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The Relationship between Platelet Adhesion on Surfaces and the Structure versus the Amount of Adsorbed Fibrinogen

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Abstract

While platelet adhesion to biomaterial surfaces is widely recognized to be related to adsorbed fibrinogen (Fg), it has remained controversial whether platelet adhesion is in response to the adsorbed amount or the adsorbed conformation of this protein. To address this issue, we designed a series of platelet adhesion studies to clearly separate these two factors, thus enabling us to definitively determine whether it is the amount or the conformation of adsorbed Fg that mediates platelet response. Fg was adsorbed to a broad range of surface chemistries from a wide range of solution concentrations, with the amount and conformation of adsorbed Fg determined by absorbance and circular dichroism (CD) spectropolarimetry, respectively. Platelet adhesion response was determined by lactate dehydrogenase (LDH) assay and scanning electron microscopy (SEM). Our results show that platelet adhesion is strongly correlated with the degree of adsorption-induced unfolding of Fg ($r^2 = 0.96$) with essentially no correlation with the amount of Fg adsorbed ($r^2 = 0.04$). Platelet receptor inhibitor studies using an RGDS peptide reduced platelet adhesion by only about 50%, and SEM results show that adherent platelets after RGDS blocking were much more rounded with minimal extended filopodia compared with the unblocked platelets. These results provide definitive proof that the conformation of adsorbed Fg is the critical determinant of platelet adhesion, not the amount of Fg adsorbed, with adsorption-induced unfolding potentially exposing two distinctly different types of platelet binding sites in Fg; one that induces platelet adhesion alone and one that induces both platelet adhesion and activation.

1. Introduction

The development of materials that are truly blood-compatible for use in cardiovascular applications (e.g., catheters, stents, vascular prostheses, hemodialysis membranes) still remains as one of the most challenging problems in the field of biomaterials. While the specific material design features that provide blood compatibility are still not well defined [1], it is widely recognized that blood compatibility is largely governed by the adsorbed proteins that rapidly coat a material surface following exposure to blood, which are somehow subsequently recognized by receptors that are active in non-activated platelets. This recognition leads to platelet adhesion, activation, and subsequent thrombus formation, which can result in many different adverse physiological responses including vascular occlusion, pulmonary embolism, organ failure, and stroke [2].

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While blood contains numerous different types of proteins, any of which may adsorb to a biomaterial surface, fibrinogen (Fg) has been identified as one of the most important types of adsorbed proteins that induces a platelet adhesion response. Although the specific mechanisms mediating platelet adhesion to adsorbed Fg remain to be fully defined, it is widely cited in the biomaterials field that the amount of adsorbed Fg is one of the most important determinants of the biocompatibility of a given biomaterial [3-6]. Others, however, have found evidence that the conformational state of adsorbed Fg may actually be the more important mediator of platelet adhesion response [7-9]. This belief is further supported by the simple fact that non-activated platelets do not strongly interact with Fg when it is in its soluble, native conformation in the blood stream, but readily adhere and activate after contacting this same protein when Fg is adsorbed on a surface. This implies that adsorption induces some conformational change in Fg that exposes otherwise hidden sites that are recognized by platelet receptors.

One of the primary problems in differentiating between these two factors (i.e., amount versus conformation of Fg) is that surfaces that tend to strongly adsorb Fg, leading to a large amount of protein on the surface, also tend to induce a large degree of conformational unfolding of the protein when it adsorbs. Although commonly coupled in this manner, it is very important to differentiate between these two potential mediators of platelet adhesion because they lead to two very different approaches for biomaterials design to promote blood compatibility. If the amount of adsorbed Fg mediates platelet adhesion, then biomaterial surfaces should be designed to minimize the amount of Fg adsorbed irrespective of its conformational state. Alternatively, if platelet adhesion is mediated by the degree of adsorption-induced Fg unfolding, then surfaces should be designed to adsorb Fg in a manner that maximally retains its native-state structure irrespective of the amount of Fg that is adsorbed.

To address this critical issue, we conducted a set of platelet adhesion studies that were specifically designed to uncouple the amount of adsorbed Fg from the degree of adsorption-induced Fg unfolding and thereby definitively determine which of these two factors is the most important mediator of platelet adhesion response. This was accomplished by adsorbing Fg to a broad range of surface chemistries from a wide range of solution concentrations to obtain a large set of systems with differing amounts and conformational states of adsorbed Fg. The amount of Fg adsorbed and its degree of adsorption-induced unfolding was then determined by absorbance and circular dichroism (CD) spectropolarimetry, respectively, with a lactate dehydrogenase (LDH) assay and scanning electron microscopy (SEM) used to assess the platelet response. Additional studies were conducted using Arg-Gly-Asp-Ser (RGDS) peptides in the platelet suspension to block $\alpha_{IIb}\beta_3$ platelet integrins, to assess how adsorption-induced unfolding of Fg influences the involvement of this receptor in the overall platelet adhesion response.

