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Application Note C100

Cardiovascular: Platelet; Thrombosis; Adhesion Assay

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Introduction

Cellix Ltd. have developed a novel microflow system consisting of Mirus Evo nanopump with microfluidic biochip and flow sensor controlled by a PC using dedicated software. The Mirus Evo nanopump allows very accurate flow rates to be achieved that are more reproducible and consistent than anything currently available. Importantly, flow rates are very low (5 pL min^{-1} to 10 µL min^{-1}) and the shear stress levels that the pump can mimic (up to 30 dyne/cm^2) are equivalent to those found in blood vessels in vivo. The biochips are comprised of eight different channels and can be manufactured so that the dimensions of the channel are similar to the blood vessel being assessed. Currently, the channels can be coated with recombinant human adhesion proteins for use in inflammation studies. However, it should soon be possible to culture a desired cell line on the channel surface, allowing for more physiologically relevant assays to be completed. The Mirus Evo nanopump is vital to the use of small diameter channels as standard syringe pumps are incapable of delivering the required low flow rates.

Overview

First of all, the cell type to be analysed must be determined, followed by establishing how to harvest such cells, e.g. culturing in growth media, or isolation from in vivo fluids. Secondly, the assay itself should be outlined, including whether live cells or proteins will be coating the channels of the biochip. If it is the former, protocols for culturing the cells both outside and inside the biochip channel must be established. Thirdly, the adhesion profile of the cells to be passed through the coated channel should be determined. Next, if exogenous compounds are being analysed, these should then be introduced to the system and their effect on the adhesion profile assessed. This should include calculation of required concentrations and pre-incubation conditions, before introduction to the system. Finally, the images taken via the digital camera attached to the microscope should be masked and analysed using the Image Pro Premier software.

Methods

1. Choice of Cell Type and Harvesting Protocols

A microfluidic assay assessing the adhesion profile of platelets on various matrix proteins was developed. Blood was collected from healthy volunteers who were not taking any medication and were free from aspirin and other anti-platelet agents within the preceding 2 weeks. The blood was drawn by venepuncture into tubes containing a 1:10 volume of 3.8% (wt/vol) trisodium citrate, which is a calcium chelator, and gently mixed. The anti-coagulant agent D-phenylalanyl L-propyl-L-arginine chloromethyl ketone dihydrochloride (PPACK), which is a thrombin inhibitor, was added to a final concentration of 80 µM in whole blood. The first 5 ml of drawn blood was discarded. Whole blood can be kept in 50 ml polypropylene tubes during assay procedures. Platelets in whole blood were labelled by incubating anti-coagulated blood with the fluorescent dye DiOC6 (1

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µM) for 10-20 mins, (blood platelet count varied from 136-273 x 10^3 platelets μl^{-1}).

2. Assay Outline, Including Biochip Coating Procedures

Vena8 Fluoro+ biochip channels were coated overnight in humid conditions at 4°C with either vWF (von Willebrand Factor, 100 µg/ml) or collagen (HORM Equine Fibrillar Collagen, 200 µg/ml). All channels were then coated with BSA (Bovine Serum Albumin, 10 µg/ml) to occupy non-specific binding sites. An additional channel was coated with BSA (10 µg/ml) for 2 hrs at room temperature. Prior to shear experiments, all channels were washed thrice with PBS.

3. Adhesion Profiles

Whole blood was infused into the vWF, collagen and BSA coated channels under a defined shear stress, ranging from 0 to 120 dyne/cm^2, 2 min per shear stress level. Images at the indicated shear stress level were captured using Venaflux assay software (1 frame per second). Adhesion of platelets and thrombus formation was evaluated by monitoring platelet migratory behaviour in real time with images captured via a liquid chilled Quantix 10-57 CCD camera connected to the Zeiss Axiovert 200 inverted epi-fluorescence microscope.

4. Image Analysis

Images for analysis have been extracted from video at frames 1, 30, 60 and 120. The thrombus profile was recorded using Image Pro Premier software. Data was exported into Excel for interpretation. Data was presented as mean±SEM.

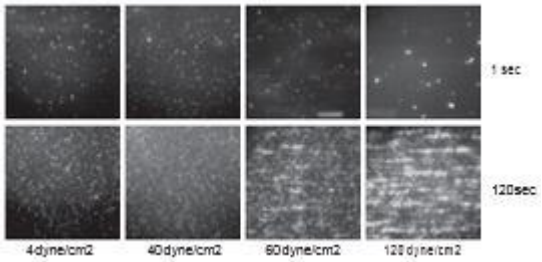

Figure 1

Figure 1: Platelet adhesion profile on vWF at indicated time points (1 and 120 s), subjected to gradient shear stress of (4, 40, 60 and 120 dyne/cm^2).

