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Effects of Plasma Proteins on *Staphylococcus epidermidis* RP62A Adhesion and Interaction with Platelets on Polyurethane Biomaterial Surfaces

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ABSTRACT

Plasma proteins influence the initial adhesion of bacteria to biomaterials as well as interactions between bacteria and blood platelets on blood-contacting medical devices. In this paper, we study the effects of three human plasma proteins, albumin, fibrinogen (Fg), and fibronectin (Fn), on the adhesion of *Staphylococcus epidemidis* RP62A to polyurethane biomaterial surfaces, and also address how these three proteins affect bacterial interactions with human platelets on materials. Measurements of bacterial adhesion on polymer surfaces pre-adsorbed with a variety of proteins demonstrate that Fn leads to increased bacterial adhesion, with the order of effectiveness being Fn $\gg$ Fg > albumin. Immuno-AFM (atomic force microscopy) was used to assess the Fn adsorption/activity on surfaces and bacterial cell membranes by looking at molecular scale events. A correlation between molecular scale Fn adsorption and macroscale bacterial adhesion was observed, with an increased numbers of Fn-receptor recognition events measured on cell surfaces as compared to Fg-receptor recognition events, suggesting Fn is an important protein in bacterial adhesion. Monoclonal antibodies recognizing either the carboxyl-terminus or amino-terminus of Fn were coupled to AFM probes and used to assess the orientation of Fn adsorbed on a surface, with an increased amount of Fn carboxyl-terminus availability corresponding to higher bacterial adhesion. Interactions between bacteria and platelets were demonstrated with fluorescence and AFM imaging on the polyurethane surfaces, with albumin inhibiting bacteria-platelet interaction and platelet activation, and both Fg and Fn promoting adhesion of bacteria to platelets and apparent platelet activation, resulting in bacteria/platelet aggregation.

Keywords: Bacterial Adhesion; *Staphylococcus epidermidis*; Fibronectin; Bacteria-Platelet Interactions

1. Introduction

Bacterial adhesion to biomaterials causing microbial infection and poor tissue integration is one of the main problems associated with the use of blood-contacting devices. Bacteria adhere to a material surface, where they proliferate and colonize to form biofilms on implanted devices, eventually leading to a biomaterial associated infection. These infections are extremely difficult to treat by use of antibiotics alone due to the formation of biofilm, which consists of a microbial community entrapped within a polymer matrix secreted by the adherent microbes, and serves to protect the community from antimicrobial agents [1,2]. The increase in antibiotic resistance of bacterium has also contributed to the increase in infections that are refractory to treatment [3,4]. Thus, surgical removal and replacement of the implanted devices is often the only treatment, causing significant morbidity and mortality [5,6].

Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, rank first among the causative agent of nosocomial infections and represent the most common source of infections associated with the use of implanted medical devices such as intravascular and peritoneal dialysis catheters, prosthetic heart valves or orthopedic prostheses [7]. The defined virulence associated with *S. epidermidis* is its ability to colonize and form biofilm on biomaterials [8]. Pathogenic bacteria associated with biomaterial-centered infections have the potential to enter the human circulatory system where they can interact with platelets, resulting in platelet activation/aggregation. For example, *S. aureus*, another important staphylococcal bacterium found in nosocomial infection, leads to platelet activation/aggregation [9-12] and subsequently leads to abnormal blood function such

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as blood coagulation and thrombosis [13,14]. While interactions of *S. aureus* and platelets have been intensively studied, less information is available on *S. epidermids*. Further knowledge of the interactions between *S. epidermidis* and blood contact devices as well as the interactions between bacteria and platelets is crucial in developing effective strategies for preventing biomaterial-centered infection and its subsequent complications.

Bacterial adhesion is the critical step in the pathogenesis of biomaterial associated infection. Biomaterial surface chemistry characteristics have been shown to influence the initial adhesion and aggregation of *S. epidermidis* on biomaterials [15]. However, when a biomaterial is implanted in contact with blood, plasma proteins can rapidly adsorb onto the material surface to form a "conditioning film". This adsorbed protein layer may minimize the effect of biomaterial surface properties on bacterial adhesion [16,17] so that interactions between the bacteria and the proteins mediate bacterial adhesion. In vitro studies have shown that the presence of serum proteins generally suppresses initial bacterial adhesion due to the lack of a specific interaction between albumin and bacteria [18,19] while the effect of plasma proteins fibrinogen (Fg) and fibronectin (Fn) on bacterial adhesion is inconclusive. It has been reported that Fg or Fn coated substrata enhanced the adhesion of *S. epidermidis* [20-22] while there were reports also reporting that both proteins inhibited or had no effect on bacterial adhesion [23-25]. The increase in bacterial adhesion related to adsorbed Fg or Fn is regarded to be due to specific ligand/receptor events between plasma proteins and bacterial cell surface proteins known as the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) [26-29]. Multiple MSCRAMM have been found on *S. epidermidis* surface to promote adhesion of bacteria. These molecules include proteins such as SdrG [20,30], SdrF [29,31], and Embp [28,32], which were identified to bind Fg, collagen, and Fn, respectively. It has also been demonstrated that SdrG promotes platelet adhesion/activation and aggregation [33].