2. Materials and methods

2.1 Gold substrates

18 mm square cover glasses (VWR Scientific, Catalog No. 48368-040) were used as substrates for the platelet adhesion experiments, while quartz slides ($0.375'' \times 1.625'' \times 0.0625''$, Chemglass) were used for CD experiments. All substrates were cleaned at 50°C by immersion in a piranha solution (7:3 v/v H_2SO_4/H_2O_2) for at least 30 minutes, followed by a Radio Corporation of America (RCA) basic wash (1:1:5 v/v $NH_4OH/H_2O_2/H_2O$), and this procedure was repeated twice. These substrates were then rinsed with 200-proof ethanol (Pharmco-Aaper; Catalog No. 11100200), followed by nanopure water and then dried under a stream of nitrogen gas.

The cleaned cover glasses were then coated with a 50 Å chromium adhesion layer followed by 1000 Å of gold, while the quartz slides for CD were coated with 30 Å of chromium and 100

Å of gold, via a thermal vapor deposition (TVD) evaporator (Model E 12 E, Edwards High Vacuum Ltd.), prior to SAM formation on these substrates.

2.2 Formation of self-assembled monolayers (SAMs) of alkanethiols

The following alkanethiols were used for creating the SAM surfaces:

- 1-Dodecanethiol ($\text{SH}-(\text{CH}_2)_{11}\text{CH}_3$; Aldrich; CH_3),
- 11-(2,2,2-Trifluoroethoxy) undecane-1-thiol $\text{SH}-(\text{CH}_2)_{11}\text{OCH}_2\text{CF}_3$; Aldrich; CF_3),
- 11-Amino-1-undecanethiol, hydrochloride ($\text{SH}-(\text{CH}_2)_{11}\text{NH}_2\text{HCl}$; Prochimia; NH_2),
- 11-Mercaptoundecanoic acid ($\text{SH}-(\text{CH}_2)_{11}\text{COOH}$; Aldrich; COOH), and
- 11-Mercapto-1-undecanol ($\text{SH}-(\text{CH}_2)_{11}\text{OH}$; Aldrich; OH).

SAM surfaces were prepared as per the established protocols [10,11] described earlier [12]. Pure alkanethiol solutions (1.0 mM) were prepared in 200-proof ethanol (Pharmco-Aaper; Catalog No. 111000200). Prior to immersion in the alkanethiol solutions for 24 hours, the gold substrates were cleaned by dipping them for 1 minute each in a modified piranha wash (4:1 v/v $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$), followed by a Radio Corporation of America (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 then rinsed copiously with 100% ethanol.

All SAM surfaces were cleaned to remove any traces of hydrophobic contaminants on their surface prior to surface characterization and protein adsorption [13]. The CH_3 and OCH_2CF_3 SAMs were cleaned by sonication in ethanol, hexane and ethanol, and then rinsed with nanopure water. The NH_2 , COOH , and 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), order to block off any hydrophobic defect sites (e.g. grain boundaries), and then rinsed thoroughly with acetone, ethanol and nanopure water to remove loosely- bound Triton.

2.3 Contact angle measurement

The advancing contact angle measurements on the SAM surfaces were carried out using a CAM 200 Optical Contact Angle/Surface Tension Meter (KSV Instruments Ltd.) and the CAM 200 software provided with the instrument. The cleaned SAM surfaces were mounted on the stage of the instrument, and the contact angles for six separate drops of nanopure water were measured on each surface.

2.4 Buffers

The protein adsorption experiments were carried out using a 25 mM potassium phosphate buffer (PBS, pH 7.4), which was prepared by combining appropriate amounts of the mono- and dibasic salts (Sigma-Aldrich) to maintain the pH at 7.4. 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 0.1U/mL apyrase [14]. 2.5 mM CaCl_2 and 1.0 mM MgCl_2 was added to the PSB to give a platelet suspension buffer with metal ions (PSB+MI).

