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The Role of Fibrinogen Spacing and Patch Size on Platelet Adhesion Under Flow

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Abstract

Platelet adhesion to the vessel wall during vascular injury is mediated by platelet glycoproteins binding to their respective ligands on the vascular wall. In this study we investigated the roles that ligand patch spacing and size play in regulating platelet interactions with fibrinogen under hemodynamic flow conditions. To regulate the size and distance between patches of fibrinogen we developed a photolithography based technique to fabricate patterns of proteins surrounded by a protein repellant layer of poly(ethylene glycol). We demonstrate that when mepacrine labeled whole blood is perfused at a shear rate of 100 s^{-1} over substrates patterned with micron-sized wide lines of fibrinogen, platelets selectively adhere to the areas of patterned fibrinogen. Using fluorescent and scanning electron microscopy we demonstrate that the degree of platelet coverage (3% – 35%) and the ability of platelet aggregates to grow laterally was dependent upon the distance (6 – 30 μm) between parallel lines of fibrinogen. We also report on the effects of fibrinogen patch size on platelet adhesion by varying the size of the protein patch (2 – 20 μm) available for adhesion, demonstrating that the downstream length of the ligand patch is a critical parameter in platelet adhesion under flow. We expect that these results and protein patterning surfaces to be useful in understanding the spatial and temporal dynamics of platelet adhesion under physiologic flow, and in the development of novel platelet adhesion assays.

Keywords

Platelet; Adhesion; Fibrinogen; Micropatterns; Blood

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1.0 INTRODUCTION

Platelet adhesion to natural (e.g. blood vessel wall) or artificial surfaces plays a crucial role in a number of biological processes (e.g. thrombosis, hemostasis, atherosclerosis) as well as the body's response to implanted devices. It is well established that adhesion of free flowing platelets to sites of vascular injury can be mediated by a variety of adhesion receptors (e.g. GPIb/V/IX, GPIIb/IIIa, or $\alpha_2\beta_1$) on the platelet membrane surface and their respective ligands (e.g. von Willebrand factor[1, 2], collagen[3, 4], fibrinogen[1, 5, 6], fibronectin[7], laminin[8]). In addition, the nature of the receptor-ligand pair mediating the initial platelet adhesion is dependent on the local shear environment. At high shear rates, platelet adhesion is primarily mediated through binding of platelet glycoprotein (GP) receptor GPIb/V/IX to von Willebrand Factor (vWF)[1, 2], while at low shear rates adhesion can be mediated by a variety of interactions (i.e. $\alpha_{IIb}\beta_3$:fibrinogen; $\alpha_{IIb}\beta_3$:fibronectin, $\alpha_6\beta_1$:laminin)[1, 5, 6, 9, 10].

Despite the considerable progress that has been made in our understanding of the molecules involved in platelet adhesion, limited information exists on the role(s) that ligand spacing or ligand patch size plays in regulating platelet adhesion and thrombus formation. The potential importance of ligand patch size in platelet adhesion and thrombosis is highlighted by recent studies in the absence of fluid flow. Kastrup et al.[11] demonstrated that initiation of blood clotting was dependent upon the clotting stimulus patch size. Patches of a photochemically produced acid with diameters $> 100 \mu\text{m}$ initiated clotting in 3 minutes, while patch diameters $< 50 \mu\text{m}$ did not initiate clotting. Similarly clotting times for factor XII and tissue factor[12] in the absence of flow were also dependent on patch size. This group also demonstrated that the spatial distribution of bacteria is critical in the initiation of blood coagulation[13].

With respect to platelet adhesion and spreading, microcontact printing has been the preferred method of protein patterning. Basabe-Desmonts et al[14], used microcontact printing to generate well defined patterns of fibrinogen, von Willebrand Factor (vWF), and anti-CD42b in the shape of dots, squares, or tear drops to isolate platelets from whole blood and to study how protein patch area affects platelet adhesion and spreading. Similarly Corum et al.,[15] used microcontact printing to generate randomly placed micron-sized islands of fibrinogen to demonstrate the role of protein pattern shape and size on platelet adhesion and spreading. Likewise Kita et al.,[16] used microcontact printing to generate micron sized lines of fibrinogen to demonstrate the effect of ligand patch geometry on platelet spreading at the single cell level, and the ability of platelets to extend filopodia to bridge and spread between fibrinogen patches. Finally Okamura et al.,[17] developed a thrombin-coated nanosheet surface to show that < 10 thrombin molecules were sufficient to cause platelet adhesion and spreading.

While the studies described above have demonstrated that the size and shape of protein patches can regulate platelet adhesion and spreading under static conditions, they did not assess platelet adhesion and behavior to these patterns under well-defined flow. The presence of flow is a key parameter in platelet adhesion, since it determines (i) the amount of time for a platelet receptor to bind its ligand; (ii) the force on the receptor ligand bond which can affect bond lifetime; (iii) the recruitment of additional platelets to the initial adhesion site; and (iv) the convection of platelet activation agonists spatially in relation to the adhesion site. As pointed out by Kita[16], the integration of flow into assays of platelet adhesion on micropatterned substrates is an important aspect.

Despite the importance of flow, only a few studies have investigated platelet adhesion to micropatterned protein substrates under well-defined flow. Okorie and Diamond[18]

employed a robotic spot microarrayer to fabricate ~175 μm patches of protein to demonstrate the importance of ligand patch composition (i.e. collagen vs vWF) during thrombus formation under physiological shear rates (100 – 500 s^{-1}). Alternatively, we previously employed a microfluidic patterning technique to capture platelets from flowing blood (100–200 s^{-1}) onto ~150 μm wide lines of vWF[19]. A potential limitation of these flow-based studies is that the size of the patterns generated was substantially larger than the size of an individual platelet (2–4 μm).

