a suspension of non-activated washed human platelets, as described in our previous studies^{5, 6}. Briefly, 25.0 mL of blood was collected from healthy, non-smoking, volunteers of both genders, in BD Vacutainer tubes (Becton-Dickinson, Catalog No. 364606) containing an acid-citrate dextrose (ACD) anti-coagulant, as per protocols approved by the Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) at Clemson University. The donors denied having

taken any medication, including aspirin, during the 2 weeks prior to donating blood. It is important to note that the first few mL of blood was discarded, as it is rich in clotting factors, and then 25 mL of blood was collected. Also, although there may be inter-individual differences in platelet function, primarily with regard to their activation response to agonists⁴⁷, and in response to anti-platelet therapy based on their gender⁴⁸, we expect to have minimized any potential differences in the platelet function of donors by using non-activated washed platelets pooled from blood drawn from healthy, non-smoking volunteers of both genders, who were medication-free for at least 2 weeks prior to blood donation.

The blood collected was then centrifuged (225g, 15 min, 25°C) to generate platelet-rich plasma (PRP), and platelets were separated from the PRP via a gel separation method⁴¹, using a liquid chromatography column (Sigma-Aldrich, Catalog No. C4169) containing Sepharose 2B (Sigma-Aldrich, Catalog No. 2B-300). The Sepharose column was equilibrated with PSB, prior to layering the PRP on the column. PSB was then added to the column from a reservoir, with the column running. Fractions were collected from the bottom of the column, with the platelets being identified by increased effluent turbidity, and the platelet-rich fractions were pooled. Platelet concentration was measured using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA), and the platelet count was adjusted to 10^8 platelets/mL with PSB. CaCl_2 and MgCl_2 were added to give 2.5 mM and 1.0 mM concentrations of these salts, respectively. The non-activated washed platelet suspension was allowed to rest for 30 min, and the platelet adhesion step was carried out on the protein-coated SAMs for 1 h at 37°C.

At the end of the platelet adhesion step, the platelet suspension was aspirated from each well, and the non-adherent platelets, which may have settled or been deposited on the surface on the Alb-coated surfaces due to gravity, were rinsed away by filling and aspirating the wells five times with PBS. The substrates were then removed to a fresh well-plate, containing 1.0 mL PSB in each well, for carrying out the lactate dehydrogenase (LDH) assay to quantify the platelet adhesion levels.

Measurement of platelet adhesion using lactate dehydrogenase (LDH) assay

A CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI) was used for quantification of the platelet adhesion levels on the Alb-coated SAMs by measuring the lactate dehydrogenase (LDH) released when the adherent platelets were lysed with a Triton-PSB buffer, (100 μL ; 2% v/v Triton-X-100 in PSB) following standard methods^{3, 32, 49–53}. A calibration curve was constructed by concurrent measurement of the LDH released from a known number of non-activated platelets suspended in 1.0 mL PSB in eppendorf tubes, incubated at 37°C for 1 h, and then lysed similarly with 100 μL Triton-PSB buffer. The platelet adhesion to Alb adsorbed on the SAM surfaces was determined from this calibration curve.

Aging studies for adsorbed Alb on SAM surfaces

CD and platelet adhesion studies were performed to measure the conformation of the adsorbed Alb layers on the SAM surfaces and the platelet adhesion response to the adsorbed Alb at day 0. The Alb-coated SAMs were then re-immersed in fresh 25 mM potassium phosphate buffer, supplemented with 0.1% cellgro antibiotic/antimycotic solution (Mediatech Inc., Catalog No. MT30-004-CI; contains penicillin, streptomycin, and amphotericin B). These substrates were then stored for up to 6 months at 37°C in a sterile incubator, with the buffer (supplemented with antibiotic/antimycotic solution) being replenished with fresh buffer solution regularly at two-week intervals. Plain buffer was used as the aging media for these initial studies rather than an albumin-containing solution in order to simplify the system and directly address the specific questions of this study

regarding whether a given layer of irreversibly adsorbed albumin would undergo structural unfolding as a function of time and, if so, subsequently support platelet adhesion response. CD and platelet adhesion analyses were performed after aging the adsorbed Alb layers at 3-month and 6-month time points following initial Alb adsorption. Prior to conducting the CD and platelet adhesion studies, the protein-coated aged samples were infinitely diluted and rinsed copiously with nanopure water, to remove all traces of the antibiotic/antimycotic solution.

Statistical analysis

The results we present are the mean values with 95% confidence intervals (CI). Statistical significance of differences between mean values for different samples and conditions was evaluated using a Student's t-test, with $p \leq 0.05$ considered as statistically significant.