2.5 Protein adsorption

Human Fg (FIB3, plasminogen, von Willebrand factor and fibronectin depleted; Enzyme Research Laboratories, South Bend, IN) was dissolved in phosphate buffer solution (pH 7.4), to prepare the protein stock solutions, and protein adsorption was carried out, as described previously [12]. Briefly, protein adsorption on the SAM surfaces was carried out at three different protein concentrations of 0.1 mg/mL, 1.0 mg/mL, and 10.0 mg/mL, in order to obtain adsorbed Fg layers with a wide range of surface conformations and coverages. These are

essential in delineating the effect of secondary structure and surface coverage of the adsorbed Fg layer on the SAM surfaces on platelet adhesion.

The cleaned SAM surfaces were incubated in phosphate buffer (pH 7.4) in a six-well-plate (Corning Costar, Catalog No. 3506) and then a suitable amount of protein stock solution was added, ensuring that the tip of the pipette was held below the air-water interface to avoid denaturation of the protein at this interface. The SAM surfaces were maintained fully immersed in the protein solution for 2 h, after which an infinite dilution step was carried out to wash away the bulk protein solution in addition to any loosely adherent protein prior to removal of the SAMs from the buffer solution. Following this infinite dilution step, the SAM surfaces were able to be safely removed from the pure buffer solution without dragging the surfaces through the denatured protein film that can be expected to present at the liquid-air interface if the protein solution had not been replaced with pure buffer prior to surface removal.

The SAM surfaces with preadsorbed protein were then either used for CD studies to analyze the structure of the adsorbed layer, or incubated with the platelet suspension.

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

CD spectropolarimetry was done using a Jasco J-810 spectropolarimeter (Jasco, Inc., Easton, MD) to determine the native and adsorbed secondary structures of Fg, as well as the surface coverage of adsorbed protein, as described earlier [12]. Special high-transparency quartz cuvettes (Starna Cells, Inc., Atascadero, CA) were used for determining the native solution structure, while the adsorbed structure of Fg on the SAM surfaces was determined using a cuvette custom-designed for maximizing the signal-to-noise ratio [12]. The ellipticity (θ , in mdeg) was converted to standard units of $\text{deg}\cdot\text{cm}^2/\text{dmol}$ (designated as $[\theta]$) using the following equation [15,16]:

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

where θ is the molar 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 nm [17], we used the height of this absorbance peak (A_{195}) for constructing a calibration curve of A_{195} vs. C_{soln} for various known concentrations of Fg, as described in our previous work [12]. 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 $\text{mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (or cm^2/g) and L is the path length of the cuvette.

The term “ $C_{\text{soln}} \cdot L$ ” in eq. 2 (above) has units of g/cm^2 , which is equivalent to the amount of protein adsorbed per unit area. Assuming that the absorbance is dependent on the total amount of protein present per unit area through which the beam of light 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 amount of Fg adsorbed per unit area on the SAMs (i.e., Q_{ads}). The validity of this method for measuring the surface coverage of adsorbed protein has been

confirmed by independent measurement of Q_{ads} from the thickness of the adsorbed protein film obtained by ellipsometry [12] using de Feijter's formula [18].

Hence, while calculating the molar ellipticity for the adsorbed Fg layer on the SAMs, the term " $C_{\text{soln}} \cdot L$ " in eq. 1 can be replaced by the term Q_{ads} to give the following equation:

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

The CD spectra (molar ellipticity vs. wavelength) thus obtained were deconvoluted using the SP-22X algorithm and analyzed using the SELCON and CONTIN/LL software packages to quantify the percentage of α -helix and β -sheet content of the native/adsorbed protein [19].

2.7 Platelet adhesion

All protocols pertaining to the use of whole blood and platelets were approved by the IRB (Institutional Review Board) and IBC (Institutional Biosafety Committee) at Clemson University. The blood (25 mL) was collected via venipuncture from healthy, aspirin-free human volunteers at the Redfern Health Center at Clemson University in BD Vacutainer tubes (Catalog No. 364606, Becton-Dickinson, Franklin Lakes, NJ) containing an acid-citrated dextrose (ACD) anticoagulant. It is important to note that the first few mL of blood was discarded, as it is rich in clotting factors, and then the remaining 25 mL was collected.

Platelet rich plasma (PRP) was generated by centrifuging the ACD-anticoagulated blood (225 g, 15 min, 25°C) using a Beckman Coulter Allegra 6R centrifuge (Beckman Coulter, Fullerton, CA). Platelets were separated from the PRP via a gel separation method [14]. A liquid chromatography column (Sigma-Aldrich, Catalog No. C4169) was filled Sepharose 2B (Sigma, Catalog No. 2B-300) and the column was allowed to equilibrate with PSB. The PRP was then carefully pipetted onto the column and then allowed to pass into the column. PSB was added to top of the column from a reservoir, while the setup was kept running. Fractions were collected from the column, with the platelet-rich fractions being identified by their increased turbidity. The platelet-rich fractions that had maximum turbidity were pooled, and then the platelet concentration was measured using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA). The platelet concentration was adjusted to 10^8 platelets/mL with PSB, and CaCl_2 and MgCl_2 were added to yield 2.5 mM and 1.0 mM final concentrations of these salts in the platelet suspension, respectively. The platelet suspension was allowed to rest for 30 min at 37°C, and then added to the protein-coated SAM surfaces and allowed to adhere for 1 h at 37°C.