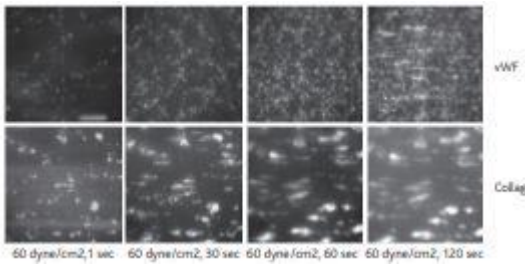

Figure 3

Figure 3: Platelet adhesion profile to vWF and collagen at constant shear stress of 60 dyne/cm^2, at indicated time points.

Results

The adhesion profile of platelets was recorded with respect to vWF, collagen and BSA (data not shown). Whole blood infused into vWF-coated channels at a range of shear stresses show rolling/adhesion of platelets after just 1 s. At 120 s, lower shear stress (4 and 40 dyne/cm^2) result in platelets rolling/adhesion, whereas, at higher stress (60 and 120 dyne/cm^2) platelet thrombus formation appears (Figure 1). A threshold limit of ~60 dyne/cm^2 is required for thrombus formation, conditions that occur in microcirculation. Chain-like structures of platelets aligned in the direction of flow

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(120 s at higher shear stress) result in the number of individual platelets interacting with immobilised vWF to be reduced. Using Image Pro Premier software, the thrombus formation profile was analysed at 120 s, and the average length of the aggregates (thrombus) was calculated. A 5.9-fold increase in thrombus formation from 4 to 120 dyne/cm^2 was recorded (Figure 2(i)). The varying length (maximum, minimum and average) of the distributed aggregates is represented in Figure 2(ii).

Collagen is a unique agonist of platelets, acting as an immobilised legend that only causes platelet activation after stable adhesion. Activated platelet thrombus formation is evident as early as 1 s when whole blood is infused into the collagen-coated channel at a shear stress of 60 dyne/cm^2, intensifying over the course of 120 s (Figure 3). Distinct thrombus formation is noted on collagen- in contrast to vWF-coated channels.

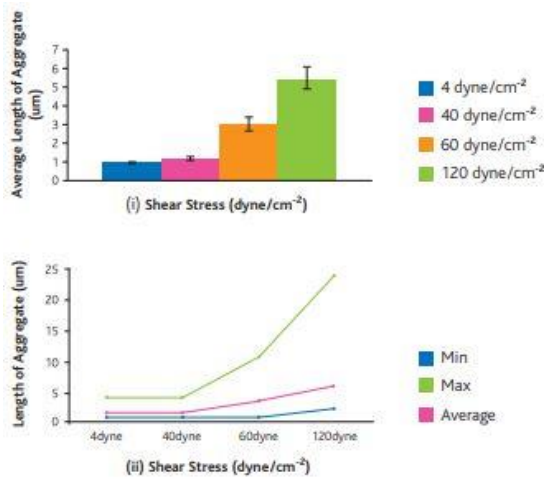

Figure 2

Figure 2: Platelet adhesion to vWF showing (i) the average length of the aggregates (thrombus) at various shear stresses and (ii) the minimum, maximum and average length of the aggregates at the various shear stresses.

Assay Conditions

The following solutions were used during the above procedure: JNL Buffer (wash buffer for inlets/outlets tubing)
JNL Buffer (100 mls) = JNL A (10 ml), JNL B (10 ml), JNL D (10 ml) and JNL E (1 ml)
[JNL A: 60 mM dextrose, JNL B: 1.3 M NaCl, 90 mM Na Bicarb, 100 mM Na citrate (tribasic, dehydrate), 100 mM Tris base, 30 mM KCl, JNL D: 8.1 mM KH_2PO_4 (monobasic anhydrate), JNL E: 90 mM $MgCl_26H_2O$]
Adjust pH 7.35 with 49 ml acid citrate dextrose (citrate acid anhydrous 38 mM, Sodium citrate 75 mM, dextrose 136 mM). Incubate at room temperature for 5 mins. Add 20 ml H_2O, incubate at RT.

Acknowledgements

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References

Williams, V.; Kashanin, D.; Shvets, I.V.; Mitchell, S.; Volkov, Y., & Kelleher, D. (2002). Microfluidic enabling platform for cell-based assays. J. Assoc. Lab. Automation, 7, 135–141.