Results and Discussion

Time-dependent conformational changes in adsorbed Alb on SAM surfaces

The percentage α -helix and β -sheet content of Alb adsorbed on the CH_3 - and OH-functionalized alkanethiol self-assembled monolayer surfaces (SAMs) from 10.0 mg/mL bulk Alb solution concentration, determined using adsorbed-state CD spectropolarimetry, are presented in Fig. 1.

At the initial, day-0 time point, the adsorption process induced relatively minor amounts of unfolding of Alb (as indicated by a small decrease in α -helix and increase in β -sheet content), with the hydrophobic CH_3 SAM surface inducing a significantly greater degree of unfolding compared to the hydrophilic OH SAM surface, as expected based on our previous studies^{6, 35}. The results then show a distinct loss in α -helix of adsorbed Alb (usually accompanied with increased β -sheet) with increasing residence time at both the 3-month and 6-month time points for both the OH and CH_3 SAMs, thus indicating that the adsorbed Alb layer underwent a significant degree of aging-induced structural unfolding over this 6-month time period. The increased unfolding on the hydrophobic CH_3 SAM as compared to the hydrophilic OH SAM may be attributed to the strong thermodynamic driving force on hydrophobic surfaces to cause proteins to unfold and cover any remaining exposed surface in order to minimize the overall solvent-accessible surface area of the system^{35, 54}. This effect continues to occur over this relatively long period of time and aging-induced increased unfolding occurs on the OH SAM surface even in the absence of this driving force.

Surface coverage of adsorbed Alb over residence times up to six months

The surface coverage of Alb on the CH_3 and OH SAMs, measured at day 0, 3 months, and 6 months, is listed in Table 1. The values for the amount of Alb adsorbed were comparable to those obtained in our earlier study⁶, which were $2.71 \pm 0.18 \mu\text{g}/\text{cm}^2$ for the CH_3 SAM, $1.77 \pm 0.49 \mu\text{g}/\text{cm}^2$ for the OH SAM. The surface coverage remained consistent over the entire 6-month residence time, indicating that the adsorbed Alb layer did not desorb on its own over this period, nor did it undergo chain lysis to the degree of releasing detectable amounts of protein fragments. This is not surprising, given that protein adsorption is generally considered to be an irreversible phenomenon and considering the fact that the elutability of proteins using surfactants like SDS has been found to decrease with increasing residence time^{31, 33, 34}, indicating that the strength of protein binding on surfaces tends to only increase with time. The combined data presented in Table 1 and Figure 1 indicate that the surfaces remained saturated with protein during this entire period, as expected for an irreversibly adsorbed protein layer, with the proteins in this layer experiencing an aging process that caused them to undergo further unfolding while occupying the same net surface

area per adsorbed protein molecule; either through some reorganization of the protein's orientational states on the surface or perhaps by limited aging-induced chain scission of the polypeptide chain.

One concern with respect to carrying out these long-term aging studies was whether or not the SAM surface itself would be stable on the gold underlying layer for 6 months in PBS solution. The long-term stability of alkanethiol SAMs on gold, especially under physiological conditions, is not well understood⁵⁵, and a recent study⁵⁶ suggested that alkanethiol monolayers may be unstable over a 35-day incubation period in physiological media due to the oxidation of thiolate head-groups, with subsequent desorption into the media. In our studies, however, instability in the underlying SAM layer would be expected to have led to the desorption of the adsorbed Alb layer as well. The consistent Alb surface coverage measured on both SAMs over the duration of the study, however, clearly demonstrates that our Alb layer was not getting desorbed or otherwise released from the surface, suggesting that the underlying SAM remained sufficiently intact over 6 months to support this layer of protein.

Effect of residence-time-dependent changes in adsorbed Alb on platelet adhesion

Based on our previous results⁶, the primary aim in this study was to examine whether an irreversibly adsorbed Alb layer would undergo any further structural changes for residence times of up to six months. And, if so, whether this would begin to cause platelet adhesion to ensue if these structural changes exceeded the same critical level of unfolding needed to support platelet adhesion that was determined from our prior studies.