At the end of the platelet adhesion step, the suspension was aspirated from each well, and the non-adherent platelets were rinsed away by filling and aspirating the wells five times with PBS. Platelet adhesion levels were quantified using a lactate dehydrogenase (LDH) assay, and the morphology of the adherent platelets was visualized using scanning electron microscopy (SEM).

A control platelet adhesion study was also performed with the PSB being supplemented with 4 mg/mL bovine serum albumin (BSA; Sigma-Aldrich), which is conventionally used as a blocking agent for non-specific interactions, in order to examine its effect on the adhesion of the washed platelets to the adsorbed Fg on the SAM surfaces. The results from this study showed that the addition of BSA had no significant effect on platelet adhesion (see Figure S2 of the Supporting Information section).

2.8 Measurement of platelet adhesion using lactate dehydrogenase (LDH) assay

Platelet adhesion was quantified by measuring the lactate dehydrogenase (LDH) released when the adherent platelets were lysed with a Triton-PSB buffer (2 % v/v Triton-X-100 in PSB), using a CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI). This is a modification of the methods used previously [20,21]. A calibration curve was constructed using a known number of platelets, counted using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA), and the platelet adhesion on the SAM surfaces was determined from this calibration curve.

2.9 Scanning electron microscopy (SEM)

The samples for SEM were fixed for 30 min at room temperature using a special fixing buffer (3% glutaraldehyde, 0.1 mM sodium cacodylate, pH 7.4), after which they were rinsed with PBS thrice. To dehydrate the adherent platelets, the samples were incubating in ascending ethanol:water mixtures (50%, 60%, 70%, 80%, 90% and 99% ethanol) for 10 min. The samples were finally treated with 0.02 mL of hexamethyldisilazane (Sigma) and allowed to dry overnight. The morphology of the adherent platelets was examined using a FESEM-Hitachi S4800 scanning electron microscope (Electron Microscopy Facility, Advanced Materials Research Laboratory, Clemson University, Pendleton, SC).

2.10 SDS-PAGE analysis of platelet suspension

It is essential to ensure that the platelet suspension was free of any plasma proteins, which may have been retained in the platelet suspension from blood plasma or released from the platelets due to platelet activation during the separation process. The platelet suspensions obtained from two separate gel separation runs were centrifuged at 1200 rpm, 1500 rpm, and 1800 rpm, and the supernatant was aspirated and analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for any traces of residual plasma proteins. Silver staining was done using a Silver SNAP Stain Kit II (Pierce, Rockford, IL), which provides a rapid and highly sensitive detection system for sub-nanogram levels of proteins in polyacrylamide gels.

2.11 Inhibition of platelet-protein interactions using Arg-Gly-Asp (RGD)-containing peptides

In order to inhibit interactions between RGD-binding platelet receptors and Fg, the platelet suspension was incubated for 30 min at room temperature with 300 μ M of an Arg-Gly-Asp-Ser (RGDS) peptide (Calbiochem, La Jolla, CA), which has been shown to be sufficient to inhibit platelet aggregation [22]. These platelets were then allowed to adhere on the CH₃ and OH SAMs preadsorbed with 0.1 mg/mL protein solution, after which the platelet adhesion was quantified using an LDH assay and the morphology of the adherent platelets was examined using SEM.

As a control, platelets incubated with 300 μ M of an Arg-Gly-Glu-Ser (RGES) peptide (American Peptide Company, Sunnyvale, CA) were allowed to adhere on the SAM surfaces preadsorbed with Fg, and the adhesion characteristics were analyzed.

2.12 Study using polyclonal antibodies against Fg and Alb

Polyclonal antibodies against human Fg and Alb (Anti-Fg/Alb Rabbit pAb; Calbiochem, La Jolla, CA) at a concentration of 4 μ g/mL were incubated for 30 min at room temperature, on the Fg coated surfaces in order to examine the role of any non-specific platelet binding to the adsorbed Fg layer. Platelet adhesion was characterized using an LDH assay.