In this study we report a photolithography-based technique to generate micron sized patterns of protein surrounded by a protein resistant background (Figure 1) to determine the effect(s) of ligand patch spacing (6–30 μm), size (2–20 μm), and orientation on the adhesion of free-flowing platelets from whole blood. We chose to study this range of protein patch sizes and spacing since similar length scales have been shown to mediate platelet adhesion and aggregation in several experimental and theoretical models of vascular injury and thrombus growth. For example, in vivo models of vascular lesions produced by laser-induced endothelial damage can be as small as 1 μm in diameter [20]. Similarly, in vitro models of platelet adhesion and aggregation have employed micron sized polystyrene beads coated with fibrinogen (5 μm diameter)[21] or vWF (2–15 μm diameter)[22]. Finally mathematical simulations of thrombi growth have employed thrombi lengths of 10 – 20 μm with separation distances of neighboring thrombi of 20 – 200 μm [23]. We believe that the data obtained in the current study and future studies with similar patterned substrates can be employed to (i) elucidate platelet adhesion dynamics, (ii) refine our understanding of pharmaceutical receptor antagonists (e.g. $\alpha_{\text{IIb}}\beta_3$ inhibitors including Tirofiban), (iii) assist in determining acceptable blood-contacting medical device surface irregularities, and provide additional data to refine computation models of platelet adhesion and aggregation.

2. EXPERIMENTAL SECTION

2.1 Chemicals and Materials

Anhydrous toluene, sulfuric acid, hydrogen peroxide, and mepacrine were purchased from Sigma Aldrich (St. Louis, MO). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). Shipley MICROPOSIT® S1805®, MF-319, and Remover 1165 were purchased from MicroChem (Newton, MA), while 2-methoxy(polyethylenoxy)propyltrichlorosilane was obtained from Gelest (Morrisville, PA). Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). AlexaFluor647-labeled bovine serum albumin (BSA) and AlexaFluor647-labeled fibrinogen were obtained from Invitrogen (Carlsbad, CA). Function-blocking anti- $\alpha_{\text{IIb}}\beta_3$ mAb HIP8 was purchased from BD Pharmingen (San Diego, CA). Human serum albumin (HSA) was purchased from Gemini Bio (Sacramento, CA).

2.2 Preparation of Photolithographic Substrates

Photoresist patterns were fabricated on clean glass coverslips by standard photolithography techniques. Glass coverslips (45 × 50 mm) were cleaned in piranha solution (3:1 sulfuric acid, 96%: hydrogen peroxide, 30%) for 15–30 min followed by a DI water rinse and blown dry under nitrogen. Coverslips were then dehydrated at 150°C for 15 min prior to spin coating. A 0.65 μm thick layer of Shipley MICROPOSIT® S1805® was applied by spinning at 2000 rpm for 40s. Resist films were then baked at 115–125°C for 120–180 s and exposed to UV illumination at 365 nm, 14 mW/cm^2 for 3s with a Karl Suss MJB3 Mask Aligner system. Substrates were developed in MICROPOSIT® MF-319 developer for ~1min, rinsed in DI water and blown dry under nitrogen.

2.3 PEG Grafting

A thin layer of poly(ethylene glycol) (PEG) was selectively grafted around the photoresist pattern template by a method similar to that of Kannan et al.[24] Briefly, photoresist patterns were subjected to an air plasma at 400 mT for 120s at medium power (10.5 W) in a plasma cleaner PDC-32G (Harrick Ossining, NY). Substrates were exposed a second time to air plasma at 400 mT for 30 seconds at low power (6.8 W). Substrates were then immediately immersed in anhydrous toluene and placed in a glove bag under nitrogen atmosphere at room temperature (~20°C) with 10–12% humidity. PEG was added at 2mM and allowed to covalently graft to the surface for 2–4 h. Upon completion, substrates were rinsed in additional toluene for 12 min to remove any ungrafted PEG moieties and dried under nitrogen. The photoresist was removed in a dual bath of Remover 1165 for 15 min under strong agitation. Substrates were dried under flowing nitrogen, and the remover residue was dissolved from the substrate surface by an isopropyl alcohol (IPA) rinse for 6 min and dried under flowing nitrogen. After a 1hr bake at 80°C, substrates were then stored in a vacuum desiccator for up to 1 week prior to use.

2.4 Protein Adsorption

AlexaFluor647-labeled Fibrinogen was dissolved in HBSS at concentrations of 20 and 100 mg/ml. Aliquots (25–50 ml) of protein solutions were dispensed on the PEG patterned surface and incubated for 2 hr at room temperature in a dark humidified environment to allow protein to adsorb onto the uncoated glass regions (Figure 1). Following protein incubation, the substrates were rinsed with HBSS containing 0.5% HSA to remove any unbound protein. Non-specific binding sites were blocked by incubation with a 0.5% HSA solution in HBSS for 30 min.

2.5 Substrate Characterization

Photoresist line patterns and protein patterned substrates were characterized by fluorescent and bright field microscopy with a Zeiss Axiovert 200 inverted microscope equipped with a CoolSnap cf digital camera and analyzed with ImageJ image analysis software. A selection of substrates were also Au/Pd coated in a Hummer VI sputtering system so that the line width and thickness of the photoresist patterns could be evaluated by scanning electron microscopy (SEM) in a Zeiss DSM 960A. The Au/Pd coating is used to reduce charging artifacts in the SEM.

