

# Microfluidics and Coagulation Biology

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## Keywords

thrombosis, hemostasis, platelet function, von Willebrand factor, collagen, fibrin

## Abstract

The study of blood *ex vivo* can occur in closed or open systems, with or without flow. Microfluidic devices, which constrain fluids to a small (typically submillimeter) scale, facilitate analysis of platelet function, coagulation biology, cellular biorheology, adhesion dynamics, and pharmacology and, as a result, can be an invaluable tool for clinical diagnostics. An experimental session can accommodate hundreds to thousands of unique clotting, or thrombotic, events. Using microfluidics, thrombotic events can be studied on defined surfaces of biopolymers, matrix proteins, and tissue factor, under constant flow rate or constant pressure drop conditions. Distinct shear rates can be generated on a device using a single perfusion pump. Microfluidics facilitated both the determination of intraluminal thrombus permeability and the discovery that platelet contractility can be activated by a sudden decrease in flow. Microfluidic devices are ideal for multicolor imaging of platelets, fibrin, and phosphatidylserine and provide a human blood analog to mouse injury models. Overall, microfluidic advances offer many opportunities for research, drug testing under relevant hemodynamic conditions, and clinical diagnostics.

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## 1. INTRODUCTION: COAGULATION BIOCHEMISTRY AND PLATELET FUNCTION UNDER FLOW

Blood has several physiological functions, which include the transport of nutrients, hormones, and cellular waste; immunological surveillance; and coagulation, which initiates wound healing in the event of an injury. The precise regulation of these functions is critical to health and achieved largely through mechanisms inherent to the blood. Human blood is composed of a proteinaceous liquid phase called plasma, and cellular components that include platelets, white blood cells (leukocytes), and red blood cells (erythrocytes). Although dysregulated blood coagulation is central to diseases such as heart attacks, strokes, and hemophilia, regulated blood coagulation is also critically important to maintain health during acute events such as surgery or trauma.

Blood coagulation is a collaborative process between plasma and blood cells. Within plasma, major protein-based coagulation pathways include extrinsic coagulation [triggered by tissue factor (TF)] and contact-activated coagulation [triggered by generation of coagulation Factor XIIa (XIIa)]. Blood cells help to facilitate coagulation at sites of injury by first undergoing heterotypic aggregation to injury sites, followed by homotypic aggregation to generate a temporary plug to stop blood loss. Formation of aggregates on surfaces represents a spatially dependent deposition process and is distinguished, both kinetically and mechanically, from bulk aggregation within a flowing suspension. Aggregates then facilitate protein coagulation reactions by providing a surface upon which the enzymatic generation of coagulation enzymes can occur, which helps to increase the rate of coagulation enzyme generation while localizing coagulation to the injury site where it is needed. In health, blood coagulation at sites of injury initiates wound healing and is termed hemostasis. In thrombosis, blood coagulation is dysregulated, aggregates and coagulation products progress to occlude the lumen of a blood vessel or dislodge (embolize) from the injury site only to occlude a downstream vessel. Vessel occlusion cuts off blood flow to tissues downstream of the vessel, resulting in ischemia and tissue death if blood flow is not restored.

The study of hemostasis and thrombosis, the intersection of these cell-based and protein-based processes, was revolutionized by the introduction of open-system flow chambers. This technique allowed investigators to study the roles of convective transport and shear stress, for example, to reveal new dynamics that could not have been predicted using closed systems. Flow chambers paved the way for modern day theoretical studies in the field of blood systems biology and advanced microfluidic techniques that use a minimum of blood in well-defined hemodynamic environments to maximize experimental control.

