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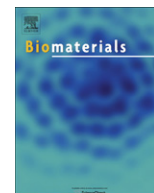

The effect of upstream platelet–fibrinogen interactions on downstream adhesion and activation

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ABSTRACT

Circulating activated platelets roll and make transient contacts before ultimately adhering to a substrate. However, despite the dynamic nature of platelet adhesion, most *in vitro* adhesion and activation studies have focused on establishing local cause and effect relationships. Here, we determined the effect of exposing platelets to immobilized upstream human fibrinogen on downstream adhesion and activation. Microcontact printing was used to prepare substrates that contained well defined fibrinogen priming regions. Washed platelets were perfused over the substrates and adhesion and activation in a downstream capture region were compared with samples that did not contain a fibrinogen priming region. It was found that samples containing an upstream priming region resulted in higher adhesion, platelet spreading areas and aggregation than samples that lacked the priming region. Also, when the priming region was selectively blocked with a polyclonal anti-fibrinogen antibody, the platelet response was attenuated. To characterize this phenomenon further, flow cytometry was used to assess bulk platelet activation following fibrinogen priming. The expression of two activation markers, PAC-1 and P-selectin were quantified. Expression of both activation markers was found to be higher after perfusion over fibrinogen versus albumin-coated substrates.

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1. Introduction

Platelet adhesion and activation on the surface of synthetic blood contacting biomaterials continues to be a challenge for vascular devices. Activation stimulates the local activation of plasma coagulation factors and eventually leads to formation of a fibrin clot. An adverse platelet response to vascular implants can lead to many complications including occlusion, neointimal hyperplasia, and embolism. Consequently, a considerable amount of effort has been devoted to developing materials that minimize the platelet response [1–4].

The general methodology used to study the blood compatibility of a biomaterial is to examine the direct local effects of a material property on platelet adhesion and activation [5–7]. However, surface induced platelet adhesion and activation is a dynamic process. Platelets attach/detach and roll [8–10], before ultimately forming stable adhesive interactions. In fact, most platelet-surface contacts are transient [10]. Even though transient interactions do not result in local platelet adhesion and aggregate formation, it is unlikely that they leave the activation state of the platelet unaffected. With each surface contact there is the opportunity to interact with adsorbed plasma protein agonists such as fibrinogen and vWf, through specific interactions with integrin $\alpha_{IIb}\beta_3$ and GPIb-IX-V membrane receptors respectively [11–13]. Also, platelets may interact with exposed subendothelial collagen due to injury at the anastamoses of the implanted vascular device [14]. Furthermore, previous studies have found that the platelet-surface response is changed when the upstream environment is varied [15]. Taken together, upstream platelet-surface interactions may affect downstream adhesion and activation. Specifically, upstream interactions with protein agonists may "prime" platelets for downstream adhesion and activation.

In this study, we characterized the effect of upstream platelet–fibrinogen interactions on downstream adhesion and activation. Microcontact printing (μCP) was used to covalently immobilize fibrinogen priming regions onto chemically reactive substrates and the downstream platelet response was observed. Adhesion, activation and aggregation were found to be significantly higher on samples containing a fibrinogen priming region compared with control samples. Also, the increase in downstream adhesion was attenuated when the priming region was blocked with a polyclonal antibody for fibrinogen suggesting fibrinogen is, in fact, capable of inducing a downstream response. The effect of transient platelet-surface contacts on bulk platelet activation was

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assessed by quantifying P-selectin and active $\alpha_{IIb}\beta_3$ using flow cytometry. An increase in bulk platelet activation was observed after perfusion over samples prepared with covalently immobilized fibrinogen versus albumin. These results suggest that platelets are capable of being primed for downstream adhesion and activation by upstream immobilized protein agonists. These findings have implications for both the design of vascular devices as well as the design of *in vitro* platelet adhesion and activation assays.

2. Methods

2.1. Preparation of polydimethylsiloxane (PDMS) stamps for µCP

PDMS stamps were prepared from masks with randomly distributed µm-sized features that were defined to cover 85% of the stamp surface area (Fig. 1A). Mask patterns were developed by generating a 500 × 500 array of randomly distributed black and white pixels using Mathematica (Wolfram). Patterns were transferred to chromium coated silica wafers using conventional photolithography. First, the pattern was uploaded into a mask making software, L-Edit (Tanner), where each pixel was defined to be 25 µm × 25 µm. An Electromask MM250 (Interserv Technology) pattern generator was used to produce the first mask (A_{mask} = 1.25 cm × 1.25 cm and A_{pixel} = 25 µm × 25 µm). This was followed by two 5× image reductions and one repeat step to produce a final mask with a 20 × 20 pattern array of randomly distributed micron sized features (A_{mask} = 1 cm × 1 cm and A_{pixel} = 1 µm × 1 µm). Sylgard 184 silicone elastomer (Dow Corning) was mixed with curing agent in a 10:1 ratio and poured over the patterned mask. PDMS was degassed by placing the samples under vacuum for 30 min. The samples were cured for 30 min at 100 °C and carefully peeled away from the mask to remove the patterned stamps. This was followed by an additional 60 min of curing at 60 °C. After curing the stamps were incubated overnight in hexane to remove any polymer that did not crosslink. To eliminate swelling that occurred after hexane incubation, stamps were rinsed for 30 min in a 95% ethanol solution, 30 min in Milli-Q water, and dried for 30 min at 60 °C.