2.6 Blood Collection and Preparation

After informed consent according to methods approved by the University of Oklahoma Institutional Review Board, human venous blood was collected by 21 gauge needle venipuncture from normal, healthy volunteers and anticoagulated with heparin (20 U/ml). To fluorescently label platelets, whole blood was incubated with mepacrine (10 µmol/L) for 10 min at 37°C prior to perfusion.

2.7 Platelet Adhesion Assays

Adhesion of platelets to fibrinogen lines under flow was investigated by assembling the micropatterned surfaces into a parallel-plate flow chamber (Glycotech, Rockville, MD) and performing fluorescent video microscopy with a Zeiss EC Plan-NEOFLUAR 40× objective. During perfusion, mepacrine labeled whole blood was withdrawn through the flow chamber by a syringe pump (Harvard Apparatus). To reduce any potential activation and adhesion of platelets due to continuous fluorescent illumination, the fluorescent lamp port was opened for 5 seconds every 30 or 60 seconds to collect the images for subsequent analysis. Fluorescent images were captured at 30 frames per second with a cooled CCD camera (DAGE-MTI CCD-300) and recorded on an S-VHS recorder for later analysis. In some

experiments, reflection interference contrast microscopy (RICM) was performed with a Zeiss Antiflex Plan-NEOFLUAR 63× objective (NA = 1.25) to observe the dynamic adhesive interactions of platelets with the patterned fibrinogen substrates. RICM is a technique that is based upon the destructive and constructive interference between light reflected from the substrate-buffer interface and the buffer-cell interface which produces image contrast due to differing separation between the two interfaces when in close proximity (< 200 nm) [25, 26]. When a cell adheres to the substrate or is in close contact, the light waves reflected from the substrate-buffer and buffer-cell interfaces cancel each other out, which produces the darkest (black) regions in the image. As the distance between the cell membrane and the substrate increases, the interference effect becomes attenuated with more distant regions appearing dark grey or white [27].

2.8 SEM Preparation and Imaging of Adherent Platelets

In some experiments, substrates were prepared for SEM imaging at the end of the adhesion assay. With the flow chamber intact, samples were rinsed with 0.5% HSA in HBSS before a 10 min. fixation with 5% paraformaldehyde in HBSS at a shear rate of 100 s^{-1} . Following fixation, excess paraformaldehyde was removed by perfusion with 0.5% HSA in HBSS. The glass substrates were then removed from the flow chamber, placed in petri dishes, and incubated in the dark at 4°C with 0.5% OsO₄ for 1 hour. Samples were rinsed with DI water before a graded ethanol dehydration and CO₂ critical point drying in a Tousimis Autosamdri-814. Finally the samples were sputter coated with Au/Pd in a Hummer VI Sputtering System. Samples were viewed on a Zeiss DSM 960A or JEOL JSM-840A scanning electron microscope with an accelerating voltage of 10kV.

2.10 Image Analysis

To quantify the amount of platelet coverage, VHS video was digitized using MetaMorph image analysis software. At each time point, single frames were analyzed to measure the amount of substrate area covered by adhering platelets. Images were first binarized by manual thresholding. For each set of experiments the optimal threshold value was determined manually by setting a single threshold value for which single platelets could be differentiated from the background in an early, middle, and end time point image. This optimized threshold value was then applied to every image acquired for that day's experiments. Following thresholding and binarization, the percentage of white area, which corresponds to the percentage of platelet coverage area in the field of view, was measured and recorded in an Excel spreadsheet. The data from multiple separate experiments (n) were analyzed and reported as the mean ± the standard error of the mean (S.E.). SEM images were used as a qualitative measure of the consistency of platelet adhesion over a large area of the pattern and for characterizing adherent platelet morphology.

2.11 Statistics

All values in the figures are presented as means ± standard errors of the mean. Significant differences between the means of the total platelet surface coverage (or platelet coverage normalized to the protein area) for the different substrates were determined by a oneway analysis of variance (ANOVA). If a significant different ($P < 0.05$) was indicated by ANOVA, a Student-Newman-Keuls (SNK) post hoc test was used to compare between group means. P values < 0.05 were assumed to indicate statistically significant differences in the mean values.

3. RESULTS

3.1 Fabrication and Characterization of Protein Patterned Substrates

Patterns of protein lines were fabricated on glass coverslips by a three-step procedure (Figure 1): (1) well-defined lines of photoresist were fabricated by standard photolithography techniques; (2) a protein-resistant PEG layer was covalently immobilized to the bare glass between the photoresist lines through silane chemistry; and (3) following photoresist removal, gaps in the PEG layer were backfilled with fluorescent protein. Prior to the perfusion studies, line patterns were characterized by a combination of light and electron microscopy techniques.

Two types (varying patch spacing vs varying patch size) of protein pattern substrates were fabricated to independently study the effect of ligand patch spacing and ligand patch size on the adhesion of free-flowing platelets from whole blood. To study the effect of ligand spacing on platelet adhesion, we fabricated a set of three complimentary line patterns with varying patch spacing. Each pattern contained 100 parallel lines having a constant line length (1 cm) and width (3 μm) but with line spacings of 6, 15, or 30 μm . Figure 2 shows both brightfield images (Figures 2A–2C) of the initial photoresist patterns, and fluorescent images (Figures 2D–2F) of the resultant protein patterns for the three different substrates. Both the bright field and fluorescent images show high fidelity patterns, and line scan measurements (Figures 2G–2I) of the fluorescent intensity suggested that protein adsorption to the line patterns was uniform.