### 1.1. Coagulation and Platelet Aggregation

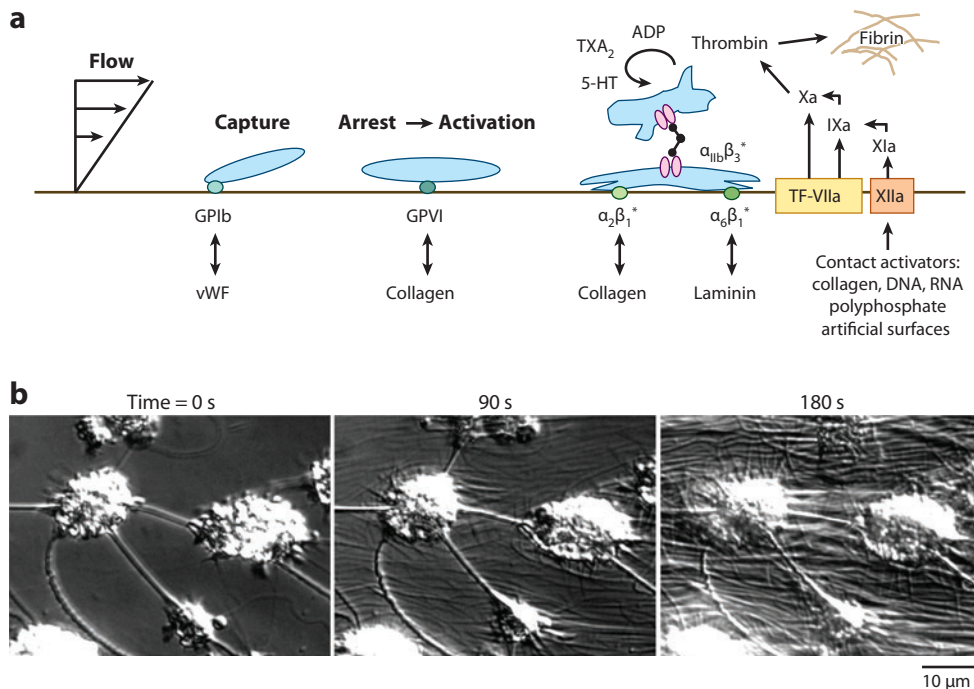
Coagulation is a surface-bound enzymatic reaction whose initiation is isolated to locations of exposed TF and whose propagation requires coagulation factors to transport from bulk plasma to the vessel wall. To be procoagulant, TF requires a phospholipid surface to initiate coagulation (53). In the case of a vessel injury, where the blood flows past the site of TF exposure, convective mass transport supplies coagulation factors from the blood to the lipid membrane, which contains TF.

The generation of thrombin, a trypsin-like serine protease protein, is central to the study of pathological blood clot formation (thrombosis) and cessation of bleeding (hemostasis). Thrombin activates platelets and triggers the cleavage of fibrinogen to a fibrin monomer, which then undergoes polymerization via protofibril extension followed by lateral aggregation to form a fibrin clot. Two pathways lead to the formation of a fibrin clot: the extrinsic and the contact pathways. TF, a membrane protein that binds VIIa to activate X to Xa and IX to IXa, is the fundamental trigger of the extrinsic pathway. The generation of XIIa triggers the contact pathway and is central to biomaterial thrombosis. The role of contact pathway XIIa and XIa during *in vivo* thrombosis is supported by studies of the XIIa knockout mouse and the role of thrombin-mediated feedback activation of XIa (**Figure 1a**).

Platelets contain numerous receptors for and are activated by collagen, thrombin, adenosine diphosphate (ADP), thromboxane (TXA<sub>2</sub>), serotonin, histamine, and epinephrine. Additionally, platelets can bind collagen via  $\alpha_2\beta_1$ , laminin via  $\alpha_6\beta_1$ , von Willebrand factor (vWF) via glycoprotein (GP) Iba and  $\alpha_{IIb}\beta_3$  (i.e., GPIIb/IIIa), and fibrinogen via  $\alpha_{IIb}\beta_3$  (**Figure 1a**) (1, 2). Platelet activation may be inhibited endogenously by endothelial production of prostacyclin and nitric oxide. Additionally, pharmacological inhibition of platelet activation is achieved with aspirin (targeting COX1), P2Y<sub>12</sub> inhibitors (metabolized and nonmetabolized forms), and an anti- $\alpha_{IIb}\beta_3$  inhibitor (ReoPro). Pharmacological inhibition of coagulation is achieved with thrombin inhibitors (heparin, dabigatran), direct Xa inhibitors (apixaban, rivaroxaban), and coumarins (Warfarin), which inhibit the vitamin K-dependent modification of multiple coagulation factors. Because the transport rate of reactive molecular and cellular species to and from a developing thrombus or site of bleeding as well as the shear forces controlling adhesion and embolization are controlled by prevailing hemodynamics, flow devices are central to the *in vitro* study of blood biology.