The platelet adhesion levels on the OH and CH₃ SAMs preadsorbed with Alb from 10.0 mg/mL bulk solutions for residence times of 0 day, 3 months, and 6 months are shown in Fig. 2. As shown, there was minimal platelet adhesion at the 0-day time point for both types of surfaces, with no statistically significant difference between them. The difference in the adhesion levels on the two SAMs, became apparent after 3 months and strongly apparent after 6 months of aging, with these results following the same general trend observed for the effect of aging time on the adsorbed conformation of the Alb layer (as shown in Fig. 1); i.e., more pronounced changes on the hydrophobic CH₃ SAM compared to the hydrophilic OH SAM for both 3 and 6 months of aging and an increased degree of unfolding with time. The platelet adhesion response on the OH SAM preadsorbed with Alb and aged over 6 months showed a significant increase compared to the two earlier time points (which were not statistically significantly different from each other). Similarly, there was a strong increase in the platelet response at the 6 month time point compared to the values at 3 months and day 0 on the CH₃ SAM, with a statistically significant increase in platelet adhesion also occurring between the 0 and 3 month time periods. Coupled with the information on the conformation of the adsorbed Alb layer on the SAM surfaces from Fig. 1, these results suggest that increased platelet adhesion to the adsorbed Alb layers with increasing residence times may be driven by the time-dependent conformational changes in the adsorbed Alb layer.

To examine the correlation between platelet adhesion and the degree of aging-induced unfolding and compare it with our prior studies⁶, the platelet adhesion levels on these aged Alb layers were plotted as a function of the degree of time-dependent unfolding as represented by the measured percentage loss of α -helix, with these data points overlaid on a graph of our previous data set⁶. It should be noted that the degree of unfolding in the adsorbed Alb from our previous data was induced by adsorbing the Alb from different solution concentrations on different surface chemistries, not by aging the adsorbed protein (i.e., previous data represents a 0-day aging condition). As seen in Fig. 3, the relationship between platelet adhesion and aging-induced unfolding of adsorbed Alb actually follows the previous trends remarkably well. When the degree of aging-induced unfolding of the

adsorbed Alb was below the previously determined 34% critical level, platelet adhesion was minimal, in close agreement with the previously observed results⁶. However, under conditions where the aging-induced degree of unfolding increased above this level, platelet adhesion was substantially increased, with the net trend generally following the previously designated relationship. Given the presence of the various proteases and other reactive species in the blood stream, in vivo conditions can be expected to be more severe than the conditions represented in this in vitro study, leading to even more rapid degradation of the adsorbed Alb layer with a likely onset of a platelet adhesion response.

Unfortunately, adsorbed state CD only provides information related to the secondary structural composition of adsorbed proteins. While this information provides important insight into one level of structure, it obviously is not sufficient to fully understand how the aging process influenced the adsorbed albumin, which could also have undergone various degrees of other types of structural rearrangements, including aggregation⁵⁷ or polymerization⁵⁸ on the surface, and the relationship between these processes and the behavior an irreversibly adsorbed protein layer in vivo as it ages. While further studies are certainly necessary to address these more complex issues, the results of these present studies clearly show that the aging of irreversibly adsorbed Alb on these surfaces over a 6-month period induced substantial changes in the secondary structure of the protein, with the degree of these changes correlating well with increased platelet adhesion compared to the non-aged Alb layer.

Although alkanethiol SAMs on gold represent some of the most widely used model surfaces for the study of protein adsorption behavior because of the very well characterized and controlled surface chemistries that they provide, they are not used in cardiovascular biomaterial applications. However, the SAM surface chemistries used in these studies (i.e., CH₃ and OH surface groups) represent very commonly encountered functional groups for many different types of polymeric biomaterials and they represent general extremes of hydrophilicity, ranging from the highly hydrophobic CH₃ SAM surface (static water contact angle = $101^{\circ} \pm 2^{\circ}$) to highly hydrophilic OH SAM surface (static water contact angle = $18^{\circ} \pm 2^{\circ}$)³⁵. The results of this study can thus be considered to be potentially relevant to a broad range of polymeric biomaterial surface chemistries under the conditions applied (i.e., adsorbed Alb aged for up to 6 months in phosphate buffer at 37°C). Further testing is required, of course, to definitively address how broadly the protein adsorption behavior determined from this study applies to actual biomaterial surfaces, as well as to other proteins and other solution conditions.

Conclusions

This study is the first one to our knowledge to examine long-term, aging-induced conformational changes in an adsorbed plasma protein and their effect on platelet adhesion. The results conclusively illustrate that an irreversibly adsorbed, tightly packed layer of Alb that is aged in phosphate buffer solution becomes structurally altered with increasing residence time, leading to enhanced platelet adhesion. The platelet adhesion levels on the CH₃ and OH SAMs agree well with the results from our previous study⁶, which suggest that Alb is capable of mediating platelet adhesion, but only if it undergoes unfolding beyond a critical degree. Additional studies are of course warranted to evaluate if the same behavior that we observed under these studies will be found if the adsorbed Alb is aged in a solution containing physiological levels of Alb (and/or other proteins), with the adsorbed Alb then possibly being continually replaced by the Vroman effect, thus avoiding individual adsorbed proteins from undergoing the same aging phenomenon. However, we hypothesize that once tightly adsorbed, an adherent Alb layer would be stable with little tendency to be displaced by Alb or other proteins in solution. Obviously, in vivo conditions are much more complex,

with numerous other proteins being in the system in addition to the presence of proteolytic enzymes, which may also substantially influence the structure of the adsorbed protein layer. Further research is needed to address these other very interesting issues.