To study the effect of protein patch size on platelet adhesion, we fabricated individual substrates that contained a seven line pattern that consisted of lines with short dimensions of 2, 3, 4, 6, 8, 10, and 20 μm and a constant long dimension of 8 mm. The seven line pattern was repeated three times (Figure 3A) and the spacing (30 μm) was constant between each line. Figures 3B and 3C show a fluorescent image of the initial seven line pattern and corresponding line scan measurements. Similarly to the results of Figure 2, the line patterns with varying widths show high fidelity.

3.2 Spacing Between Fibrinogen Lines Affects Adhesion of Free-flowing Platelets and Growth of Platelet Aggregates

To examine the effect of ligand spacing on the adhesion of free-flowing platelets, mepacrine labeled whole blood was perfused at 100 s^{-1} over fibrinogen line patterns with different spacings and platelet adhesion was followed for ~9 minutes by fluorescent video microscopy. Upon perfusion of whole blood, free-flowing platelets adhered directly to the fibrinogen within seconds. With time, free-flowing platelets were also captured by platelets that had previously attached to the surface and local platelet aggregates developed (Figure 4). Platelet coverage increased with time on both patterned and un-patterned (control) fibrinogen. When fibrinogen lines were closely spaced together (6 μm) platelets initially adhered only to the fibrinogen lines. However with time, as new platelets were incorporated into growing aggregates, we observed the lateral growth of the aggregates. This lateral growth led to the formation of aggregates that bridged between neighboring fibrinogen lines across the PEG layer and the formation of irregularly shaped platelet aggregates similar to those formed on unpatterned fibrinogen substrates. When the spacing between lines was increased to 15 μm we still observed the presence of platelet aggregates that bridged between neighboring lines, however the frequency of bridging was significantly reduced and a majority of the aggregates observed had a linear shape. A further increase of the line spacing to 30 μm resulted in lines of platelets with virtually no aggregate bridging.

To confirm that the observed platelet adhesion was mediated by an interaction between the patterned fibrinogen and its platelet glycoprotein receptor $\alpha_{\text{IIb}}\beta_3$, two complimentary

control experiments were performed. The first control involved measuring platelet adhesion to patterned substrates with 3 μm lines of BSA with 6 μm spacings. As shown in Figure 4, there was little to no adhesion to the BSA lines, demonstrating that the platelets' adhesion was fibrinogen dependent. The second control involved treating platelets with a blocking mAb to $\alpha_{\text{IIb}}\beta_3$ prior to blood perfusion over substrates patterned with 3 μm fibrinogen lines with 6 μm spacings. There was little to no adhesion to the fibrinogen lines (Figure 4) providing evidence that platelet adhesion to the patterns was mediated by $\alpha_{\text{IIb}}\beta_3$.

To quantify differences in the amount of platelet adhesion to substrates with varying ligand spacing, a time-course analysis of the increase in platelet surface coverage on the unpatterned and patterned substrates was performed (Figure 5). Control samples (unpatterned fibrinogen) had the highest total platelet coverage (~30%), while samples with 6, 15, and 30 μm spacing between lines had approximately 20%, 10%, and 3% total platelet coverage respectively (Figure 5A). A one-way ANOVA showed that the differences in platelet coverage on the different substrates were statistically significant ($P < 0.001$). Multiple comparison by the Student-Newman-Keuls test ($P < 0.05$) demonstrated that the area of platelet coverage on the control substrate $> 6 \mu\text{m}$ spacing $> 15 \mu\text{m}$ spacing $= 30 \mu\text{m}$ spacing.

Since the total area of fibrinogen available for platelet adhesion is decreased with increased spacing, platelet adhesion area was normalized to protein area for each pattern (Figure 5B). When platelet coverage was normalized by protein area, we observed that the both the 6 μm and 15 μm spaced patterns had similar amounts of adhesion. Surprisingly these values were higher than normalized platelet coverage of the unpatterned fibrinogen controls. This increase in normalized platelet coverage on the 6 μm and 15 μm substrates was related to the bridging of platelet aggregates across lines or in areas where there was no fibrinogen adsorbed. A one-way ANOVA showed that the differences in platelet coverage on the different substrates were statistically significant ($P < 0.001$). Multiple comparison by the Student-Newman-Keuls test ($P < 0.05$) demonstrated that the platelet coverage on the 30 μm spacing was statistically less than the 6 μm spacing and 15 μm spacing.

To verify that the platelet aggregates that bridged between adjacent lines were not due to non-specific adhesion of platelets to the PEG layer we performed reflection interference contrast microscopy (RICM) to image the contact area between the adhering platelets and the glass slide. As shown in Figure 6A, fluorescent images of the platelet coverage on substrates patterned with 6 μm spacing between fibrinogen lines showed irregular shaped platelet aggregates that spanned neighboring fibrinogen lines, while the adhering platelets on the 30 μm spacing were primarily confined to the fibrinogen lines. The fluorescent images of the substrates having 6 μm spacing might suggest direct adhesion of platelets to the PEG layer. However, RICM imaging of the surfaces showed dark regions that were fairly linear with limited dark regions connecting them (Figure 6B). When RICM images of platelet adhesion were overlayed on fluorescent images of fibrinogen lines (Figure 6C) the dark regions of RICM aligned with the fluorescent fibrinogen lines. Similar alignment of RICM imaging of platelet adhesion with fluorescent fibrinogen lines was observed on surfaces with 30 μm spacing. Overlays of the RICM images with the corresponding fluorescent platelets (Figure 6D) show that not all of the adhering platelets achieve close contact with the substrate.