2.2. Covalent immobilization of fibrinogen to reactive surfaces

PDMS Stamps were “inked” with human fibrinogen in PBS (c_{Fgn} = 1 mg/ml, pH 8.5) for 15 min, rinsed in Milli-Q water and dried with N_2 gas. The fibrinogen coated stamps were placed in contact with commercially available Nexterion-H (Schott) reactive slides with 490 Pa of pressure applied evenly using a 5 g weight. These slides contain a reactive coating in which a cross-linked poly(ethylene glycol) PEG layer is functionalized with NHS esters providing means for covalent protein immobilization through the terminal amine group [16]. A fibrinogen “priming” region was printed in the upstream region on test samples and a platelet capture region was printed 10 mm downstream of the priming region (Fig. 1B). Fibrinogen coated stamps were allowed to react with the surface for 1 h. After fibrinogen was

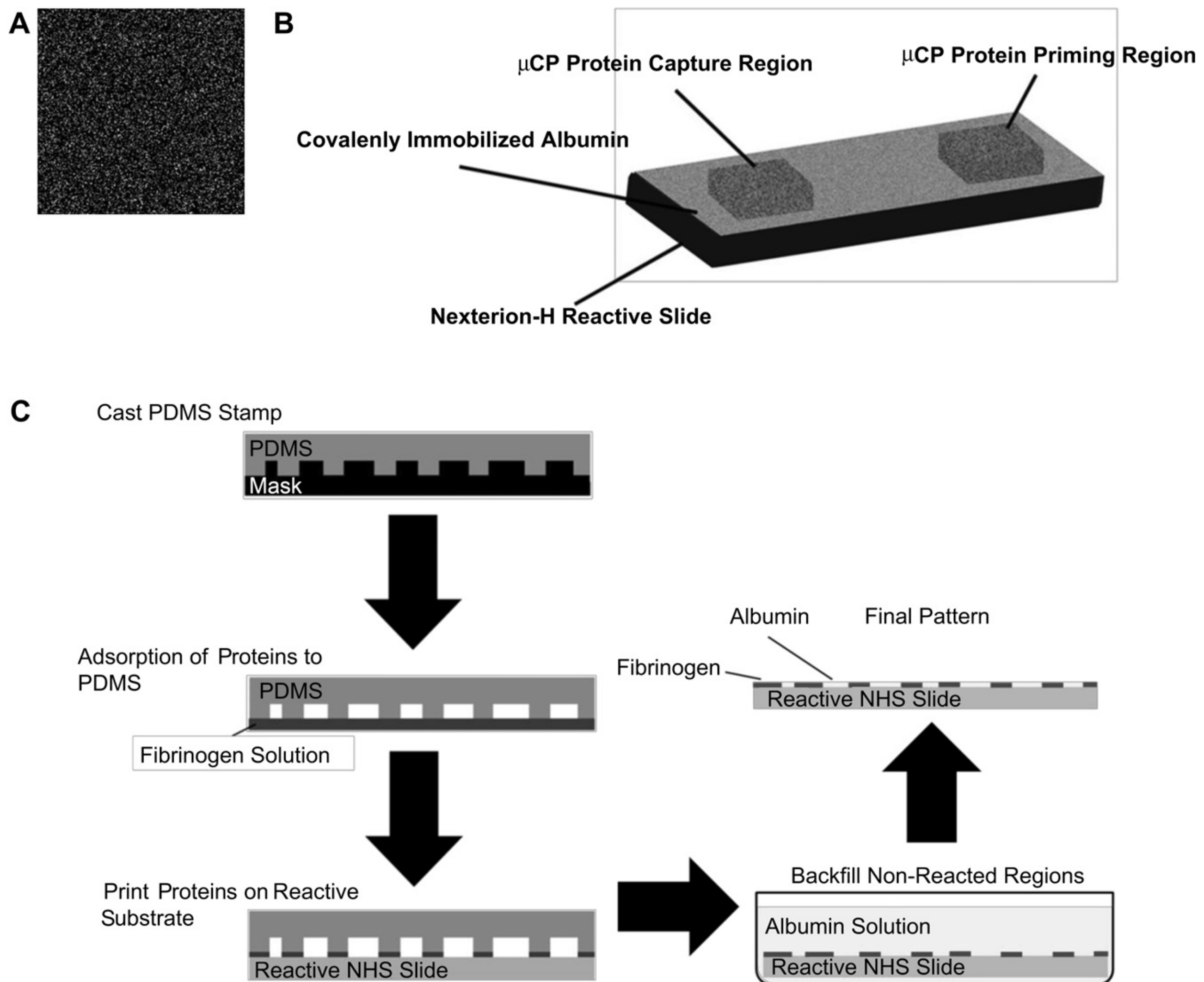

Fig. 1. Sample preparation using µCP: (A) Image of a random pattern used to prepare PDMS stamps with an 85% relative coverage area. (B) Nexterion-H samples were prepared with covalently immobilized protein patterns using µCP. (C) A schematic representation of the µCP process. First PDMS stamps are cast and cured in patterned masks. The stamps are transferred to a protein solution where they are “inked” by allowing the protein to adsorb to the surface. The protein coated stamp is placed in contact with the reactive surface allowing the protein transfer to occur. On Nexterion-H substrates, the printed surface was incubated in an albumin solution to passivate the unpatterned regions.

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