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PLATELET FUNCTION ASSESSMENT IN A MICROFABRICATED DEVICE

Ameya Kantak, Yuri Lvov, David K. Mills, James G. Spaulding, Steven A. Jones

Program in Biomedical Engineering
Institute for Micromanufacturing
and School of Biological Sciences
Louisiana Tech University
PO Box 10348
Ruston, LA 71272

Introduction

Although platelets are small and simple in shape, they are complicated in their physiologhy. Their alpha granules and dense bodies secrete a large number of agents that are involved in haemostasis, and the glycoproteins on their surfaces form the linkages with proteins like fibrinogen, fibronectin, collagen and von Willebrand factor that are necessary for adhesion and aggregation (Frojmovic, 1998). Diseases such as heart attack (Meade, 1992), stroke (Harker, 1998) and eclampsia (Schindler et al., 1990), can be the result of pathologies in platelets. Although devices have been recently developed to diagnose platelet function (Nicholson et al., 1998), there is still a need for devices that can examine the multiple factors of platelet physiology that affect thrombus formation. Because fluid shear is a key factor in platelet adhesion and aggregation (Colantuoni et al., 1977), it is necessary to test platelet function under conditions of flow. Also, because of the large number of adhesion receptors and secreted agents that make up a platelet's physiology, a complete diagnosis of platelet function should be performed on a variety of substrates.

A simple way to test platelet function is to immobilize substrates on a flow channel, induce flow over the substrate, and measure the percentage of surface covered by platelet adhesion. The use of several substrates can allow the assessment of a variety of platelet functions.

A practical platelet analyzer should 1) be capable of distinguishing multiple aspects of platelet behavior, 2) require a small amount of blood, 3) examine platelet function under shear flow conditions, 4) be relatively simple to use, and 5) be relatively easy to manufacture. Modern micromanufacturing methods are readily available for the construction of the microchannels that can be filled with volumes of blood on the order of ?L. However, a technique is still needed to coat these channels with the appropriate protein substrates.

Recently, a process called layer-by-layer assembly (Lvov et al., 2000) has been developed that allows well-controlled protein coatings to be adsorbed onto charged surfaces. The process takes advantage of the charge already on the surface to lay down, in alternating layers, positively and negatively charged ions. The combination of layer-by-layer assembly and micromanufacturing can thus form a basis for the device being sought. Key questions to be answered are whether it is possible to assemble the needed substrates and whether the platelet adhesion patterns behave as expected. For example, the amount of adhesion should be dependent on the substrate and the fluid shear stress. Because fibrinogen is a key protein in both platelet adhesion and platelet aggregation, this was selected as the first protein to be examined.

Methods

The microchannels were first created as ridges on a silicon wafer. The photopolymer SU-8 was spun onto the wafer and exposed through a mask under ultraviolet light to allow the polymer to cure. Afterwards, the wafer was developed in SU-8 developer, leaving SU-8 ridges only where the surface was exposed to light. The cured wafer was then used as a mold for polydimethylsiloxane (PDMS), an optically clear polymer that cures at room temperature in three days. The raised portions on the silicon then become microchannels in the PDMS.

The PDMS microchannels were coated by the layer-by-layer self-assembly technique to provide controlled nanometer-thick layers of fibrinogen. A plexiglass plate was created to cover the channels, and inlet and outlet ports were incorporated into the cover to allow the injection of blood. A syringe injector (Cole Parmer) was used to inject platelet rich plasma at a controlled flow rate. Anticoagulated platelet rich plasma labeled with both acridine orange and a fluorescin isothiocynate-tagged anti-GpIIb/IIIa-antibody was passed through the microchannels. Several experimental runs for different shear rates were carried out. To estimate shear, Couette flow was assumed in the microchannels. Control experiments were performed on bare PDMS surfaces. Images were recorded with a fluorescent microscope. For each image, background subtraction was applied, followed by a thresholding technique to distinguish dark areas (platelets) from light areas (substrate with no adhesion). From these steps, the extent of

platelet adhesion to the fibrinogen substrate was determined for each of the shear rates.