To confirm the fluorescent microscopy results and to characterize platelet adhesion over the entire surface, some samples were fixed and imaged by SEM. Figures 7A–7C show a single field of view of platelets adhering to the 3 μm wide fibrinogen lines patterns where bridging is seen for narrow spacings (6 μm) but adhesion is limited to lines on wider spacings (15 μm and 30 μm). Figures 7D and 7E are high magnification images (5000 $\times$) of the rectangular

regions of Figures 7A and 7B, and demonstrate (i) the morphology of platelet bridging between lines (7D) and (ii) platelet filopodia often extended in a direction perpendicular to the protein line (7E). Montage wide view SEM images (Figures 7F–7H) show platelet adhesion is uniform over large areas of the pattern. Similarly, bridging is limited to narrow spacing patterns.

3.3 Adhesion of Free-flowing Platelets Depends on the Downstream Length of Fibrinogen Patches

To study the effect of ligand patch size on the adhesion of free-flowing platelets, whole blood was perfused over a seven line pattern (Figure 3) that consisted of varying line dimensions (2–20 μm) with a 30 μm line spacing. A line spacing of 30 μm was chosen to reduce the likelihood of platelet aggregates from bridging between lines. The coating concentration of fibrinogen was increased to 100 $\mu\text{g/ml}$ since initial experiments with 20 $\mu\text{g/ml}$ showed minimal platelet adhesion. To determine whether the lateral (i.e. perpendicular relative to the direction of blood flow) width or the downstream length dimensions were important in regulating the adhesion of free-flowing platelets to fibrinogen, we perfused blood either parallel or perpendicular to the line patterns (Figure 8). *Parallel line patterns* are defined as lines having a constant downstream length of 8 mm and varying lateral widths (2–20 μm) that are oriented parallel to the direction of blood flow. *Perpendicular line patterns* are defined as lines having a constant lateral width of 8 mm and a varying downstream length (2–20 μm) that are oriented perpendicular to the flow of blood.

When blood was perfused over parallel line patterns, free-flowing platelets adhered directly to all the fibrinogen lines of different lateral widths within seconds (Figure 9A). Total platelet coverage on each line width increased with time similar to our results with constant line widths of 3 μm (Figure 3). Analysis of platelet coverage with time showed that total platelet coverage per line generally increased with the line's lateral width (Figure 9B). A one-way ANOVA showed that the differences in platelet coverage on the different substrates were statistically significant ($P < 0.001$). Multiple comparison by the Student-Newman-Keuls test ($P < 0.05$) demonstrated that the area of platelet coverage on the 20 μm line $>$ 10 μm line $=$ 8 μm line $>$ 6 μm line $=$ 4 μm line $=$ 3 μm line $=$ 2 μm line. In contrast, normalization of platelet coverage to the amount of fibrinogen area available did not show any clear trends (Figure 9C). Although one-way ANOVA showed that the differences in normalized platelet coverage on the different substrates were statistically significant ($P < 0.05$), multiple comparison by the Student-Newman-Keuls test ($P < 0.05$) demonstrated that the normalized area of platelet coverage differed only between the 3 μm and 6 μm lines. Finally, SEM images of the surfaces after perfusion demonstrated that platelet adhesion was fairly uniform along the downstream length of the lines and that little to no bridging of platelets between the lines occurred. The observation that platelets adhered similarly well to all the line lateral widths suggests that platelet adhesion was not limited by the line lateral width minimum of 2 μm that we tested.

Since the lateral width of fibrinogen had little effect on platelet adhesion, we investigated whether the downstream length of fibrinogen impacted platelet adhesion. When blood was perfused over perpendicular line patterns, significant differences in platelet adhesion were observed as compared to the parallel line pattern flow results (Figure 10A). While platelet adhesion still occurred on the large lines (i.e. downstream lengths of 8, 10, & 20 μm) adhesion to the smallest lines (downstream lengths of 2 – 4 μm) was nearly eliminated. Furthermore, even with the large lines there was a ~50% reduction in total platelet adhesion (Figure 10B). It should also be noted that there was an increased amount of platelet aggregates that bridged between neighboring fibrinogen lines. One-way ANOVA showed that the differences in platelet coverage on the different substrates were statistically significant ($P < 0.001$). Multiple comparisons by the Student-Newman-Keuls test ($P < 0.05$)

demonstrated that the area of platelet coverage on the 20 μm line > 10 μm line = 8 μm line > 6 μm line = 4 μm line = 3 μm line = 2 μm line.

Normalization of platelet coverage to protein area (Figure 10C) showed a general trend of decreasing platelet adhesion with decreasing downstream length of the line. It should be noted that the spike observed in the normalized platelet coverage on the 2 μm wide line experiment at 4 minutes was due to the adhesion of a large platelet aggregate in a single experiment that broke in half at 5 minutes and completely detached by 6 minutes. One-way ANOVA showed that the differences in normalized platelet coverage on the different substrates were statistically significant ($P < 0.001$), multiple comparisons by the Student-Newman-Keuls test ($P < 0.05$) demonstrated that the normalized area of platelet coverage on the 20 μm line = 10 μm line = 8 μm line > 6 μm line = 4 μm line = 3 μm line = 2 μm line.

SEM images of the substrates agreed with the fluorescent imaging results in showing limited, discontinuous platelet adhesion on small lines and increasing amounts of adhesion on the larger lines. It also should be noted that the SEM images indicated that platelet adhesion on the 2 μm lines for the 2nd and 3rd pattern repeat was increased when they were preceded by a 20 μm line upstream. These results suggest a potential influence of upstream structures on platelet adhesiveness and velocity, but will require additional studies beyond this report to elucidate.

