Core and shell platelets of a thrombus: A new microfluidic assay to study mechanics and biochemistry

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Abstract

Background: Hemostatic clots have a P-selectin positive platelet core covered with a shell of P-selectin negative platelets.
Objective: To develop a new human blood microfluidic assay to interrogate core/shell mechanics.

Methods: A 2-stage assay perfused whole blood over collagen/± tissue factor (TF) for 180 seconds at 100 s⁻¹ wall shear rate, followed by buffer perfusion at either 100 s⁻¹ (venous) or 1000 s⁻¹ (arterial). This microfluidic assay used an extended channel height (120 µm), allowing buffer perfusion well before occlusion.

Results: Clot growth on collagen stopped immediately with buffer exchange, revealing ~10% reduction in platelet fluorescence intensity (at 100 s⁻¹) and ~30% (at 1000 s⁻¹) by 1200 seconds. Thrombin generation (on collagen/TF) reduced erosion at either buffer flow rate. P-selectin–positive platelets were stable (no erosion) against 1000 s⁻¹, in contrast to P-selectin negative platelets. Thrombin inhibition (with D-Phe-Pro-Arg-CMK) reduced the number of P-selectin-positive platelets and lowered thrombus stability through the reduction of P-selectin–positive platelets. Interestingly, fibrin inhibition (with H-Gly-Pro-Arg-Pro-OH acetate salt) increased the number of P-selectin–positive platelets but did not lower stability, suggesting that fibrin was only in the core region. Thromboxane inhibition reduced P-selectin–positive platelets and caused a nearly 60% reduction of the clot at arterial buffer flow. P2Y1 antagonism reduced clot size and the number of P-selectin–positive platelets and reduced the stability of P-selectin–negative platelets.

Conclusion: The 2-stage assay (extended channel height plus buffer exchange) interrogated platelet stability using human blood. Under all conditions, P-selectin–positive platelets never left the clot.

Keywords
platelets, P-selectin, shear stress, thrombin, thrombosis
1 | INTRODUCTION

Under physiological conditions, platelets are kept quiescent by endothelial cell production of molecules such as nitric oxide and prostacyclin.\(^1\) In the event of vascular injury or inflammation, this inhibition is suppressed and results in platelet activation and accumulation.\(^2\) Agonists of varying potencies such as adenosine 5'-diphosphate (ADP), thromboxane A\(_2\) (TxA\(_2\)), thrombin, and collagen activate platelets through receptor-mediated signaling.\(^3\) Alongside platelet activation, tissue factor (TF) triggers fibrin generation via thrombin production,\(^4\) resulting in a thrombus composed of platelets linked via fibrinogen with an interspersed fibrin mesh. As a thrombus is formed, platelets are activated nonuniformly,\(^5\) and heterogeneous intracellular calcium mobilization\(^6\) results in differing populations of platelets with respect to P-selectin expression, granule release, and phosphatidylserine exposure. Highly activated platelets undergo pseudopod formation and α-granule release,\(^5,7\) whereas less activated platelets retain their discoid morphology.\(^5,8\)

In vivo mouse models drive a core/shell hierarchy during the hemostatic response to injury.\(^9-12\) This organization consists of a tightly packed, highly activated core of platelets that are P-selectin positive and a loosely packed, less activated shell of P-selectin negative platelets. Similar core/shell architecture composed of a P-selectin positive core localized at the collagen surface surrounded by a P-selectin negative shell is observed with human blood perfused over prothrombotic surfaces.\(^13\) Transthrombus pressure gradients from the lumen to the interstitial space that reduce local thrombin also reduce the thickness of the P-selectin positive core region.\(^13\) The stability of a thrombus can influence whether it will grow to occlusion or not. Importantly, as a clot grows into the flow field, the shear forces increase on the clot surface as the lumen is reduced until the approach of the vessel occlusion when flow ceases. Causes of (in)stability have been examined for a wide variety of proteins, including receptors and membrane proteins,\(^14-16\) plasma proteins,\(^17-20\) and intracellular signaling proteins.\(^21,22\) and the importance of clot retraction and fibrin.\(^23,24\) Even though there is overlap in which pathways appear to be important in both thrombus stability and growth, there appear to be differences between the two. Therefore, we have developed a 2-stage microfluidic assay to measure the mechanics of the core/shell clot architecture using human blood.