2.13 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.

3. Results and Discussion

3.1 Amount of Fg adsorbed on SAM surfaces

The amount of Fg adsorbed on alkanethiol self-assembled monolayer (SAM) surfaces on gold functionalized with OH, COOH, NH₂, CF₃, and CH₃ function groups under solution concentration conditions of 0.1, 1.0, and 10 mg/mL of Fg was measured spectroscopically by absorbance at 195 nm [12]. The results presented in Figure 1 show that the amount of Fg generally increased with increased surface hydrophobicity (see Table 1), and indicate that the amount of Fg adsorbed was dependent on the solution concentration from which it was adsorbed, with significantly greater amounts of Fg being adsorbed on a given SAM surface for each increasingly higher solution concentration. This can be explained by the fact that at a high solution concentration, the rate of diffusion of protein molecules to the surface is higher than that from a more dilute solution [23], and has been shown to be independent of the surface chemistry by Wertz et al. [24]. Therefore, an adsorbed protein molecule has less time to unfold and spread out over the surface before other protein molecules adsorb next to it, blocking further spreading on the surface [23,25], leading to higher surface coverages of adsorbed Fg.

The minimum amount of Fg adsorbed under any our conditions was 0.8 $\mu\text{g}/\text{cm}^2$, as shown in Figure 1. Given that the Fg molecule has dimensions of about $5.0 \times 5.0 \times 47.0 \text{ nm}^3$ [26], which yields a theoretical surface coverage at saturation between 0.24 $\mu\text{g}/\text{cm}^2$ and 2.26 $\mu\text{g}/\text{cm}^2$ for side-on versus end-on adsorption [24], respectively, the amounts of Fg adsorbed on the SAM surfaces from each bulk solution condition clearly show that our surfaces were saturated with the adsorbed protein. The values of surface coverage obtained for preadsorption with 0.1 and 1.0 mg/mL Fg lie between the theoretical maxima for side-on and end-on adsorption of Fg, suggesting a tight monolayer coverage. Adsorption from the 10.0 mg/mL Fg solution concentration led to surface coverage values well beyond the theoretical maximum for monolayer coverage of Fg, indicating multilayer adsorption.

We expect the Fg molecules to adsorb on the SAMs in a wide range of orientations, which would play a role in the platelet adhesion response, but it is difficult to get accurate information on the orientation of the adsorbed Fg layer using CD spectropolarimetry. Overall, our results indicate that the Fg molecules preadsorbed from 0.1 mg/mL and 1.0 mg/mL bulk solution concentrations are arranged in a mixture of side-on and end-on orientations, since the surface coverage lies between the theoretical maxima for these two orientations. A recent study of Fg adsorption showed that the majority of Fg molecules are adsorbed end-on when adsorption is carried out at Fg solution concentrations greater than 0.2 mg/mL [27], which suggests that as the Fg solution concentration for preadsorption increases, the fraction of Fg molecules adsorbed in an end-on orientation will increase, thereby increasing the surface coverage at higher Fg solution concentrations.

3.2 Conformation of adsorbed Fg on SAM surfaces

The secondary structural composition (percentage α -helix and β -sheet content) of Fg adsorbed on the SAM surfaces, which was determined by adsorbed-state CD spectropolarimetry [12], is presented in Figure 2, with the native/solution structure included for comparison. As shown, the combined variation of surface chemistry and protein solution concentration provided a wide range of average conformational states of the adsorbed Fg on the SAM surfaces. As the surfaces became more hydrophobic (see Table 1), Fg underwent a higher degree of adsorption-induced

conformational change (i.e., loss of α -helix, accompanied by increase in β -sheet), which is consistent with observations made by other groups [28,29]. This effect can be understood based on the increasingly strong thermodynamic driving force to induce protein unfolding on a surface to reduce the overall solvent accessible surface area of the system as a surface becomes increasingly hydrophobic [12]. Similarly, the degree of adsorption-induced loss of α -helix was more prominent when the protein was adsorbed from lower solution concentrations. This effect is particularly evident when Fg was adsorbed from the 10.0 mg/mL Fg solutions (Figure 2C), in which case the Fg adsorbed so rapidly that it had little time to unfold before the surface became saturated, resulting in minimal conformational changes such that the secondary structure of the adsorbed protein was much closer to the native solution structure of Fg.

As with the amount of Fg adsorbed, this concentration effect can be understood to be due to the relationship between the kinetics of transport of the protein to the surface by diffusion compared to its rate of unfolding on the surface after it adsorbs. At lower protein solution concentrations, the transport of protein molecules to the surface has been shown to be much slower [24], thus providing adsorbed protein molecules with more time to unfold and spread out on the surface before they come in contact with neighboring adsorbed protein molecules, which inhibits further spreading [23,25].

