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The relationship between platelet adhesion on surfaces and the structure versus the amount of adsorbed fibrinogen

Balakrishnan Sivaraman, Robert A. Latour*

Department of Bioengineering, 501 Rhodes Engineering Center, Clemson University, Clemson, SC 29634, USA

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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].

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

* Corresponding author. Tel.: +1 864 656 5552; fax: +1 864 656 4466.
E-mail address: latourr@clemson.edu (R.A. Latour).

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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 min, 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 ($SH\text{-}(CH_2)_{11}CH_3$; Aldrich; CH_3),
11-(2,2,2-Trifuoroethoxy) undecane-1-thiol) $SH\text{-}(CH_2)_{11}OCH_2CF_3$; Aldrich; CF_3),
11-Amino-1-undecanethiol, hydrochloride ($SH\text{-}(CH_2)_{11}NH_2HCl$; Prochimia; NH_2),
11-Mercaptoundecanoic acid ($SH\text{-}(CH_2)_{11}COOH$; Aldrich; COOH), and
11-Mercapto-1-undecanol ($SH\text{-}(CH_2)_{11}OH$; 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 h, the gold substrates were cleaned by dipping them for 1 min each in a modified piranha wash (4:1 v/v H_2SO_4/H_2O_2), followed by a Radio Corporation of America (RCA) basic wash (1:1:5 v/v $NH_4OH/H_2O_2/H_2O$), 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), in 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 0.1 U/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 deg cm^2/dmol (designated as [θ]) using the following equation [15,16]:

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

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 "$\epsilon_{protein} \cdot L$" from Beer's Law, which can be written as:

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

where $\varepsilon_{protein}$ is the extinction coefficient of the protein in mL g^{-1} cm^{-1} (or cm^2/g) and L is the path length of the cuvette.

The term "$C_{soln} \cdot L$" 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