To compare the differences in the normalized platelet adhesion coverage between parallel and perpendicular line patterns, we plotted the end point measurements (i.e. 9 min) from Figures 9 and 10 in a single bar graph (Figure 11). From this figure it can be seen that platelet adhesion to the lines parallel to the flow was between 29–90% greater than adhesion to the perpendicular lines of equal area. One-way ANOVA between the parallel and perpendicular measurements showed that these differences were statistically significant ($P < 0.001$). For the parallel line pattern, the amount of platelet adhesion did not appear to have a dependence on the normalized line's lateral width. In contrast, it appeared that the amount of platelet adhesion to the perpendicular line pattern was dependent on each line's downstream length when 6 μm and smaller. Taken together these results suggest that the downstream length of protein patches is a significant parameter in regulating platelet adhesion from the free-flow.

4. DISCUSSION

In this study we report a method for fabricating micron scale patterns of protein on a bio-inert background using basic photolithography techniques. Using this technique we were able to generate discrete line patterns of fibrinogen surrounded by a protein-repellant layer of PEG with high fidelity down to 2 μm . Incorporation of these novel protein patterned substrates into standard flow chamber systems allowed us to investigate the role of protein patch size on the adhesion of free-flowing platelets from whole blood. Using both real-time fluorescent imaging and end-point SEM imaging to characterize the interaction of platelets with these novel fibrinogen patterned substrates we were able to identify two new aspects of platelet adhesion under low flow conditions: (i) the amount of platelet adhesion under flow conditions was dependent upon the lateral spacing between patches of protein; and (ii) the degree of platelet adhesion from the free-flow was dependent upon the downstream length of the protein patch.

Our studies on the role of *lateral spacing* (6–30 μm) between patches of fibrinogen extend recent studies[14, 28] of platelet adhesion onto micropatterned substrates. Basabe-Desmonts[14] reported that a 6 μm separation between spots was sufficient to avoid the simultaneous adhesion of a single platelet to neighboring protein dots. Likewise, Kita et

al[28]. reported that individual platelets could not span gaps $> 5 \mu\text{m}$ between stripes of protein. Initially these results would appear to be in disagreement with our lateral spacing results where we observed platelet bridging on 6 and 15 μm gaps but not on 30 μm gaps.

There are several important differences between the studies (e.g. type of anticoagulant, protein site density, incubation time) that may explain these different observations. Probably the most important difference between the studies is that we conducted our experiments under continuous well-defined flow of heparinized whole blood. In contrast the study by Basabe-Desmonts employed a gentle rocking motion of citrated whole blood for 30 minutes to capture platelets onto the protein patterned surfaces, while Kita et al, used isolated platelet suspensions purified from acid-citrate dextrose (ACD) whole blood with a 1–2 hour incubation period.

The issue of continuous flow vs static (or rocking) conditions on platelet adhesion and growing platelet aggregates is an important distinction for a couple of reasons. First, the presence of continuous flow meant that a continuous supply of free-flowing platelets was delivered to the surface and these platelets could be captured by previously adherent platelets to form growing aggregates. As new platelets were incorporated into the aggregate, growth of the platelet aggregate occurred both in the downstream and lateral direction. The growth of platelet aggregate in both of these directions (i.e. downstream and lateral) has been observed by others on surfaces coated homogeneously with fibrinogen.[29–31] Thus the fact that platelet aggregate grew downstream in the experiments in which blood was perfused parallel to the lines (Figure 9) was to be expected since the line geometry provided an adhesive surface for downstream accumulation. A surprising finding was the lateral growth of platelet aggregates on the patterned substrates since the line width ($\sim 3 \mu\text{m}$) of fibrinogen is on the same order of size of an individual, unactivated platelet diameter (i.e. 2 – 4 μm). The fact that platelet aggregates grew laterally over the PEG regions suggests that lateral growth of platelet aggregates can occur in the absence of an adhesive surface.

We speculate that there are a couple of mechanisms for the lateral growth of platelet aggregates over the nonadhesive PEG regions of the substrate. One possible mechanism is the lateral stacking of platelets on top of each other. The RCM imaging of the surfaces (Figure 6) in which platelets showed a close contact or direct adhesion (dark regions) primarily to the fibrinogen areas and a more distant or non-existent adhesive interaction (white regions) to areas lacking fibrinogen provides some evidence that the lateral platelet aggregate growth occurred due to lateral stacking of platelets and not direct adhesion to the PEG. The fact that bridging of platelets was routinely observed on 6 μm spacing, limited on the 15 μm spacing and essentially non-existent on the 30 μm spaced patterns suggests that there is a limit to the lateral distances platelet aggregates can grow in the absence of an underlying adhesive substrate. This may suggest that the drag force on a growing platelet bridge may influence its ability to span a non-adhesive gap during formation. This space dependence of the lateral growth could also suggest an alternative growth mechanism involving the extension of platelet filopodia. Studies have reported that when platelets adhere to fibrinogen they become activated, extend filopodia, and spread out[29]. Previous measurements of filopodia (or pseudopodia) lengths for activated platelets have been reported to range from 2–5 μm [28, 32, 33]. Thus one possible explanation for the growth of platelet aggregates between the 6 or 15 μm spaced fibrinogen lines is that platelets on neighboring fibrinogen lines could initially interact with each other by their extended filopodia. Thus if two platelets extended $\sim 5 \mu\text{m}$ long filopodia in the direction of each other they could easily span a distance up to 10 μm in the absence of an underlying adhesive ligand. Subsequently these extended filopodia would provide an adhesive binding site for the capture of other free-flowing platelets. However, further studies will require high resolution imaging to determine the dominant mechanism. A final possibility is that of non-

specific adhesion of platelets to the PEG regions. Although we cannot completely rule this mechanism out, there are three observations that would argue against this. First, when we perfuse whole blood over substrates coated with PEG alone and no fibrinogen, we see little to no platelet adhesion to the surface, which suggests that the PEG layer reduces and or eliminates non-specific adhesion. Second, the control experiments with lines of BSA also show little to no platelet adhesion on either the BSA or PEG regions. Finally, the observation that lateral growth of platelet aggregates between lines primarily occurs on lines that are closely spaced (6 μm) and not larger spacing (> 15 μm) would also suggest that nonspecific adhesion is not the cause.