Results

Platelet adhesion was readily visible on the fibrinogen substrate and less visible in the uncoated PDMS channels. Figure 1 shows images taken on both substrates at a shear stress of 20 dynes/cm^2.

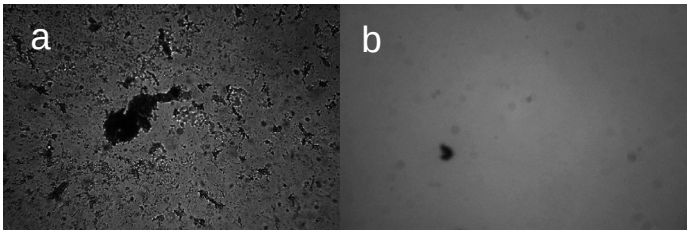

Figure 1: Platelet adhesion on the fibrinogen substrate at a shear stress of 20 dynes/cm^2. (a) Surface with fibrinogen demonstrating substantial adhesion. (b) Control with no fibrinogen, demonstrating minimal adhesion.

The percentage of surface coating was dependent on shear rate in the channel. Figure 2 shows the percentage of surface coverage as a function of shear stress. The coverage for the uncoated PDMS is an order of magnitude lower than that for the fibrinogen-coated surface. There is a peak in the data at 20 dynes/cm^2.

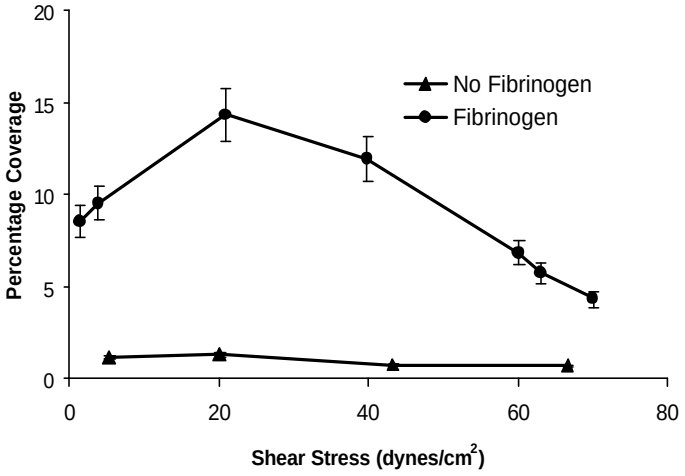

Figure 2: Percent surface coverage on the uncoated PDMS and on the fibrinogen coated PDMS as a function of shear stress.

Discussion

This study demonstrated that differences between adhesion on fibrinogen-coated and uncoated PDMS microchannels could be detected, and that the adhesion was also a function of shear stress.

The reason for the shear dependence has not been established directly in this study. One explanation is that von Willebrand factor, either released from activated platelets or already present in the PRP, attaches to the fibrinogen and the GPIb and/or GPIIb/IIIa receptors on the platelets. Since von Willebrand factor-mediated adhesion is shear dependent (Girma et al., 1995), it naturally leads to the type of behavior shown in Figure 2. For low levels of shear, von Willebrand factor is not effective, and thus adhesion is minimal. For higher levels of shear, von Willebrand factor anchors the platelets to the fibrinogen substrate, tending to increase the surface coverage. At even higher shear stresses, the shear is high enough that either insufficient time is available for the necessary adhesion bonds to form, or the bonds do form but are torn apart by the stress. It is also possible that the shear removes part of the fibrinogen layer itself. Although experiments by Lvov et al. (2000) indicate that self-assembly in microchannels is not affected by these levels of forces, this result still needs to be verified for the case studied here.

A second explanation for the shear dependence is simply that the shear alters the transport of platelets to the channel wall. However, because there are no red blood cells present in the injected PRP, the enhanced diffusion effect will not be present.

Conclusion

These studies support the idea that a device can be developed that allows functional assessment of platelets through measurements of adhesion over microchannels coated with various proteins. With such a device, it will be possible to measure natural platelet activation and suggest treatment criteria for platelet-specific diseases. The same principle can be used to study biomaterial interactions.

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