Although it would be ideal to know the full structural composition of the adsorbed protein layers in each of these cases, this information cannot be obtained using CD studies. Results obtained via CD represent the single average secondary structural content of the adsorbed Fg, and CD studies cannot distinguish between discrete sub-populations of states and a uniform distribution of a single population. Hence, we cannot rule out the possibility that the multilayer observed for preadsorption with 10.0 mg/mL Fg solution may be composed of more than one population, with varying degrees of unfolding.

3.3 Platelet adhesion to adsorbed Fg on SAM surfaces

Prior to conducting platelet adhesion experiments, SDS-PAGE analysis was carried out to test for traces of residual proteins in the platelet suspension that could complicate our study by potentially displacing the preadsorbed Fg through Vroman effects [25,30]. As seen in Figure S1 in the Supporting Information section, this analysis shows that residual plasma proteins in the platelet suspension were below detectable limits, thus providing confidence that the measured platelet adhesion response was directly mediated by the preadsorbed layer of Fg.

Figure 3 shows the platelet adhesion levels to preadsorbed Fg on each of the five SAM surfaces at each of the three different Fg solution concentrations. Each platelet adhesion value represents the mean of a set of six independent measurements ($n = 6$), with each measurement being conducted using a different blood sample. As is clearly evident, the platelet adhesion levels observed were strongly influenced by both the surface chemistry as well as the solution concentration from which the protein was adsorbed. The platelet adhesion response increased with increasing surface hydrophobicity for each solution concentration and decreased with increased solution concentration for each surface chemistry, except for the OH-SAM surface where solution concentration did not significantly influence the platelet adhesion response.

To investigate whether platelet adhesion was more strongly mediated by the amount of adsorbed Fg or the conformation of adsorbed Fg, the data presented in Figures 1-3 were re-plotted to show the platelet adhesion response versus the amount of adsorbed Fg (Figure 4A) and versus the degree of adsorption-induced Fg unfolding as represented by the percent loss in α -helix content of the protein (Figure 4B). As shown in Figure 4A, platelet adhesion appears to correlate fairly strongly with the amount of Fg adsorbed when the protein is adsorbed from a given solution concentration. However, when the universal data set is considered, the correlation between platelet adhesion and the amount of adsorbed Fg is essentially zero ($r^2 =$

0.04). In distinct contrast to this, the platelet adhesion response correlates very strongly with the adsorption-induced loss in α -helix ($r^2 = 0.96$), as seen in Figure 4B, when the results for all surfaces and Fg solution conditions are combined. These results strongly indicate that under these experimental conditions platelet adhesion is actually not in response to the amount of Fg adsorbed, but rather it is controlled by the conformational state of the protein on the surface, and provide an explanation for why many previous studies have reported results that imply that platelet adhesion is in response to the amount of adsorbed Fg. Referring to the data presented in Figure 4A, platelet response does correlate with the amount of adsorbed Fg for a given solution concentration. However, close evaluation for each of the three individual data sets shown in Figure 4A for each solution concentration in comparison with Figure 4B reveals that within a given solution concentration, the change in the conformation of the adsorbed protein also tracks very closely with the adsorbed amount of Fg. It is only when the results are presented as a universal data set combining the results from each different solution concentration that these two effects are clearly separated, which then reveals that platelet adhesion is actually responding to the change in adsorbed structure of the Fg with little influence from the amount of Fg adsorbed. This situation is particularly demonstrated by comparing the platelet response to the COOH and OH SAMs with Fg preadsorbed from the 10.0 mg/mL Fg solution in comparison to the platelet response to the CH₃ SAM with Fg preadsorbed from the 1.0 mg/mL Fg solution (see associated data points in Fig. 4A). In each of these cases, the amount of adsorbed Fg was nearly the same while the degree of change in conformation of the adsorbed Fg on the CH₃ SAM was much greater than on either the COOH or the OH SAMs (see associated data points in Fig. 4B). If platelet adhesion was primarily governed by the amount of adsorbed Fg, then the platelet response would be expected to be nearly the same for each of these conditions. However, the platelet response on the CH₃-SAM (with the much greater degree of structural change in the adsorbed Fg) is nearly four times greater than on the COOH and OH SAMs. This clearly illustrates that the platelets are responding to something other than simply the amount of Fg adsorbed on the surface. The strong correlation shown in Fig. 4B indicates that the degree of structural unfolding of the adsorbed Fg is the much greater determinant of the platelet response than the amount of Fg adsorbed.