### 1.2. Blood Systems Biology

Blood coagulation and thrombus formation are complex processes that are often experimentally analyzed by visual determination of coagulation product formation. As such, dissecting out the contribution of individual components involved in functional blood coagulation is aided by computational modeling. Modeling of blood systems biology includes reaction kinetics, flow, and transport physics and helps to delineate the effects of homotypic and heterotypic aggregation/fragmentation in linear shear fields or complex flows (1–4), coagulation as a pseudohomogeneous cascade [TF triggered (5) or TF/XIIa triggered (6)] or as a platelet surface-dependent coagulation cascade



**Figure 1**

Autocatalytic deposition of platelets on an injured vascular wall and generation of coagulation proteases. (a) Adhesion of platelet to vWF mediates capture under arterial flow conditions and is followed by platelet activation via GPVI. Once activated, the platelet integrins can bind collagen, laminin, and fibrinogen. Platelet activation is also associated with release of ADP and serotonin, synthesis of thromboxane, and exposure of phosphatidylserine, which facilitates thrombin generation. Thrombin production is triggered primarily by TF, with contact activation via coagulation Factor XIIa playing a secondary role in thrombosis. Thrombin also triggers the polymerization of fibrinogen to fibrin. (b) Video microscopy of platelet aggregates forming on a surface with generation of fibrin strands. Abbreviations: 5-HT, serotonin; ADP, adenosine-di-phosphate; GP, glycoprotein; TF, tissue factor; TXA<sub>2</sub>, thromboxane; vWF, von Willebrand factor.

under no-flow or flow conditions (7–10), fibrin polymerization under flow (11), fibrinolysis under flow conditions (12), reactive platelet deposition with or without coagulation (7, 9, 13, 14), and shear-induced changes in vWF conformation (15). These physics-based models seek to identify, quantify, or deconvolute kinetic or mechanical subprocesses that occur within complex reactive blood flows.

### 1.3. Adhesion Biology and Receptor Mechanics

The requirement that cells bind to subendothelial matrix proteins while they are entrained in the bloodstream places stringent physical demands on the receptors involved in the adhesion and arrest of blood cells and platelets. Specifically, the on-rate of a platelet receptor binding to a subendothelial matrix protein, which describes how quickly binding events occur, must be fast enough to allow efficient adhesive interactions within the time frame that a free-flowing platelet is in the proximity of the exposed subendothelial matrix. In humans, blood circulates at different flow velocities depending on anatomical location and presence in the arterial versus venous circulation.

Flow velocity is a direct determinant of shear rate for fully developed, Newtonian flow and directly influences the residence time of a platelet above a subendothelial matrix protein. In mammals, shear rates span two orders of magnitude, from 50–60  $s^{-1}$  in the vena cava to 1,000–5,000  $s^{-1}$  in the arterioles (16, 17). In humans, the only receptor-ligand interaction with a high enough on-rate to cause platelet adhesion at shear rates above 500  $s^{-1}$  is vWF/GPIb $\alpha$  (18). The adhesion of bound platelets in the presence of blood flow places a hydrodynamic shear stress on the receptor-matrix protein bond, which may determine the duration that the formed bond persists (i.e., off-rate). Subsequently, native vWF binds GPIb $\alpha$  only when subjected to high fluid shear rates or when vWF is first bound to collagen (19), suggesting that other receptor-ligand interactions mediate platelet adhesion at lower shear rates. This possibility is further supported by the observation that platelets roll on vWF surfaces at higher velocities under lower shear rates (20). These characteristics are identical to catch-slip bonds previously described for leukocyte-expressed selectins (21), and sustained arrest of platelets requires additional contributions from other receptor-matrix protein interactions. Stable adhesion to collagen involves contributions from collagen receptors  $\alpha_2\beta_1$  and GPVI (22). In addition, fibrin(ogen) deposition has been observed at sites of subendothelial matrix exposure, and platelets can bind to fibrin(ogen) through  $\alpha_{IIb}\beta_3$ , which can also form homotypic bonds with other platelets to facilitate the aggregation of platelets, a requirement for forming a hemostatic plug *in vivo*.

#### 1.4. Anticoagulation for Ex Vivo Research

Selection of an appropriate microfluidic device requires careful consideration of anticoagulation, which depends on the intent of the investigation. Use of phosphate buffers is always avoided owing to the resulting formation of calcium phosphate precipitate, and the first 5–10 mL of drawn blood is often discarded to avoid TF contamination during venipuncture. The following approaches are the most commonly used, each having its own advantages and disadvantages.