Microscopy analysis and quantification of adherent bacteria are generally used to evaluate bacterial adhesion on material surfaces. This approach reveals cellular and macroscopic scale phenomena of bacterial adhesion under a variety of conditions and provides useful information on the relationships between bacterial adhesion and various experimental parameters. However, the direct measurement of interactions between material surfaces, plasma proteins, and the bacterial cell surface at the molecular scale is particularly important for understanding the mechanisms of bacterial adhesion and pathogenic infection. Atomic force microscopy (AFM) is a powerful tool in studying bacterial adhesion, not only for imaging of bacterial cells under physiological conditions, but also for probing the nano-Newton (or less) interaction forces between bacteria and various substratum surfaces or biological molecules [34]. Méndez-Vilas *et al*. [35,36] characterized the surfaces of slime covered *S. epidermidis* and the nano-mechanical properties of cell walls, showing the importance of cell surface properties to adhesion. Successful coating of bacteria on the AFM probe made it possible to directly measure the molecular interaction forces between bacterium and surfaces, indicating the probability of adhesion. Liu *et al*. [37] measured the adhesion forces between *S. epidermidis* and self-assembled monolayers surfaces in the presence of proteins and found that molecular adhesion forces between bacteria and Fn were much greater than the forces between bacteria and fetal bovine serum. Other investigators [38] measured the time-dependent bacterial adhesion forces of *S. epidermidis* to hydrophilic and hydrophobic surfaces using a similar approach and found different bond-strengths for staphylococcal adhesion to surfaces with different wettability.

In this paper we studied the effects of plasma proteins on adhesion of *S. epidermidis* as well as bacteria interacttions with platelets on microphase-separated polyurethane (PU) biomaterial surfaces, an important material used for blood-contacting devices for over 30 years. Three plasma proteins (albumin, Fg, and Fn) were pre-adsorbed on PU surfaces and bacterial adhesion was measured. AFM was used to detect the molecular-scale Fn adsorption/orientation and to measure the interaction forces between proteins and bacterial cell surfaces to reveal the role of protein in bacterial adhesion. The correlation between molecular scale results and macroscale bacterial adhesion yields important information for understanding the mechanisms of bacterial adhesion and biological responses to materials.

2. Material and Methods

2.1. General

Phosphate buffered saline (PBS, 0.01 M, pH 7.4, Sigma) was prepared using purified water (18 MΩ) from a Millipore Simplicity 185 system. Human fibrinogen (Fg, 100% clottable) from Calbiochem (La Jolla, CA), human serum albumin (HSA, >99%) and human fibronectin (Fn) from Sigma Inc were used as received. A polyclonal anti-Fn antibody was obtained from Abcam (Cambridge, MA). Monoclonal antibodies (MAb) anti-amino (N-terminus) (MAb1936) and anti-carboxy (C-terminus) (MAb-1935) of human Fn were purchased from Millipore (Billerica, MA). MAB1936 specifically recognizes the N-terminal fibrin and heparin binding 29 kDa domain. MAB1935 recognizes the C-terminal domain containing

the second fibrin binding site [39].

2.2. Polyurethane Film Preparation

A BioSpan® MS/0.4 segmented polyurethane urea (PUU), having 22 wt% hard segments, was obtained from the Polymer Technology Group (Berkeley, CA). PUU films were prepared by a drop of solution casting onto round glass coverslips (15 mm dia, Ted Pella Inc., CA) and dried in a vacuum oven at 65˚C overnight. PUU films on glass coverslip were soaked in purified water over night and equilibrated in PBS for 1 hr before use.

2.3. Bacterial Strain Culture and Adhesion

Strain *S. epidermidis* RP62A (ATCC 35984) was cultured in tryptic soy broth (TSB, BD) at 37˚C for 24 hrs and collected by centrifuge at 1360 g for 10 min. The pellet was resuspended in PBS and the concentration of bacteria was measured by a spectrophotometer at 600 nm. PUU films on glass coverslip hydrated in H_2O for 24 hr were incubated in protein solutions at the desired concentration for 15 min in 12-well tissue culture plate (BD). After adsorption of proteins, PUU films were rinsed with PBS three times and incubated in bacterial solution at a concentration of 1×10^8 cfu/ml for 1 hr with shaking at 250 rpm. The samples were rinsed in PBS 3 times and fixed in 2.5% glutaraldehyde for 2 hrs, then the bacteria were stained with Hoechst 33258 (Invitrogen) for 30 mins. Adherent bacteria on PUU films were imaged under a fluorescent optical microscopy with magnification of 1000× (Nikon, Eclipse 80i) at six random locations. The DAPI filter was used for the Hoechst 33258 (excitation/emission wavelengths of 352/461 nm). Cell numbers were quantified using Image J software (NIH, Bethesda, MD). Adhesion was measured on three replicates of each protein concentration and presented as average ± standard deviation.

2.4. Modification of AFM Probes

All AFM experiments were performed using a Multimode AFM equipped with a Nanoscope IIIa controller system (Veeco Instruments, Santa Barbara, CA). AFM probes having long-narrow Si_3N_4 triangular cantilevers (Veeco Instruments, Santa Barbara, CA, nominal k = 0.06 N/m) were modified with anti-Fn antibodies, purified Fn or purified Fg. Probes were treated by glow discharge plasma at 100 W power for 30 min and then incubated in a 1% (v/v) solution of aminopropyltriethoxysilane (Gelest Inc., PA) in ethanol for 1 hr to provide reactive amine groups on the tip. After thoroughly rinsing with Millipore water, the probes were reacted with 10% glutaraldehyde in aqueous solution for 1 hr. The probes were again rinsed with Millipore water and incubated in protein solution (~20 μg/ml) for 1 hr. The probes were rinsed with PBS after removal from protein solution and were stored in PBS at 4˚C until use within 2 days. This attachment method has been shown to provide sufficient mobility and flexibility for proteins to rotate and orient themselves for binding [40,41]. Multiple probes were prepared together to improve consistency between experiments. The spring constants of cantilevers (all taken from the same wafer) were determined using the thermal tuning method (Nanoscope V6.12r2) using a multimode AFM with a PicoForce attachment and Nanoscope IIIa control system (Veeco Instruments, Santa Barbara, CA).