Additionally, it is also important to keep in mind that this study was focused on comparing the role of the conformation versus the amount of adsorbed Fg in mediating the platelet response. Other factors, such as the orientation, packing arrangement, and structural heterogeneity of the adsorbed Fg layer can be expected to also influence the overall bioactive state of the adsorbed layer of protein, and in turn the platelet adhesion response. However, the results from these present studies indicate that these other variables, which were not controlled or measured in this study, do not obscure the effect of the adsorbed conformation of Fg on platelet adhesion, as indicated by the strong correlation between these two factors as shown in Fig. 4B.

3.4 The role of RGD-specific receptors in mediating platelet adhesion to adsorbed Fg

Previous studies have shown that $\alpha_{IIb}\beta_3$ platelet integrins are involved in platelet adhesion to adsorbed Fg, with this receptor being effectively blocked by Arg-Gly-Asp-Ser (RGDS) peptide [22,31]. In order to probe the extent of the involvement of this platelet receptor as a mediator of the platelet adhesion response as a function of the degree of adsorption-induced Fg unfolding, we conducted additional studies for Fg adsorbed from the most dilute solution concentration (0.1 mg/mL) on the two SAM surfaces representing the extremes in hydrophobicity (OH and CH₃ SAMs). The platelet suspensions were treated with an RGDS peptide (300 μ M solution concentration) prior to exposure to the SAM surfaces preadsorbed with Fg to determine the extent that platelet adhesion is mediated by RGD-specific receptors (e.g., $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ platelet integrins) [32], with an Arg-Gly-Glu-Ser (RGES) peptide used as a non-bioactive control. The results of these studies are shown in Figure 5.

As presented in Figure 5, treatment of the platelets with the RGDS peptide resulted in a substantial decrease in platelet binding to adsorbed Fg on both of the SAM surfaces, while the RGEs peptide had no significant effect ($p > 0.05$) on platelet adhesion to Fg for either surface. RGDS-treatment of the platelets led to a similar extent of inhibition of platelet adhesion to adsorbed Fg on both the CH₃ and OH SAMs (58.3% on CH₃, 45.7% on OH SAM) compared to the normal (unblocked) platelet control. These results indicate that RGD-specific platelet receptors play a key role in mediating the interactions with adsorbed proteins, but only account for about half of the total platelet adhesion response.

This not surprising, considering the fact that the RGDS peptide has been shown to be a partial inhibitor of platelet-Fg interactions in a previous study [22], leading to a hypothesis that RGDS-bound platelet receptors can still interact with Fg but with lower affinity compared to their unbound state. Additionally, RGDS peptides have been shown to 'activate' the GPIIb/IIIa platelet receptors [33], and a recent study indicated that RGDS-treatment of washed platelets led to powerful activation of the $\alpha_2\beta_1$ platelet integrin without overall platelet activation [34]. This suggests that the role of GPIIb/IIIa in its RGDS-bound low-affinity state, as well as other platelet receptors cannot be ruled out in mediating the adhesion to RGDS-treated platelets to adsorbed Fg.

The fact that the RGDS peptide was unable to completely inhibit platelet interactions with adsorbed Fg suggests that the remaining level of platelet adhesion was either due to (i) non-specific platelet adhesion to exposed areas of the SAM surfaces and/or (ii) a specific receptor mediated process by non-RGD-specific platelet receptors to the adsorbed Fg.

To address this issue, we treated the adsorbed Fg with polyclonal antibodies against Fg (pAb-Fg), with polyclonal antibodies against Alb (pAb-Alb) serving as a control. As seen in Figure 5, the pAb-Fg inhibited platelet interactions with adsorbed Fg almost entirely, while the pAb-Alb had no significant effect on platelet adhesion. These results thus clearly indicate that the adhesion of RGDS-treated platelets to adsorbed Fg was not due to a non-specific platelet adhesion mechanism, but rather indicates that nearly 50% of the platelet adhesion response on both SAM surface was mediated by non-RGD-specific platelet receptors. Future studies are planned to elucidate what these non-RGD-specific receptors may be and what the motifs are that they are binding to in the adsorbed, unfolded Fg.

3.5 SEM analysis of platelet adhesion to adsorbed Fg

Scanning electron microscopy (SEM) was carried out to assess the morphology of the adherent platelets to Fg on the CH₃ and OH SAMs, as well as to analyze the effect of platelet-receptor blocking with RGDS-peptides on the adherent platelet morphology.