Since Alb is the most abundant plasma protein¹¹, and has been shown in some studies to only exhibit minimal Vroman displacement by other plasma proteins once it adsorbs^{19, 20}, it can be expected to potentially be a major component of the adsorbed layer of proteins on biomaterial surfaces in vivo. The results of our present study may thus have important implications regarding the development of biomaterials with improved hemocompatibility for cardiovascular applications, as they indicate that the relationship between platelet adhesion and protein adsorption is more complex than previously understood, and that blood compatibility is not simply related to the amount of adhesive proteins, such as Fg, that adsorb to a biomaterial surface. The potential time-dependent unfolding of proteins adsorbed from plasma on biomaterial surfaces may also play a role in mediating platelet adhesion, thus possibly contributing to late-thrombotic responses, as observed on vascular stents after prolonged exposure to blood^{22, 25–27}.

Combining these results with our previous studies^{5, 6}, we propose that the concept of preventing protein adsorption altogether as a means of achieving hemocompatibility may indeed be effective, but unnecessarily restrictive. Our results suggest that design objectives should instead be focused on attaining conditions where protein adsorption is allowed to occur, but in a reversible manner that minimally perturbs the protein's native-state structure, thus not allowing aging-induced unfolding processes to occur before the protein desorbs from the surface to be replaced by other adsorbing proteins. While still challenging, this represents a much less restrictive and thus possibly much more attainable and maintainable set of conditions for the development of truly hemocompatible biomaterials for cardiovascular applications.

Acknowledgments

The authors thank numerous volunteers for kindly donating blood for these studies, as well as the staff at the Redfern Health Center at Clemson University for their assistance with the blood-draw process. We are grateful to Mr. Aby Abraham Thyparambil for technical assistance. We are also grateful to Dr. James Harriss at Clemson University, for fabrication of the gold-coated surfaces used in our studies. This project was partially supported by NIH Grant Numbers P20 RR-016461 from the National Center for Research Resources and P20 RR-021949 from the National Institute of Bioimaging and Bioengineering.

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Figure 1. Secondary structural changes in Alb adsorbed on OH (left) and CH₃ (right) SAMs, from 10.0 mg/mL bulk solution concentration for time points of day 0, 3 months, and 6 months (n=10 for each treatment condition, mean $\pm$ 95% CI). The native solution structure of Alb is included for comparison. (* denotes that mean values are not shown to be statistically different, i.e., $p > 0.05$)

Figure 2. Platelet adhesion to OH and CH₃ SAMs preadsorbed with Alb from 10.0 mg/mL bulk solution concentration, for residence times of 0 day, 3 months, 6 months ($n=6$, mean $\pm$ 95% CI). * denotes that mean values are not shown to be statistically different, $p > 0.05$.

Figure 3.

Platelet adhesion to adsorbed Alb aged on the OH and CH₃ SAM surfaces for ‘aging’ times of 0 day, 3 months, and 6 months, as a function of the degree of unfolding, as measured by the percentage loss in α-helix. These data points are overlaid for purposes of comparison on the previous results obtained by our group [6] for platelet adhesion to Alb preadsorbed from 0.1, 1.0 and 10.0 mg/mL bulk solution concentrations to induce unfolding without aging. (Each point represents the mean of six values for each SAM surface).

Table 1

Amounts of Alb adsorbed on CH₃ and OH SAM surfaces from 10.0 mg/mL bulk solution concentrations, for 0 day, 3 month, and 6 month residence times. (n= 6, mean $\pm$ 95% CI).

Surface	Day 0 [$\mu\text{g}/\text{cm}^2$]	3 months [$\mu\text{g}/\text{cm}^2$]	6 months [$\mu\text{g}/\text{cm}^2$]
CH ₃	2.65 $\pm$ 0.24	2.63 $\pm$ 0.30	2.83 $\pm$ 0.18
OH	1.63 $\pm$ 0.23	1.64 $\pm$ 0.19	1.73 $\pm$ 0.16