2.5. Protein Adsorption and Orientation Detection

An immuno-AFM technique [41] was used to detect the adsorption/orientation of Fn from mixtures with Fg or HSA on PUU film surfaces using a probe modified with anti-Fn antibodies. The orientation of Fn adsorbed on PUU surface was detected by the monocolonal antibodies (MAb) coupled AFM probes. PUU films hydrated in H_2O for 24 hr were incubated in protein solutions at the desired concentrations for 15 min, and rinsed with PBS to remove the free proteins. The PUU film was mounted on the AFM stage in an AFM fluid cell filled with PBS. An array of 32 × 32 force curves were collected by force volume image mode with a scanning area of 1×1 μm^2 at a scan rate of 1Hz and ramp size of 1 μm. As nonspecific controls for these measurements, polymer films were incubated with HSA for 10 min and used for measuring the nonspecific interactions of the antibody with protein. The individual retraction force curves were extracted and analyzed off-line with tools developed with Matlab software. The maximum debonding forces, defined as rupture force, calculated from the distance between the zero deflection value to the point of maximum deflection during probe separation from the surface for each force curve, was used as the strength of the interactions. The rupture length was calculated from the distance that the tip moves from the zero interaction force during separation to the position where the probe has separated and returned to zero deflection. Both rupture force and rupture length can be used to distinguish the specific and non-specific interactions.

2.6. Measurement of Binding Strengths between Proteins and Cell Surface

To directly measure the binding strengths between proteins and bacterial cell surface, bacteria were nonspecifically attached onto the glass coverslips coated with poly-

L-lysine for 5 min and rinsed with PBS 3 times to remove un-attached cells [37]. The cells were kept wet in PBS buffer for AFM measurement. AFM probes modified with Fg or Fn were used to measure the interaction forces between protein-probe and bacterial cell surfaces in PBS. A probe coated with HSA was used as control for non-specific interactions between protein and cell surface. An array of 32 × 32 force curves were collected by AFM force volume mode. Force curve data were extracted from AFM files and analyzed off-line with tools developed using Matlab software, and the maximum deflection of the cantilever from each retracting curve was used to calculate binding strength. The slope of approaching force curve was used to distinguish the forces curves measured on cell or substrate surfaces.

2.7. Interactions of Bacteria and Platelets

Salvaged human platelets with citrate phosphate dextrose anticoagulant were obtained from the Blood Bank at Hershey Medical Center. A previous study showed that salvaged human platelets retain functional activity [42]. The platelets were centrifuged at 200 g for 20 min to remove any remaining red blood cells. The supernatant was centrifuged at 600 g for 20 min to separate platelets into a pellet. The platelet pellet was gently resuspended in 10 ml of PBS, and the platelet count was measured by a hematology analyzer (Sysmex KX-21N, Japan). The PUU films were incubated in 2 ml of PBS solution containing bacteria (1×10^8 cfu/ml) and platelets (2.5×10^8/ml) for 1 hr with shaking at 250 rpm on a shaker plate. Plasma proteins (HSA (4 mg/ml), Fg (0.3 mg/ml), and Fn (0.03 mg/ml)) were added into solutions respectively in order to study the influences of proteins on bacteria-platelet interactions. After 1 hr, the adherent platelets and bacteria were fixed in 1% paraformaldehyde and 2.5% glutaraldehyde for 1 hr. After washing with PBS, platelets and bacteria on the PUU surface were stained and examined with a fluorescence microscopy. For platelet staining, samples were incubated in a primary antibody solution containing Ab662 anti-human $\alpha_{IIb}\beta_3$ (1.5 μg/ml) in 6% normal donkey serum (Jackson ImmunoResearch) overnight at 4˚C. Following this labeling step, samples were washed with PBS and labeled with a secondary antibody by adding 10 μl/ml of AlexaFluor555 goat anti-mouse antibody IgG (Invitrogen) in 6% normal goat serum in the dark and at room temperature for 1 hr. Samples were rinsed with PBS and incubated in Hoechst 33258 solution for 30 min to stain bacteria. After rinsing in PBS, the sample was mounted under a coverslip with antifade gel (Biomeda) and stored at 4˚C overnight. The adherent platelets and bacteria were examined under fluorescence microscopy with appropriate fluorescence filters. In another experiment, the fixed platelets and bacteria samples on PUU surfaces were washed by pure water and dried in air for AFM imaging.

2.8. Image and Data Analysis

The fluorescence images were analyzed by Image J software. Statistical analysis of bacterial adhesion data was performed by ANOVA utilizing the commercial software program GraftPad Instat (version 3.06). $p < 0.05$ was considered statistically significant. Significant differences are denoted by symbols (* or #) with one symbol denoting $p < 0.05$, two symbols denoting $p < 0.01$, and three symbols denoting $p < 0.001$.

3. Results

3.1. *S. epidermidis* RP62A Adhesion on PUU Surfaces with Pre-Adsorption of Proteins

Bacterial adhesion values for *S. epidermidis* RP62A on PUU surfaces pre-adsorbed with different proteins and different concentrations are illustrated in **Figure 1**. For surfaces adsorbed with single proteins, the bacterial adhesion was significantly increased when surfaces were pre-adsorbed with Fn (either concentration) compared to either Fg or albumin. HSA appears to impart a small inhibition of bacterial adhesion, evidenced by the lower adhesion against albumin compared to the control surface without any protein adsorption, although the result is not statistically significant. Fg produced a slight increase in adhesion compared to HSA, but adhesion to Fg was still much lower than Fn. Results showed the general trend of effect of single protein adsorption on bacterial adhesion to be in the order of Fn $\gg$ Fg > HSA. When surfaces were pre-adsorbed with dual component proteins solutions (either Fn + Fg or Fn + HSA), the Fg + Fn combination showed greater bacterial adhesion than either the control or Fn + HSA samples, as expected. It is interesting to note that increasing the amount of Fg with respect to Fn in solution had no significant effect on bacterial adhesion (perhaps even a small drop in adhesion, though not significant) while increasing Fn concentration significantly increased the bacterial adhesion (Fn 0.01 + Fg 0.3 mg/ml and Fn 0.03 + Fg 0.3 mg/ml).