The platelets adherent to both the CH₃ SAM (Figure 6A) and the OH-SAM (Figure 6C) preadsorbed with 0.1 mg/mL Fg were considerably spread, with the expression of extensive filopodia (thus showing that our platelets were sensitive to activating surface conditions), with the primary difference between these two surfaces only being the number of adherent platelets per unit area as opposed to their morphology. In distinct contrast to this, the RGDS-treated platelets adherent to Fg adsorbed on both the CH₃-SAM surface (Figure 6B) and the OH-SAM surface (Figure 6D) did not exhibit any filopodial extensions and were only moderately spread with prominent granulomeres.

The difference in morphology of the RGDS-treated platelets compared to untreated platelets also strongly suggests that there may be at least two distinct set of receptors associated with platelet adhesion to adsorbed Fg. The RGD-specific receptor set is shown to mediate adhesion along with a high degree of activation (i.e., spreading, accompanied by extension of filopodia),

while the non-RGD-specific receptor set mediates platelet adhesion with minimal activation (i.e., minimal spreading, with no expression of filopodia).

4. Conclusions

The results from these studies indicate that the platelet response correlates strongly with the adsorption-induced conformational changes in Fg, as measured by the loss of α -helix. Adsorption-induced conformational changes apparently lead to the exposure of platelet binding motifs in Fg that are otherwise not accessible to platelet receptors in the native/solution state of this protein. The facts that the RGDS peptides only partially inhibited platelet adhesion to adsorbed Fg while pAb vs. Fg nearly totally blocked platelet adhesion suggests that non-RGD-binding platelet receptor(s) are also substantially involved in mediating platelet adhesion to adsorbed Fg. Further studies are planned to elucidate the specific platelet receptors and their recognition sites in Fg that mediate platelet adhesion responses to adsorbed Fg as a function of adsorption-induced unfolding.

These results call for a re-evaluation in the biomaterials field of our understanding of how Fg adsorption influences platelet response and our approach to design surfaces for improved hemocompatibility. Based on these results, studies that use adsorbed Fg as a measure of blood compatibility of biomaterials surfaces should be based on the ability of surfaces to maximally retain the native state of adsorbed Fg as opposed to focusing merely on the amount of Fg that is adsorbed. While the determination of adsorbed conformation of a protein is more difficult to assess than the adsorbed amount, this factor should correlate much more strongly with platelet response, and thus serve as a much more sensitive indicator of the tendency of a surface to resist a platelet-induced thrombotic response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fg adsorption from bulk solutions of 0.1 mg/mL, 1.0 mg/mL and 10.0 mg/mL on SAM surfaces functionalized with OH, COOH, NH₂, CF₃, and CH₃ surface groups (n=6, mean $\pm$ 95% CI).

Figure 2.

Changes in secondary structure of adsorbed human fibrinogen (Fg) adsorbed at (A) 0.1 mg/mL, (B) 1.0 mg/mL, and (C) 10.0 mg/mL on SAM surfaces determined by CD compared to its native conformation. (n=6, mean $\pm$ 95% CI). * denotes not significant, all other values are significantly different from one another; $p < 0.05$.

Figure 3. Platelet adhesion on SAM surfaces preadsorbed with Fg, assessed by LDH assay (n=6, mean $\pm$ 95% CI). * denotes no statistically significant difference, $p > 0.05$.

Figure 4.

Platelet adhesion to Fg adsorbed on SAM surfaces as a function of (A) the amount of adsorbed Fg, and (B) the loss of α -helix. (Each point represents the mean of six values for each SAM surface.)

Figure 5. Platelet adhesion on OH- and CH₃-SAM surfaces preadsorbed with Fg from 0.1 mg/mL solutions under various blocking treatments. The blocking treatments included RGDS and RGEs peptides and polyclonal antibodies specific for human Fg (pAb - Fg) and human albumin (pAb - Alb); (n=6, mean $\pm$ 95% CI). * signifies no significant difference, $p > 0.05$.

Figure 6. Platelet adhesion to the CH₃ (top row) and OH SAMs (bottom row) preadsorbed with 0.1 mg/mL Fg, under different blocking conditions: (A,C) unblocked Fg, (B,D) platelets treated with RGDS.

Table 1

Advancing contact angle data for the SAM surfaces (mean $\pm$ 95% CI, n =7).

	OH	COOH	NH ₂	CF ₃	CH ₃
Contact Angle (°)	17.6 $\pm$ 1.9	17.9 $\pm$ 1.3	47.6 $\pm$ 1.8	90.2 $\pm$ 0.8	100.9 $\pm$ 1.9