In vitro microfluidic devices allow for precise control over flow fields, prothrombotic surfaces, and imaging resolution.\(^25\) Previous research has shown the presence of core/shell morphology in thrombi formed in both side-view\(^26\) and stagnation point devices.\(^27\) A device composed of 8 identical parallel channels has been used to study thrombus growth through the use of immunofluorescence.\(^28-31\) This 8-channel device allows for many conditions or replicates to be studied for a single donor. The goal of this study is to use in vitro microfluidics to study platelet activation, specifically core/shell morphology, in the context of thrombus stability using a 2-step process with a modified 8-channel device. The modification to the 8-channel is an extended height (120 µm vs 60 µm) to prevent occlusion from occurring at early time points.

2 | MATERIALS AND METHODS

2.1 | Blood collection and preparation

Whole blood (WB) was collected in 40 µg/mL corn trypsin inhibitor (CTI; Haematologic Technologies, Essex Junction, VT, USA) or 100 µM D-Phe-Pro-Arg-CMK (PPACK; Haematologic Technologies) from healthy donors who self-reported to be free of oral medication for at least 10 days before phlebotomy. All blood was collected under approval of the University of Pennsylvania’s Institutional Review Board. WB was treated with various reagents: 5 mM H-Gly-Pro-Arg-Pro-0H acetate salt (GPRP; Bachem Americas, Vista, CA, USA), 50 µM acetylsalicylic acid (ASA; Sigma-Aldrich, St Louis, MO, USA), or 100 µM MRS-2179 (Tocris, Minneapolis, MN, USA). Platelets were labeled with an AF488 mouse anti-human CD61 antibody (Bio-Rad Laboratories, Hercules, CA, USA) at 20 µg/mL, P-selectin was labeled with AF647 anti-human CD62P (BioLegend, San Diego, CA, USA) at 2 µg/mL. AF647-conjugated fibrinogen was added to WB at 12.5 µg/mL to observe fibrin formation.

2.2 | Device fabrication and preparation

Microfluidic devices were fabricated out of polydimethylsiloxane (Ellsworth Adhesives, Germantown, WI, USA) using previously described soft lithography techniques.\(^32\) A single-channel (250 µm wide and 60 µm high) patterning device was vacuum-sealed to a Sigmacote (Sigma-Aldrich) treated slide, and 5 µL of 1 mg/mL type I fibrillar collagen was perfused through the channel to create a prothrombotic surface. For some experiments, 5 µL of 20-µM lipitated tissue factor (TF; Siemens, Munich, Germany) was adsorbed to the collagen surface through Dade Innovin prothrombin time reagent. The TF was incubated for 30 minutes without flow and then rinsed with 20 µL of 0.5% bovine serum albumin (BSA; Sigma-Aldrich) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered saline

Essentials
- The relationship between core/shell platelet mechanics and stability is not fully elucidated.
- We designed a 2-step microfluidic assay to use shear stress to monitor platelet erosion.
- The highly activated, tightly packed core region is stable when exposed to high shear.
- Thrombin and secondary agonists, but not fibrin, are crucial for thrombus stability.
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A microfluidic device composed of 8 parallel 250-µm-wide channels with heights of either 60 µm or 120 µm measured with a P7 2 Profilometer (KLA-Tencor, Milpitas, CA, USA) were positioned perpendicular to the collagen strip and vacuum sealed to the slide. The device was incubated with 0.5% BSA for 30 minutes before the introduction of blood.