3.2. Fn Adsorption on PUU Surfaces

The Fn adsorption on polyurethane surfaces was measured by an immuno-AFM technique. Utilizing an AFM probe modified with polyclonal anti-Fn antibody (pAb), Fn on the polymer surface can be recognized by differentiating specific and non-specific interactions between antibody and adsorbed proteins [41]. Either the rupture force or rupture length (stretching of the interaction)

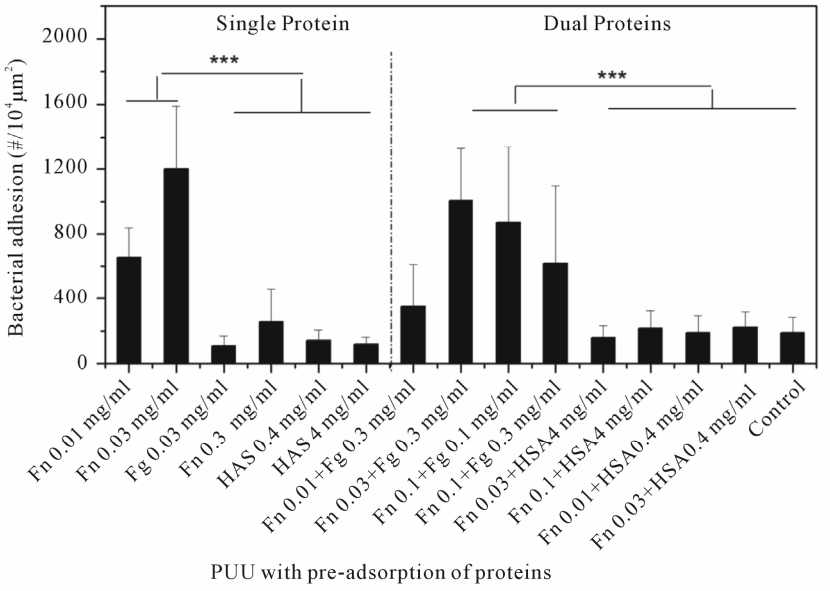

Figure 1. *S. epidermidis* RP62A adhesion on PUU surfaces after pre-adsorption of plasma proteins at varying concentrations. (n = 3; ***: p < 0.001; Fn = fibronectin; Fg = fibrinogen; HSA = human serum albumin).

from each force curve can be used to characterize the interactions between antibodies and proteins. It should be noted that non-specific interactions measured between pAb anti-Fn and HSA in this case show a wide range of rupture forces, similar to the interaction forces between pAb and Fn, however, the rupture length of non-specific interactions varied over a small range, compared to the distribution of rupture lengths of pAb and Fn (**Figure 2**). Results here suggest that rupture length is more suitable than rupture force for distinguishing non-specific and specific interactions in this system. Therefore, the distribution of rupture length of non-specific interactions between pAb and HSA was used to build a 95% confidence interval limit, similar to what we have done previously [41]. This resulted in a value of 93.6 nm as the cut-off value. Interactions with a rupture length above this limit were considered as specific interactions, and the percentage of curves across an array of force curves showing specific interactions was used to indicate the recognition of Fn on the material surfaces.

Figure 3 illustrates the molecular Fn recognition data from the dual component protein adsorption on PUU surfaces. A lower Fn fraction was recognized on the surfaces after adsorption of Fn and HSA, while higher Fn recognition was observed on surfaces with adsorption of Fn or Fn + Fg. A good correlation between molecular scale measurements of Fn adsorption and macroscale bacterial adhesion was observed, suggesting Fn plays an important role in bacterial adhesion.

To assess the orientation of adsorbed Fn on PUU surfaces, the amino (N)- or carboxy (C)-termini of Fn were detected by mAb-coupled probes using similar method to the recognition of Fn adsorption by pAb probes. Results show that Fn adsorbed from pure solutions showed more C-terminus available compared to N-terminus in same sample, and corresponded to higher bacterial adhesion, while more N-terminus was measured in the presence of HSA and with lower bacterial adhesion (**Figure 3**). Thus, results suggest the orientation of protein Fn is important in controlling *S. epidermidis* RP62A adhesion.

3.3. Binding Strengths of Bacterial Cell Receptors and Protein Ligands

The interactions of plasma proteins (e.g., Fg or Fn) with the bacterial cell surface are specific ligand/receptor type interactions [43]. Characterization of the binding strengths between cell and proteins as well as the distribution of binding sites on cell surfaces can offer insight into the mechanisms of bacterial adhesion. With a protein-modified probe, the interaction forces between proteins and surfaces (cell or substrate) can be measured by analysis of an array of force curves. **Figure 4** illustrates a representative low-resolution (32 × 32) height image of bacterial clusters attached on polymer surface along with the corresponding force map measured with an Fn-modified probe. The warmer colors in the force map represent a strong interaction force while the cooler colors represent a weak force (**Figures 4(c)** and **(d)**). The different colors in the force map of bacterial cell cluster surfaces show the heterogeneous distribution of binding sites on cell surfaces. Extracting the force data collected from cell surfaces, the binding strength distributions of