**1.4.1. EDTA or citrate, with and without recalcification.** For purely biorheological studies (e.g., those studying velocity profile blunting, platelet drift, or the Fåhræus-Lindqvist effect) without any analysis of coagulation or platelet function, EDTA is an ideal inhibitor of thrombin production and platelet adhesion. Addition of excess calcium will overwhelm calcium chelators such as EDTA or citrate; however, EDTA destroys  $\alpha_{IIb}\beta_3$  function, and citrate significantly impairs  $\alpha_{IIb}\beta_3$ , even after recalcification. Citrate also chelates  $Mg^{2+}$  and  $Zn^{2+}$  ions, which are not restored during recalcification. For studies of TF-triggered or XIIa-triggered coagulation, citrate has some relatively modest effects following recalcification (6), despite its widespread clinical use. Importantly, thrombin once formed does not require calcium, and XIIa can be generated without calcium.

**1.4.2. Corn trypsin inhibitor (CTI).** CTI, when used at 10–50  $\mu g/ml$ , can inhibit the activity of  $\beta$ XIIa, the soluble cleavage product derived from surface-bound  $\alpha$ XIIa. Because CTI does not inhibit  $\alpha$ XIIa, it provides about 40–60 min of inhibition of the contact pathway without interfering with the physiological divalent cation levels required for TF-mediated clotting. CTI is a useful inhibitor for studying blood function (albeit with an attenuated  $\beta$ XIIa pathway) under flow, provided data is collected within about 30 min of phlebotomy. Other less commonly used routes of controlling *in vitro* contact pathway activation include antibodies against XIIa and XIa (23), XIIa antagonism with infestin-4 (24, 25), and XIa antagonism with domains from protease nexin-2 (26). A low level of CTI (<5  $\mu g/ml$ ) can be used to partially delay contact activation, allowing the study of XIIa *ex vivo*, provided blood is perfused within 20–30 min of phlebotomy.

**1.4.3. PPACK, direct Xa inhibitors, or heparin.** PPACK (100  $\mu\text{M}$ ), the direct Xa inhibitor apixaban (0.25–1  $\mu\text{M}$ ), or heparin (>1–10 U/ml) provides strong and irreversible inhibition of prothrombinase or thrombin. This approach is useful for the study of platelet activation, adhesion, or aggregation in the absence of thrombin and fibrin. Heparin has been reported to interfere with some P-selectin pathways.

**1.4.4. Replacing the endothelium.** As the endothelium is often difficult to recreate within microfluidic systems, a nitric oxide (NO) donor; platelet prostaglandin-I<sub>2</sub>-receptor (IP) activators such as PGE<sub>1</sub>, PGE<sub>2</sub>, and iloprost; or soluble thrombomodulin (to activate protein C) is often added to account for the endothelium's contribution. Whereas blood flow studies with endothelium in the absence of thrombin production are not uncommon, fewer studies have perfused whole blood over cultured endothelium under conditions of coagulation and thrombin production (27–29).

## 1.5. Thrombosis and Hemodynamics

The biorheology of reactive blood flow has presented numerous challenges and opportunities for discovery. In recreating the relevant hemodynamics in a laboratory device, whole-blood perfusion is needed to create an enriched platelet-plasma layer ( $\sim 5$   $\mu\text{m}$  thick) near the vessel wall. Platelet concentrations near the wall are enhanced severalfold relative to that of whole blood. Thrombosis *in vivo* also occurs under constant pressure drop conditions, such that a growing thrombus, once nearly occlusive, results in a significant resistance to flow that diverts flow to other vessels. Experiments are often conducted at a constant flow rate using syringe pumps, which is appropriate when recreating a thrombus that is thin relative to the flow geometry. However, flows become nonphysiological ( $>10,000$   $\text{s}^{-1}$  and  $>300$   $\text{dyne/cm}^2$ ) when the clot obstructs the flow channel. Most laboratory flow devices can recreate the shear rate or shear stress at the wall, but they cannot recreate the *in vivo* Reynolds number ( $\text{Re} = Dv\rho/\mu$ , where  $D$  is the pipe diameter,  $v$  is the average velocity,  $\rho$  is the density, and  $\mu$  is the viscosity) or transitions to turbulence that occur *in vivo* with severe stenosis and pulsatile flow ( $\text{Re} > 400$ ). These flow conditions are not typically possible in microfluidic devices because the Reynolds numbers of such devices are generally less than 1. Additionally, there are differences between tube flows and flows in the rectilinear channels of laboratory devices, as phenomena in the corners of the channel are completely nonphysiological.