2.3 | Microfluidic assay

CTI-treated WB was perfused for a period of 180 seconds through the 8-channel device within 10 minutes of phlebotomy (Figure S1B). An initial wall shear rate of 100 s⁻¹ (24 µL/min) was set by a syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA, USA). After 3 minutes, the WB was swapped out for HBS, and the wall shear rate was either held constant or increased to 1000 s⁻¹ (240 µL/min, Figure S1C). Wall shear rates were determined with COMSOL Multiphysics (COMSOL Inc, Burlington, MA, USA). Platelets, fibrin, and P-selectin were detected by an epifluorescent microscope (IX81, Olympus America Inc, Center Valley, PA, USA) and a charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA) with background-corrected mean fluorescence taken from the middle 75% of the channel (Figure S2). Each replicate donor experiment was composed of multiple devices and conditions, and every individual clot was normalized to its peak platelet fluorescence intensity. Statistical analysis was done with Prism 8 (GraphPad Software, La Jolla, CA, USA) and graphs were produced with MATLAB (MathWorks, Natick, MA, USA). The normalized data were compared between conditions using an unpaired t test at the specified time point. Data are presented as mean ± SD; P < .05 was considered significant.

3 | RESULTS

3.1 | Extended-channel-height device with buffer switch to investigate clot stability

Previously, a 60-µm-high 8-channel device had been used to investigate platelet function and coagulation in WB.²⁸⁻³¹ This height allows for ~500 seconds to investigate clotting under a constant flow regime but can lead to issues when switching to HBS to probe thrombus stability. Due to a large influx of platelets when the flow rate is
increased, a thrombus can reach occlusion and embolize very quickly (Figure 1A). The clot then embolizes, and it is difficult to continue the experiment. This embolism is not physiologically relevant since our microfluidic system is run under a constant flow regime, whereas the heart pumps blood through the circulatory system under a constant pressure regime. As a clot grows inside the channel, shear stresses increase until the forces grow so large that they shear off from the collagen surface. This type of analysis gives insight into the strength of platelet-collagen interactions, but we aim to explore platelet-platelet interactions, and therefore embolism is counterproductive to our goals. Due to donor-donor variation, occlusion is also variable (Figure S3), and the lower height limits the robustness of a shear-based stability assay. To remedy this, an extended height (120 µm) 8-channel device was fabricated with the same 250 µm width. When compared to the 60-µm height, the occlusion time increased 3-fold (~500 s vs ~1500 s) for clots formed over collagen/TF surfaces (Figure 1C-E). The longer occlusion time allows for the probing of thrombus stability without the possibility of embolism during the switch to buffer (Figure 1B).

When comparing the new extended-height 8-channel device to the previously used device, we used COMSOL to determine a new flow rate to match shear rates between the 2 devices. However, there are a number of other key dimensional parameters that are affected by a change of height and flow rate. These are summarized in Table 1, with typical values seen in flow models of thrombus formation from the Scientific and Standardization Committee of ISTH. The entrance length of the new device increased, but the location of the collagen/TF strip is more than 5 mm downstream of the well, and the Reynolds number has increased but still is squarely in the laminar regime. Both the aspect ratio and velocity have increased, and the relative channel height and injury size have decreased, but the growth curves in Figure 1 suggest that they have not significantly impacted thrombus formation. With all key parameters still within typical values and very similar thrombus dynamics as the 60-µm height, we proceeded with the extended-height 8-channel device to probe thrombus stability without the possibility of embolism.

With a repeatable approach for using buffer perfusion to explore clot erosion, the effect of increased shear rates was examined.