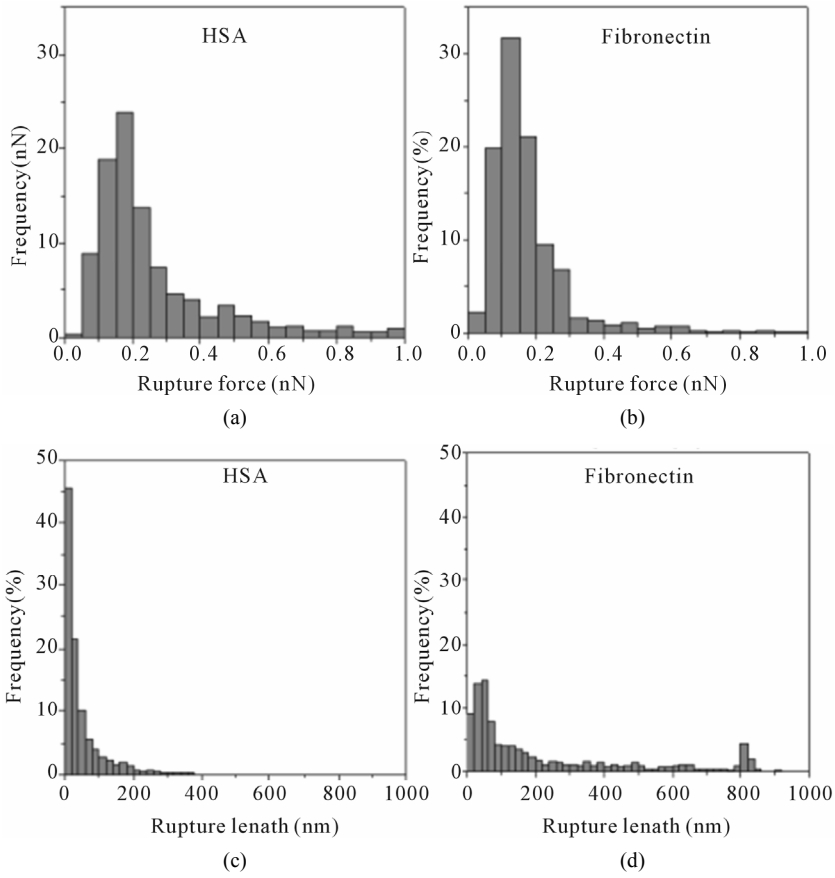

Figure 2. Histogram of (a), (b) rupture forces and (c), (d) rupture lengths measured from PUU surfaces pre-adsorbed with HSA and Fn.

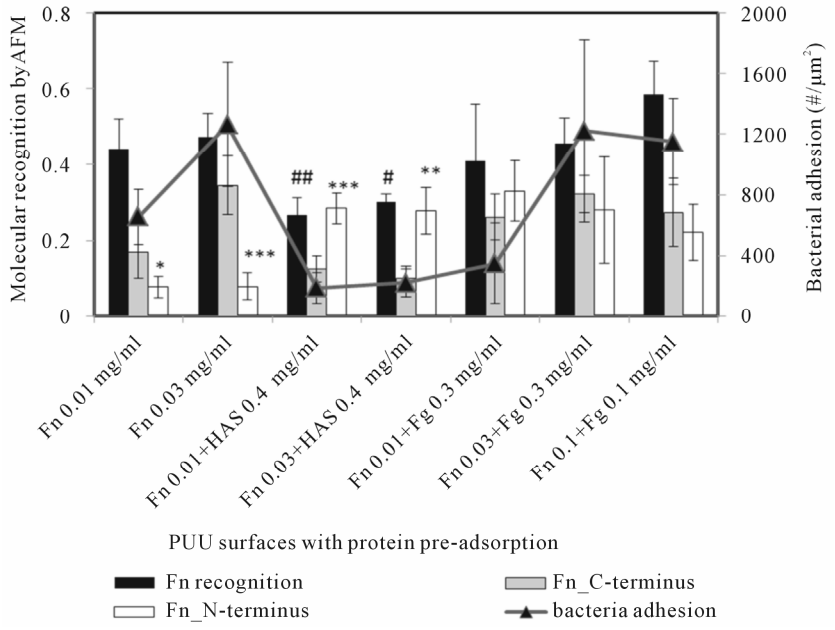

Figure 3. Correlation between molecular Fn recognition, amino (N)-, carboxy (C)-termini of Fn, and bacterial adhesion on PUU surfaces. Statistical analysis symbol # denotes the comparison of Fn recognition to surfaces adsorbed from pure Fn (0.01 mg/ml) solution, and * denotes comparing N- and C-termini on same sample.