## 1.6. Parallel-Plate/Capillary Devices for Platelet Adhesion Research

Parallel-plate flow chambers, which were introduced in the early 1970s, have a characteristic gap separation of about 100 to 200  $\mu\text{m}$  and can be operated with 1–10 mL of blood or fluid, thus limiting studies to two or three conditions per 20–60 mL blood draw. Parallel-plate studies in thrombosis have revealed a critical role of vWF for platelet adhesion under arterial flow conditions (30).

vWF circulates in blood plasma in a protein complex with VIII. The VIII/vWF complex mediates platelet adhesion to denuded arteries placed in an annular flow chamber under conditions of flow (31). In turn, the adhesion of platelets to subendothelial matrix proteins depends upon red blood cell size and deformability, the presence of divalent calcium ions, and specific multimeric vWF distribution (32–35). Patients with Bernard-Soulier disease have platelets that do not bind to collagen under flow even in the presence of VIII/vWF, indicating that platelets from these patients lack a vWF receptor (36) now identified as the 155-kDa platelet GPIIb (37–41).



Without the use of flow chambers, the correct physiological scenario by which platelets adhere to arterial vessel walls would not have been evident. vWF is not absolutely necessary for platelet binding to collagen under conditions of stasis or venous flow ( $100 \text{ s}^{-1}$  wall shear rate). Subsequent studies have identified other subendothelial matrix proteins capable of binding platelets in venous flow conditions. Fibronectin facilitates the adhesion of platelets to collagen (42–44). Additionally, laminin, the second most abundant subendothelial matrix protein, binds platelets under flow through  $\alpha_6\beta_1$  (45, 46) and vWF/GPIb (47). Studies have also demonstrated that  $\alpha_{IIb}\beta_3$  on platelets can bind to fibrinogen under flow (48). Adherent platelets can also bind to collagen through  $\alpha_2\beta_1$  (49, 50) as well as GPVI (51). Beyond collagen, fibronectin, and laminin—the major constituents of normal subendothelial matrix—other proteins have been identified in pathological portions of blood vessels. For example, Tenascin-C is enriched in atheromas, and perfusion of platelets over prepared surfaces of Tenascin-C resulted in platelet adhesion and activation, independent of collagen and laminin, suggesting a possible pathological role for Tenascin-C in atherosclerosis (52).

### 1.7. Parallel-Plate/Microcapillary Devices for Thrombosis Research

The effects of flow on blood coagulation and fibrin formation can be studied *ex vivo* with the use of flow chambers and microfluidics. As noted above, flow chambers typically require a syringe pump to perfuse liquid through the capillary. Control of the infusion rate, coupled with measurements of the capillary geometry, allows for control of shear rate at the capillary wall. Flow rate can potentiate convective mass transport, and flow is a major regulator of coagulation kinetics (54). In addition, larger regions of exposed TF or lower shear rate increases the probability that a soluble coagulation factor will bind to the surface and subsequently participate in coagulation (55). Conversely, the presence of flow can wash formed enzyme complex or activated enzymes from the vicinity of the TF-exposed site. This effectively dilutes the concentration of enzyme, mostly thrombin, and can prevent the formation of fibrin perpendicular to flow (56). Using printed microarrays mounted to a parallel-plate flow chamber, Okorie et al. (56) demonstrated a threshold surface requirement of 1 to 10 molecules of lipidated TF per  $\mu\text{m}^2$  to initiate fibrin formation under venous and arterial flow conditions. One potential drawback to using a pump-based perfusion chamber is the increase in pressure at the forming thrombus due to occlusion of the chamber lumen, a condition that would not be present *in vivo*, owing to collateral circulation. This increased pressure may be overcome with a gravity-driven capillary perfusion assay that was developed to maintain a constant pressure head with which to drive blood flow through a capillary tube (57).

Flow chambers that combine platelet adhesion with coagulation under flow have shown that the arrest of platelets at the injury site effectively seals off the initial coagulation stimulus of the surface (58). The procoagulant stimulus that maintains the growth of a thrombus following initial platelet deposition is not certain. Thrombin was discovered to activate XI, a finding which in turn led to the discovery of the thrombin-XI feedback loop by which thrombin can propagate itself, in the absence of TF, by activating XI to XIa and thereby initiating the contact pathway (59). Supporting this mechanism, inhibition of XIa was shown to reduce the formation of fibrin over collagen-coated surfaces, supporting a role for XIa in thrombus formation (60, 61). It is also possible that blood-borne TF binds to platelet aggregates and contributes to thrombus formation under flow (62–64), a pathway that may be pronounced in certain cancers (65). Furthermore, platelet-derived polyphosphates can activate XIIa (albeit very weakly compared with activation by long-chain bacterial polyphosphate), which represents another possible route for activated platelets to sustain thrombin production in a growing thrombus (66).