Our observation that the *downstream length* of the protein line effects the degree of adhesion of free-flowing platelets (Figure 10) compares well with the results of Basabe-Desmonts[14], who reported that 2 μm spots of protein (vWf, fibrinogen, or anti-CD42b antibody) allowed for the capture of single platelets while spots with a 24 μm diameter allowed for the adhesion of 12–36 individual platelets. Although we did observe some individual platelets adhering to the initial 2 μm line during perpendicular line pattern blood perfusion assay, the coverage was not uniform. In contrast with findings of platelet adhesion to protein line widths as low as 0.6 μm under static conditions[28], the influence of constant flow conditions over perpendicular line patterns in our studies yielded little platelet adhesion on line downstream lengths up to 6 μm (Figure 10). Likewise our studies in which blood was perfused over parallel line patterns (Figure 9) showed similar amounts of platelet adhesion on all line lateral widths when normalized to the protein area.

One way to reconcile the different results of platelet adhesion during perpendicular vs parallel line pattern flow assays is to consider the following. When the line was parallel to the direction of blood flow, the free-flowing platelet had multiple opportunities, or a long period of time, to make a stable adhesive interaction to the line of protein. For example if a platelet near the surface had a free flowing velocity of 100 $\mu\text{m}/\text{sec}$, it would have ~80 sec to interact/adhere along the 8 mm downstream length of the 2 μm wide line. Since we found minimal differences in the degree of adhesion when normalized to the protein area, the relevant patch size parameter in the parallel line pattern flow experiments was not the line lateral width (2–20 μm) but rather the downstream length of the line (8 mm). In contrast, when the protein line was oriented perpendicular to the flow, the platelet had a reduced amount of time to make a stable adhesive interaction with the protein line. In the case of the shortest downstream length of 2 μm , a platelet traveling at 100 $\mu\text{m}/\text{sec}$ would have ~0.02 sec to interact and adhere. It is interesting to note that SEM imaging of the surfaces in which blood was perfused over perpendicular line patterns suggested that there was increased adhesion to the 2nd and 3rd repeat of the 2 μm lines. We hypothesize that the increased platelet adhesion on these downstream 2 μm lines is related to the fact that they are located immediately downstream of a 20 μm line. Since the 20 μm line provided a protein patch 10 times greater in size then the 2 μm line, it is extremely likely that a free-flowing platelet could transiently interact with the 20 μm line, thereby slowing down its velocity to a level that allowed for a subsequent binding event to the downstream 2 μm line. An alternative explanation is that platelets are being activated while flowing over surfaces of adhered platelets that are secreting platelet agonists such as ADP and thromboxane A₂. In support of this explanation is that the phenomenon of a downstream injury affecting an upstream injury has been described mathematically[23]. Likewise it has been shown experimentally that ADP activation increases adhesion efficiency of platelets to fibrinogen[21]. Additional experiments are needed to differentiate between these mechanisms.

In summary, we have developed a method for spatially controlling the adhesion of free-flowing platelets from whole blood onto protein coated surfaces. The method is highly versatile in that the size, shape, and spacing of the protein patch as well as the protein type

can easily be changed. We have demonstrated that the space between ligand patches affects the aggregation process and the formation of platelet bridges between the patches. We have identified the downstream length of a ligand patch as an important factor in platelet adhesion under low shear conditions. We speculate that this result is representative of a minimum interaction time or length requirement for receptor-ligand bond formation. Continued investigation into these parameters should include both downstream length variations as well as shear variation. Although we demonstrate the use of these substrates to investigate the role of fibrinogen patch spacing and size on the adhesion of platelets and growth of platelet aggregates under low or venous shear conditions, these substrates can easily be used to probe the effects of vWF presentation under high shear stress conditions.

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Figure 1. Schematic of the protein patterning procedure

A glass coverslip is spin coated with photoresist (B), patterned by exposure to U.V. light (C), and developed (D). Next a layer of PEG is grafted to the glass substrate through silane chemistry around the photoresist (E) and the photoresist is removed (F). Protein is adsorbed to the bare glass regions of the PEG layer (G).

Figure 2. Characterization of patterned substrates with constant line width and variable line spacing

(A–C) Bright field images of the photoresist templates with constant line width (3 μm) but varying line spacing (6–30 μm); (D–F) Fluorescent images of the line patterns fabricated with AlexaFluor647-labeled fibrinogen. (G–I) Fluorescent line intensity (arbitrary units) graphs of the line scans figures D–F.

Figure 3. Characterization of patterned substrates with variable line dimensions and constant line spacing

(A) Bright field image of the seven line pattern of constant long dimension (8 mm), constant spacing (30 μm) and varying short dimensions (2, 3, 4, 6, 8, 10, 20 μm). The seven line pattern was repeated three times. (B) Fluorescent imaging of the first set of the seven line pattern and the corresponding line scan of the fluorescent intensity (C).