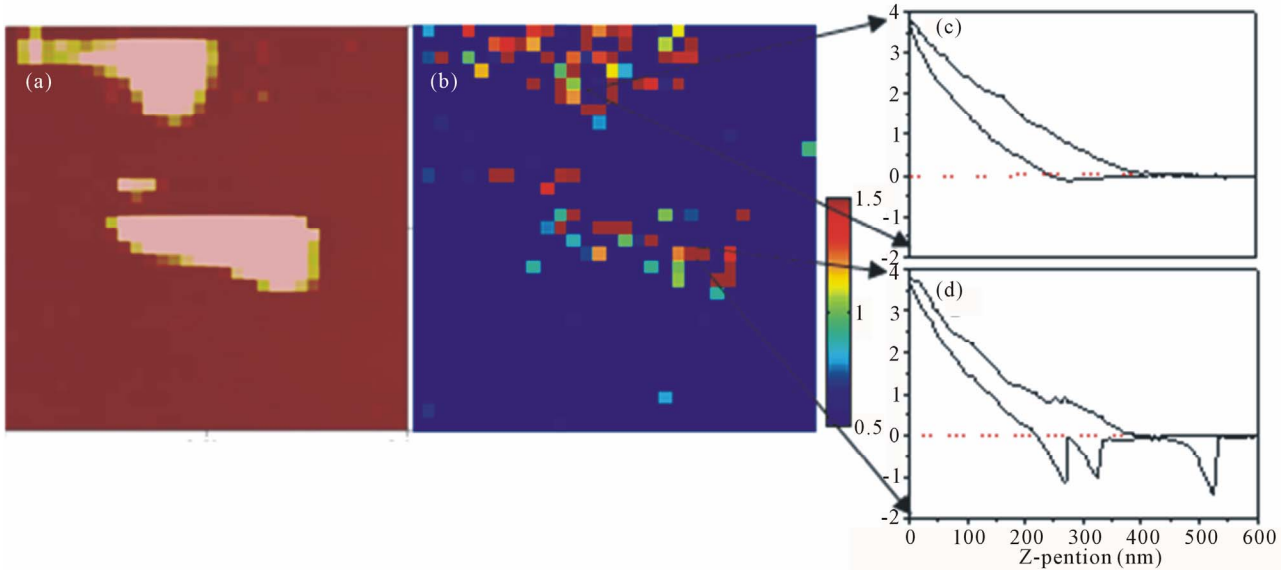

Figure 4. (a) Height image of bacterial clusters on polymer surface, and (b) corresponding force map measured by Fn-probe (32 × 32). Representative force curves measured on bacterial cell surface were shown as (c) weak interaction force corresponding to cool color in (b) and (d) strong force corresponding to warm color in (b) (AFM image size: 5 × 5 μm^2).

Fn or Fg were calculated and illustrated in **Figure 5**. To recognize the protein receptors on bacterial cell surfaces, HSA was used as a control and nonspecific interaction forces between HSA and bacterial cell surfaces were measured. The force distribution measured between HSA-probe and bacterial cell surfaces produced a 95% confidence limit at 0.36 nN. Binding strengths over this limit were considered as protein ligand and cell receptor interactions. There are approximately 48% of the measurements showing bacteria-Fg interactions while around 69% of the measurements showing an interaction with Fn-probes (**Figures 5(a)** and **(b)**). Results suggest more Fn-receptor recognition events than Fg-receptor recognition events on the cell surface.

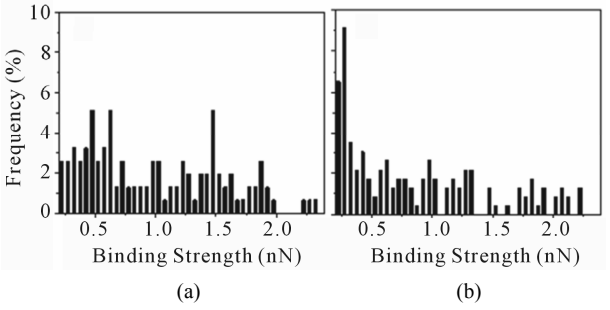

Figure 5. Histogram of binding strengths between bacterial cell surfaces and proteins (a) Fn, and (b) Fg.

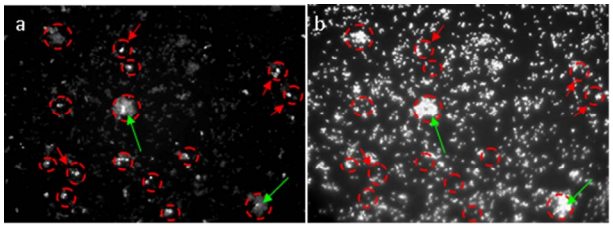

Figure 6. Fluorescent images of *S. epidermidis* RP62A bacteria and platelets interactions on polyurethane surface, (a) platelets and (b) bacteria. Red circles are drawn for comparison. (Image size: 226 μm × 169 μm).

3.4. Interaction of Platelets and Bacteria in the Presence of Plasma Proteins

Interaction of platelets and bacteria was illustrated from the distribution of platelets and bacteria adhered on PUU surfaces. Results show that bacteria adhered on surface and aggregated to form clusters. Although fewer platelets were observed compared to the number of bacteria, most platelets were found to be either entrapped in bacterial aggregates (green arrows in **Figure 6**) or adherent with bacteria (red arrows in **Figure 6**), suggesting the formation of platelet-bacteria aggregates. The platelet-bacteria aggregates on the PUU surface were analyzed and counted when plasma proteins were added in bulk solution or proteins were just pre-adsorbed on PUU surfaces (without proteins in bulk solution). Results show that Fn leads to more aggregates than either Fg or albumin in both cases, and more aggregates formed when the protein was present in bulk solution as opposed to just pre-adsorbed on the polymer surface (**Figure 7**). It is interesting to note that Fg showed a slight increase in aggregation, not significantly, compared to albumin or control.

The interactions of platelets and bacteria were further imaged by AFM. **Figure 8** shows the morphology of

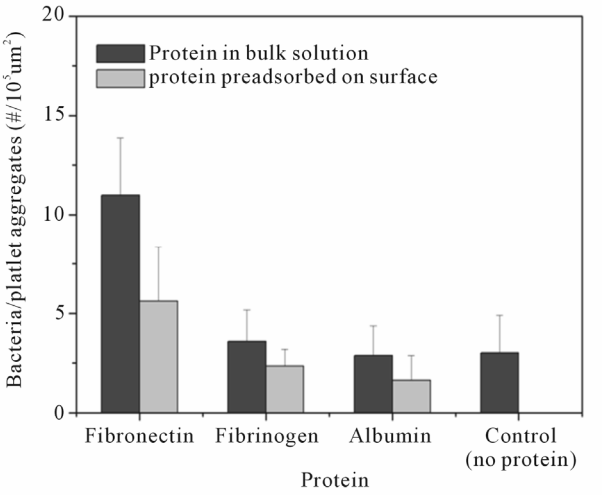

Figure 7. Bacteria-platelet aggregates on PUU surfaces observed when proteins were present.

platelets and bacteria adhered on PUU surface. Both non-activated (round) and activated (spread) platelets were found on the surface when only platelets were present in solution (**Figure 8(a)**), however, platelets were found only activated and spread on the surface when bacteria were present and interacted with platelets. Bacteria were seen to adhere with activated platelets and form aggregates (**Figure 8(b)**), suggesting that bacteria increase the platelets activation and aggregation of bacteria-platelet. When plasma proteins were present in solution, HSA appeared to decrease the number of bacteria-platelet aggregates on PUU surface (**Figure 8(c)**) while Fg and Fn increased the formation of aggregates (**Figures 8(d)** and **(e)**). Furthermore, non-activated platelets were observed on surface in the case of HSA while all platelets were activated in the cases of Fg and Fn. The images with large magnification show activated platelets either adhered to bacteria or entrapped in bacterial clusters (**Figure 9**).