TABLE 1 Important parameters for microfluidic devices used for thrombus formation for both a 60-µm and 120-µm channel height

| Parameter              | 60 µm | 120 µm | Typical values |
|------------------------|-------|--------|----------------|
| Relative channel height| 0.125 | 0.063  | 0.01-0.2       |
| Aspect ratio           | 0.24  | 0.48   | 0.1-1          |
| Relative injury size   | 4.17  | 2.08   | 0.1-10         |
| Reynolds number        | 162   | 485    | <2000 (laminar)|
| Entrance length (µm)   | 891   | 4479   | 1-1000         |

FIGURE 2 Perfusion of buffer over thrombi allows for the probing of stability and morphology in a shear-dependent manner. High-CTI WB at 100 s⁻¹ was perfused over collagen or collagen/TF followed by switching to HBS (Δ) at either an initial shear rate of 100 s⁻¹ (red) or 1000 s⁻¹ (blue). A and B, Normalized platelet fluorescence intensity (FI) calculated by normalizing to the highest intensity obtained during the experiment. Data are expressed as mean ± SD with n = 12 clots for 2 donors. C and D, Representative image of platelets in red at 3 minutes and 20 minutes. Scale bars are 100 µm. CTI, corn trypsin inhibitor; FI, fluorescence intensity; HBS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered saline; TF, tissue factor; WB, whole blood.
High-CTI WB was perfused over either collagen (Figure 2A, C) or collagen/TF (Figure 2B, D) at 100 s$^{-1}$ initial wall shear rate, followed by switching to HBS (indicated by $\Delta$) at the same initial shear rate (red/black) or increasing to 1000 s$^{-1}$ (blue/light gray). There were no differences in platelet fluorescent intensity between TF and no TF conditions for both buffer perfusion wall shear rates of 100 s$^{-1}$ and 1000 s$^{-1}$ conditions since their growth conditions were identical. These results suggest that thrombin plays an important role in stability through the activation of platelets, and higher shear rates caused greater erosion, as expected.

### 3.2 | Thrombin, but not fibrin inhibition, lowers the stability of thrombi

To further examine the role of thrombin on stability, either PPACK (100 $\mu$M, red/dark gray) to inhibit thrombin or GPRP (5 mM, blue/light gray) to inhibit fibrin but not thrombin was added to high-CTI WB for the growth period with a switch to buffer and increase in shear occurring at 180 seconds. For all conditions, platelet fluorescent intensities were the same at the end of the growth period ($P = .81$ for GPRP vs PPACK; $P = .20$ for control vs PPACK; $P = .65$ for control vs GPRP). The inhibition of thrombin production resulted in fewer platelets remaining at the end of the experiment when compared to both the control condition and GPRP condition ($P < .0001$; Figures 4A, 5A), which agrees with the data in Figure 3A. When thrombin but not fibrin was present, the same number of platelets were left in comparison to the control ($P = .79$). This suggests that fibrin may not play a significant role in stability in this assay and that fibrin resides in the core, which is always shear resistant. Even though the overall platelet intensity decreased when subjected to buffer, P-selectin fluorescence intensity did not. This suggests highly activated P-selectin positive platelets are more stable than less activated P-selectin negative platelets. P-selectin fluorescence intensity is significantly different among each of the 3 conditions ($P < .0001$; Figure 5B). Both PPACK and GPRP inhibit all fibrin formation at the concentrations used, whereas fibrin is present in the control condition (Figure 5C). Overall, these data demonstrate that the core region of a thrombus is stable when subjected to increased shear. Thrombin is a key activator of platelets, and the decreased stability in the PPACK condition that lowers P-selectin intensity can lead to more platelet erosion.