Figure 4. The effect of spacing between fibrinogen lines on platelet adhesion under low shear conditions

Whole blood treated with the fluorescent dye mepacrine was perfused over surfaces coated with either AlexaFluor647-labeled Fibrinogen or AlexaFluor647-labeled bovine serum albumin (BSA) in a parallel plate flow chamber at 100 s^{-1} . Fluorescent images of the $3 \mu\text{m}$ wide line patterns (red) with varying line spacing (6, 15, $30 \mu\text{m}$) were obtained prior to perfusion of blood. Imaging of fluorescently labeled platelets (green) adhered to the various protein patterns were acquired in real time at the indicated time points by an epifluorescent video microscopy system and overlayed onto the previously acquired images of the protein lines. In some experiments, platelets were treated with a blocking anti- $\alpha_{\text{IIb}}\beta_3$ mAb prior to perfusion over the fibrinogen line patterns.

Figure 5. Total and normalized platelet adhesion area on fibrinogen line patterns

(A) Measurement of the total platelet accumulation with time onto the various protein patterned surfaces illustrated in Figure 4. (B) To account for differences in the amount of protein available for each line pattern, the total platelet coverage of figure (A) were normalized to the area of fibrinogen available for each line. Each data point represents the mean $\pm$ S.E. for 6 separate experiments with blood from 5 different donors. Differences in the platelet coverage were tested in one-way-ANOVA and found to be statistically significant ($P < 0.001$), and a subsequent Student-Newman-Keuls test was performed to determine statistical differences ($P < 0.05$) among the different spacings.

Figure 6. Interaction of Adhering Platelets to Fibrinogen Line Patterns

(A) Fluorescence microscopic overlay images of mepacrine labeled platelets (green) adhering to the 3 μm wide line patterns of Alexa-Fluor647 Fibrinogen (red). (B) RCM images of the close contact points (dark regions) of adhering platelets to fibrinogen line patterns. (C) Overlay of RCM images of platelet contact points with fluorescent images of the fibrinogen line patterns (red). (D) Overlay of the RCM images of platelet contact points with the fluorescent images of platelets (green).

Figure 7. Scanning electron microscopy characterization of platelet adhesion

In some experiments, platelets were fixed at the completion of the adhesion assay and imaged by SEM. 1000 $\times$ magnification images show platelets adhering to 6 μ m (A), 15 μ m (B), or 30 μ m (C) line patterns. (D) A 5000 $\times$ magnification image of the rectangular region in (A) that shows the morphology of a platelet bridge. (E) A 5000 $\times$ magnification image of the rectangular region in (B) showing platelet filopodia. Figures 7F–H, are montage images of the 6 μ m spacing patterns (F), 15 μ m spacing (G), and 30 μ m spacing (H).

Figure 8. The convention of line pattern orientation

Parallel line patterns have a downstream length of 8mm and vary in lateral width from 2–20 μ m. Cells reach each parallel line simultaneously during blood perfusion assays. Perpendicular line patterns have a lateral width of 5mm and vary in downstream length from 2–20 μ m. For perpendicular lines cells reach the smallest line first and each line consecutively after the previous.

Figure 9. The effect of fibrinogen patch size in parallel line patterns on platelet adhesion over time

(A) Fluorescent images of platelet adhesion (green) over time on fibrinogen line patterns (red) of different lateral widths. Since the 30 μm spacing limited the field of view, we imaged 3 line widths at a time (e.g. 20 μm , 2 μm , 3 μm), changed the field of view to image the next set of line widths (e.g. 4 μm , 6 μm , 8 μm), and changed the field of view to image the last set of lines (e.g. 10 μm , 20 μm , 2 μm). (B) Real time analysis of the total platelet accumulation onto each line shown in figure (A). (C) To account for differences in the amount of protein available for each line, the total platelet coverage of figure (B) was normalized to the area of fibrinogen available for each line. (D) SEM shows platelet adhesion over a large area the pattern. Each data point represents the mean $\pm$ S.E. for 9 separate experiments with blood from 3 different donors. Differences in the platelet coverage were tested with one-way-ANOVA and found to be statistically significant ($P < 0.001$), and a subsequent Student-Newman-Keuls test was performed to determine statistical differences ($P < 0.05$) among the substrates.

Figure 10. The effect of fibrinogen patch size in perpendicular line patterns on platelet adhesion over time

(A) Fluorescent images of platelet adhesion (green) over time on fibrinogen line patterns (red) of different downstream lengths. Since the 30 μm spacing limited the field of view, we imaged 3 line lengths at a time (e.g. 2 μm , 3 μm , 4 μm), changed the field of view to image the next set of line lengths (e.g. 6 μm , 8 μm , 10 μm), and changed the field of view to image the last set of lines (e.g. 20 μm , 2 μm , 3 μm). (B) Real time analysis of the total platelet accumulation onto each line shown in figure (A). (C) To account for differences in the amount of protein available for each line, the total platelet coverage of figure (B) was normalized to the area of fibrinogen available for each line. (D) SEM shows platelet adhesion over a large area the pattern. Each data point represents the mean $\pm$ S.E. for 8 separate experiments with blood from 3 different donors. Differences in the platelet coverage were tested in one-way-ANOVA and found to be statistically significant ($P < 0.001$), and a subsequent Student-Newman-Keuls test was performed to determine statistical differences ($P < 0.05$) among the substrates.

Figure 11. Comparison of platelet adhesion between parallel and perpendicular line patterns Minute 9 data points for parallel line patterns (n=9) and perpendicular line patterns (n=8) from Figures 9C and 10C are plotted with linear regression trend lines and show that lines parallel to the flow collect 29–90% greater amounts of platelets than lines of equal area perpendicular to the flow. Further, the trend lines show constant coverage for all lines widths parallel to the flow, but decreasing coverage for downstream lengths of lines perpendicular to the flow. Data points are reported as the mean $\pm$ S.E. Differences in the platelet coverage between parallel and perpendicular flow were tested in one-way-ANOVA and found to be statistically significant ($P < 0.001$).