4. Discussion

Bacterial adhesion is the first step in the development of biofilm formation on implanted biomaterials. Factors that influence bacterial adhesion on a polymeric surface include the nature of environment, type of microorganism, and properties of material, and each one of these factors is in turn affected by several other parameters [43]. When a material is implanted, plasma proteins rapidly interact with the surface to form a layer of proteins. The nature of adsorbed proteins is affected by the physicochemical properties of surface, and in turn moderates the initial bacterial adhesion. *S. epidermidis* is a predominant bacterial species contributing to cardiovascular implant infection. In this study we measured the adhesion of *S. epidermidis* and interaction with platelets on polyurethane biomaterial surfaces in the presence of plasma proteins. The molecular scale measurement of protein adsorption and interaction forces between protein and cells

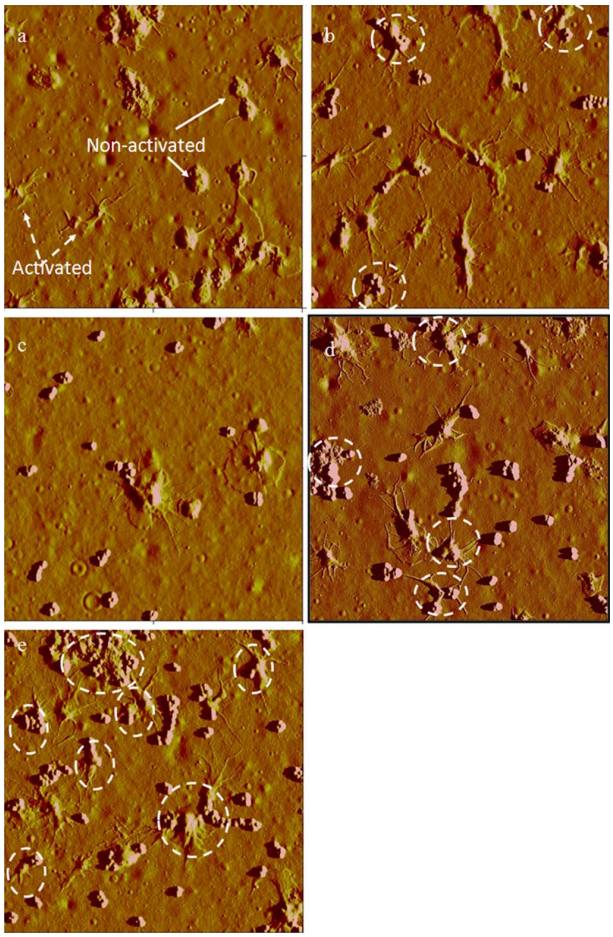

Figure 8. AFM images of platelet and bacteria interactions on PUU surfaces, (a) platelet only, (b) platelet and bacteria without plasma proteins, platelets and bacteria in the presence of (c) HSA, (d) Fg and (e) Fn. The bacteria-platelet aggregates are indicated by circles. (Image size: 50 µm × 50 µm).

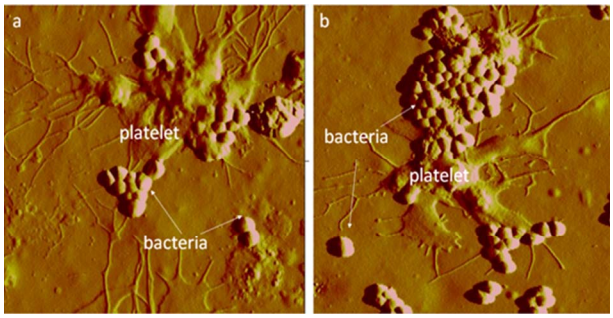

Figure 9. Aggregates of bacteria-platelets in the presence of Fn showing platelet (a) adherent or (b) entrapped with bacteria. (Image size: 20 µm × 20 µm).

were correlated to bacterial adhesion. Results provided important information to understand the roles of plasma proteins in bacterial adhesion and biological responses to implanted biomaterials.

Albumin is the most abundant plasma protein. The addition of HSA to Fn solution decreased the adsorption of Fn and subsequent bacterial adhesion to the PUU surfaces (**Figures 1** and **3**). No ligand/receptor binding event was measured between albumin and the cell surfaces. Furthermore, the presence of albumin appeared to decrease the activation of platelets and interactions with bacteria.

Fibrinogen is the most third abundant plasma protein in blood and plays a prominent role in development of surface-induced thrombosis. It serves as a ligand, binding to the platelet integrin receptor $\alpha_{IIb}\beta_3$, leading to platelet immobilization, activation, and aggregation [44,45]. It is also found to promote *S. aureus* adhesion to material surfaces [17]. The increase in adhesion of *S. aureus* with Fg was identified to be due to the Fg-binding MSCRAMM clumping factor on cell surface [27]. However, the presence of Fg shows no significant increase in *S. epidermidis* RP62A adhesion compared to HSA in this study. Lower bacterial adhesion was measured on polymer surfaces in the presence of Fg compared to Fn, although the solution concentration of Fg (0.3 mg/ml) is 10-times higher than that of Fn (0.03 mg/ml) (**Figure 1**). Results suggest that *S. epidermidis* RP62A cell surface has fewer binding sites to Fg than Fn, as evidenced by the molecular scale measurement of protein binding sites on cell surfaces, where approximately 48% of the measurements showed bacteria-Fg interactions while ~69% of the measurements showed an interaction with Fn-probes (**Figure 5**).