### 3.3 | Secondary agonists are crucial for stability of the shell region

To further explore the stability of the shell region, ASA (50 $\mu$M, red) was added to PPACK WB and incubated for 10 minutes before perfusion. ASA irreversibly blocks the formation of TxA$\_2$. During the growth period, ASA limited P-selectin positive platelets ($P < .001$), but not total number of platelets ($P = .35$; Figure 6A, B). When subjected to increased shear and buffer, the ASA-treated thrombus eroded more than the control condition. At the end of the experiment, only 40% of the clot that was treated with ASA remained versus 65% of the control condition ($P < .001$; Figure 6A). However, P-selectin positive platelets did not erode during the shear period (Figure 6B). This further supports that the core region is stable when subjected to high shear rates.
To inhibit ADP responses, MRS-2179 (100 µM, red) was added to PPACK WB to antagonize P2Y1 and prevent ADP binding. P2Y1 antagonism lowers both platelets and P-selectin positive platelets during the growth period similarly to TxA₂ inhibition (Figure 7A, B; P < .001). After switching to buffer, the MRS-2179–treated platelets were more likely to come off the thrombus compared to the control, with P-selectin positive platelets being stable in both conditions. These data, combined with the ASA data, demonstrate that treating platelets with soluble agonist inhibitors lowers thrombus growth and increases shell platelet erosion when subjected to shear.
4 | DISCUSSION

To study the contribution of the spatiotemporal heterogeneity of clotting mechanisms to thrombus stability, we developed a 2-part microfluidic assay that utilizes an extended height 8-channel device. Computational flow dynamic simulations indicate a relatively uniform shear rate for this geometry (Figure S2). This is further demonstrated by the similarity of thrombus growth dynamics when...
compared to the 60-µm height before occlusion. By increasing the channel height of the 8-channel and adjusting the flow rates to preserve dynamics, the modified device allows for a longer time period to study thrombus morphology and stability.

As clot formation proceeds, there is a temporal distribution of various agonists. Early on, thrombin and collagen drive platelet activation and clot growth, and at later time points, secondary agonists ADP and TxA2 play a larger role. One of the key benefits of this assay is that swapping in buffer stops coagulation and platelet deposition and allows for probing of the structure of the clot at a particular time point. Data presented in this paper used thrombi that have formed for 180 seconds, but the switch can occur at a variety of different time points without occlusion (Figure S5).

Previous research has shown that fibrin plays a key role in governing stability,39 but clots formed in the presence of GPRP to block fibrin formation were as stable as clots formed without GPRP present in this assay. Since the P-selectin signal was constant, core platelets did not erode, and therefore only shell platelets were affected by shear. This suggests that fibrin is present only in the core. Furthermore, inhibition of thrombin decreased P-selectin fluorescence intensity and led to more platelet erosion most likely due to less overall platelet activation. GPRP increased P-selectin fluorescence intensity most likely as a result of larger thrombin generation.34,35 However, platelet erosion was not affected suggesting that more than just P-selectin expression governs stability.

The core/shell model suggests that outside of the P-selectin positive region, very little to no thrombin is present. In this outer region, ADP and TxA2 regulate platelet activation once they are produced by platelets resulting in secondary activation. The data with secondary agonist inhibitors further supports this conclusion. Secondary agonists contribute somewhat to core formation and α-granule release but not as significantly as thrombin. Both inhibitors lowered P-selectin expression further suggesting that less P-selectin leads to more platelet erosion. However, ASA-treated blood did not significantly affect total platelet fluorescence intensity whereas MRS-2179-treated blood did. Even though the fraction of platelets left for both inhibitors are similar, ASA-treated blood had more initial platelet deposition, and therefore more platelets eroded. This suggests that TxA2 production plays a crucial role in stabilizing shell platelets, and ADP may be more important for initial platelet aggregation. Overall, secondary agonists help stabilize platelets in the shell region that are more likely to be shear eroded.

In summary, we demonstrate a new 2-part microfluidic assay using an extended-height 8-channel microfluidic device that allows for precise control of thrombus growth conditions followed by increased shear rates to examine stability and morphology.

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AUTHOR CONTRIBUTIONS

MED and SLD designed the experiments. MED conducted the experiments. MED, SLD, and LFB analyzed and interpreted data and wrote the manuscript.

RELATIONSHIP DISCLOSURE

The authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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