Fibronectin is one of the main plasma proteins responsible for forming a conditioning film on implanted biomaterials. It can bind a variety of extracellular molecules including fibrin, heparin, and collagen, and plays a key role in cell adhesion and proliferation [46,47]. Numerous studies have found that Fn facilitates bacterial adhesion to biomaterials including *S. epidermidis* [48-50]. In this study, the macroscale measurements of bacterial adhesion show higher adhesion of bacteria on surfaces preadsorbed with Fn compared to Fg or HSA, and consistent with the amounts of molecular Fn detected by anti-Fn pAb probe on polymer surfaces (**Figure 3**). Results strongly suggest that Fn plays an important role in adhesion of *S. epidermidis* to polymer surfaces. Higher adhesion of bacteria on PUU surfaces bearing Fn is believed to be due to the interaction of Fn with MSCRAMM on *S. epidermidis* RP62A cell surfaces. William *et al*. identified a giant Fn-binding protein, extracellular matrixbinding protein (embp), from *S. epidermidis* cell surface [28]. Christner *et al.* further demonstrated that embp mediates binding of *S. epidermidis* to solid phase attached Fn, constituting the first step of biofilm formation on conditioned surfaces. Embp is also a multifunctional cell surface protein that mediates attachment to host extracellular matrix, biofilm accumulation and escape from phagocytosis, promoting biomaterial-associated infections [32]. Although the Fn-binding proteins on *S. epidermidis* RP62A cell surface were not identified in this study, such proteins are expected to be present on cell surface, as evidenced by the larger binding strengths and increased binding events measured on cell surfaces by Fn-probe (**Figure 5(a)**). The binding between Fn and cell surface can be considered as a ligand/receptor interaction. Bustanji *et al.* measured the energy landscape of this binding/unbinding process through dynamic force spectroscopy under different loading rates, and revealed the molecular mechanism of Fn in bacterial adhesion [51].

The orientation of Fn influences bacterial adhesion. Fn is a dimer of two similar polypeptides linked by disulfide bonds at the carboxyl terminus, possessing several functional domains that bind to a variety of extracellular molecules such as heparin and collagen [52]. There are two particularly important relevant binding sites for *S. epidermidis*, which are located at the N-terminus and the C-terminus of Fn [39]. The immuno-AFM measurements show that C-terminus is much more available than N-terminus of Fn molecules when adsorbed from pure Fn solution. The presence of albumin appears to influence the orientation of Fn, with more N-terminus available although the total amount of Fn adsorbed appears low.

Bacteria in blood can interact with platelets. This interaction appears mediated by plasma proteins. Albumin appears to inhibit the bacteria-platelet interaction and activation/aggregation of platelets, while Fg and Fn promote the interactions of bacteria and platelets along with platelet activation, leading to bacteria/platelet aggregation (**Figure 8**). Interactions between bacteria and platelets are characterized as the binding of bacteria to platelets either directly through a bacterial surface protein or indirectly by a plasma bridging molecule that links bacteria and platelet receptors [53]. *S. epidermidis* induced platelet activation and aggregation of bacteria and platelets in the absence of plasma proteins, showing the direct mechanism mostly involved (**Figure 8(b)**), while more bacteria-platelet aggregates were observed in the presence of Fg and Fn, suggesting that both mechanisms may be involved in interaction of bacteria and platelets (**Figures 8(d)** and **(e)**). More aggregates of bacteria-platelet were measured on PUU surface when Fn or Fg were added in bulk solution compared to the case of proteins only pre-adsorbed on surface (**Figure 7**). This suggests that Fg or Fn may serve as linker in interaction

of bacteria and platelets.

Fn leads to more bacteria adhering to platelets and forming more aggregates than Fg does. This may be due to the different functions of MSCRAMM involved in bacteria-platelet interactions. Different MSCRAMM on *S. aureus* cell surface have been identified including clumping factors (Clf) and Fn binding proteins (FnBP), and they were shown to promote bacterial adhesion to and activation of platelets [11,43]. Both Clf and FnBP bind Fg, allowing an interaction with platelet GPIIb/IIIa, leading to platelet adhesion. However, less information on the interactions of platelets and *S. epidermidis* is available. Recently Brennan *et al*. reported that Fg-binding serine-aspartate repeat protein G (SdrG) from *S. epidermidis* supports adhesion to platelets and aggregation through both a direct interaction with platelet integrin receptor $\alpha_{IIb}\beta_3$, and an indirect mechanism by a bridge of Fg [33], however, the roles of other MSCRAMM, e.g., the giant Fn-binding protein (embp) from *S. epidermidis* cell surfaces, have been identified in bacterial adhesion and biofilm formation, but without report of interactions of bacteria-platelet.

5. Conclusion

Bacterial adhesion to polyurethane biomaterial surfaces as well as interactions with platelets is complex and can be mediated by plasma proteins. This study demonstrated the roles of plasma proteins (albumin, fibrinogen, and fibronectin) in the adhesion of bacteria, *S. epidermidis* RP62A, on polyurethane biomaterial surfaces. Results show that Fn leads to increased bacterial adhesion, with the order of effectiveness being Fn $\gg$ Fg > albumin. A correlation between molecular scale Fn adsorption and macroscale bacterial adhesion was observed, with increased numbers of Fn-receptor recognition events measured on cell surfaces as compared to Fg-receptor recognition events, suggesting Fn is an important protein in bacterial adhesion. Interactions between bacteria and platelets induced platelet activation and bacteria-platelet aggregation. Albumin inhibited bacteria-platelet interactions and platelet activation, while both Fg and Fn appear to serve as a linker, promote the adhesion of bacteria to platelets and platelet activation, resulting in bacteria-platelet aggregation.

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