Ex vivo coagulation experiments must exercise caution to limit the confounding effects of XII activation leading to the contact pathway of blood coagulation. Surfaces can be chemically treated with low surface tension polymers to limit the charged surface activation of XII. Further, high-energy surfaces may be blocked with native blood proteins, such as albumin, to limit XII activation. Moreover, there is mounting evidence that subendothelial matrix proteins, such as collagen and laminin, also have the ability to activate XII, suggesting yet another possible contribution to blood coagulation in vivo (67). In contrast to flow chambers that require many centimeters of tubing to connect a blood reservoir, microfluidic devices minimize the travel distance from the blood reservoir to the site of the thrombotic event to only a few millimeters.

The adhesion of white blood cells, such as neutrophils, and their role in coagulation have also been considered in square glass microcapillaries. Goel & Diamond (68, 69) demonstrated enhancement of coagulation through neutrophil release of Cathepsin G on platelet monolayers, upon which fibrin generation was visualized in real time. Capillaries have been functionalized with P-selectin, as in the visualization of neutrophil tether formation performed by Schmidtke et al. (70) or with P-selectin-coated beads, and used to assess neutrophil deformation and bond lifetimes after collision. Furthermore, in an effort to mimic microvasculature, microcapillaries have been used to culture endothelial cells to confluence. For example, Nash and colleagues (71), who pioneered this technique, used these surfaces to describe a mechanism by which neutrophils can be transferred to the surface of intact endothelium when coated with activated platelets (72).

## 2. MICROFLUIDICS AND COAGULATION BIOLOGY

### 2.1. Closed Systems

Closed microfluidic systems may be as simple as plates of small-microliter- or submicroliter-volume wells. Closed blood systems do not undergo changes in volume and, once initiated, do not exchange components with the external environment. Although the test tube represents the classical milliliter-scale basic batch reactor format, advances in robotic liquid handling now allow the use of 96-, 384-, and 1,536-well plate formats for investigations of clotting and platelet function at a 1–200- $\mu$ L scale without significant fluid flow or mixing. These methods offer the advantage of high throughput and high replicate number as well as dynamic readouts of kinetic processes. Such reactions are generally isotropic initially but may develop gradients due to cell settling, cell wall-dependent phenomena, or evaporation over the course of an hour-long experiment.

Important analytical techniques to monitor thrombin include the use of fluorogenic substrates in 96-well plates (73) and 384-well plates (6). Fibrin generation is a dual measure of thrombin production and fibrinogen level and can be gauged in closed systems by clot rigidity as measured by oscillatory rheometry such as thrombelastography (TEG) or thromboelastometry (ROTEM) (74), magnetorheometry, ultrasound (75), or turbidity in clotting plasma. Platelet function can be measured by aggregometry or by calcium mobilization in 384-well plate assays (76). Beyond the well-plate approach, microfluidic platforms used for nanoliter-scale reactions include printed arrays of fluorogenic substrates (77–83) or fluorogenic substrates deployed in micropatterned wells or channels. Label-free reactions of thrombin function have also been conducted on matrix-assisted laser desorption/ionization (MALDI) plates for mass spectrometry (MS) interrogation (84).

Cone-and-plate viscometry, a closed system that includes a defined bulk shear rate and linear shear flow, is mostly used to study processes in bulk suspensions such as platelet aggregation, vWF aggregation, or shear-induced platelet aggregation (85, 86). Aggregometry measures platelet aggregation rates in the presence of a chaotic stir-bar-induced flow. Despite its poorly defined hemodynamics, aggregometry is very useful for monitoring receptor function and platelet signaling.



## 2.2. Open Systems

Open microfluidic devices for the study of platelet aggregation and coagulation offer the ability to spatially and temporally control flow, surface, and perfused blood chemistry (**Figure 2**). They allow for high replicate number and use low volumes of blood. Microfluidic devices allow control of the local flow environment by providing well-defined geometries and command of sample flow rates. Physiological venous ( $100\text{--}200\text{ s}^{-1}$ ) or arterial forces ( $1,000\text{--}2,000\text{ s}^{-1}$ ) may be achieved in microchannels, as well as in pathological shear environments ( $10,000\text{--}100,000\text{ s}^{-1}$ ) (**Figure 3**). Because most microfluidic models support only single-pass perfusion, the transport and kinetic properties of these systems are preferable to closed system methodologies. For straight rectangular channels, the  $x$ -directed velocity field for steady flow over a domain of  $-w/2 \leq y \leq w/2$  and  $0 \leq z \leq b$  is given by:

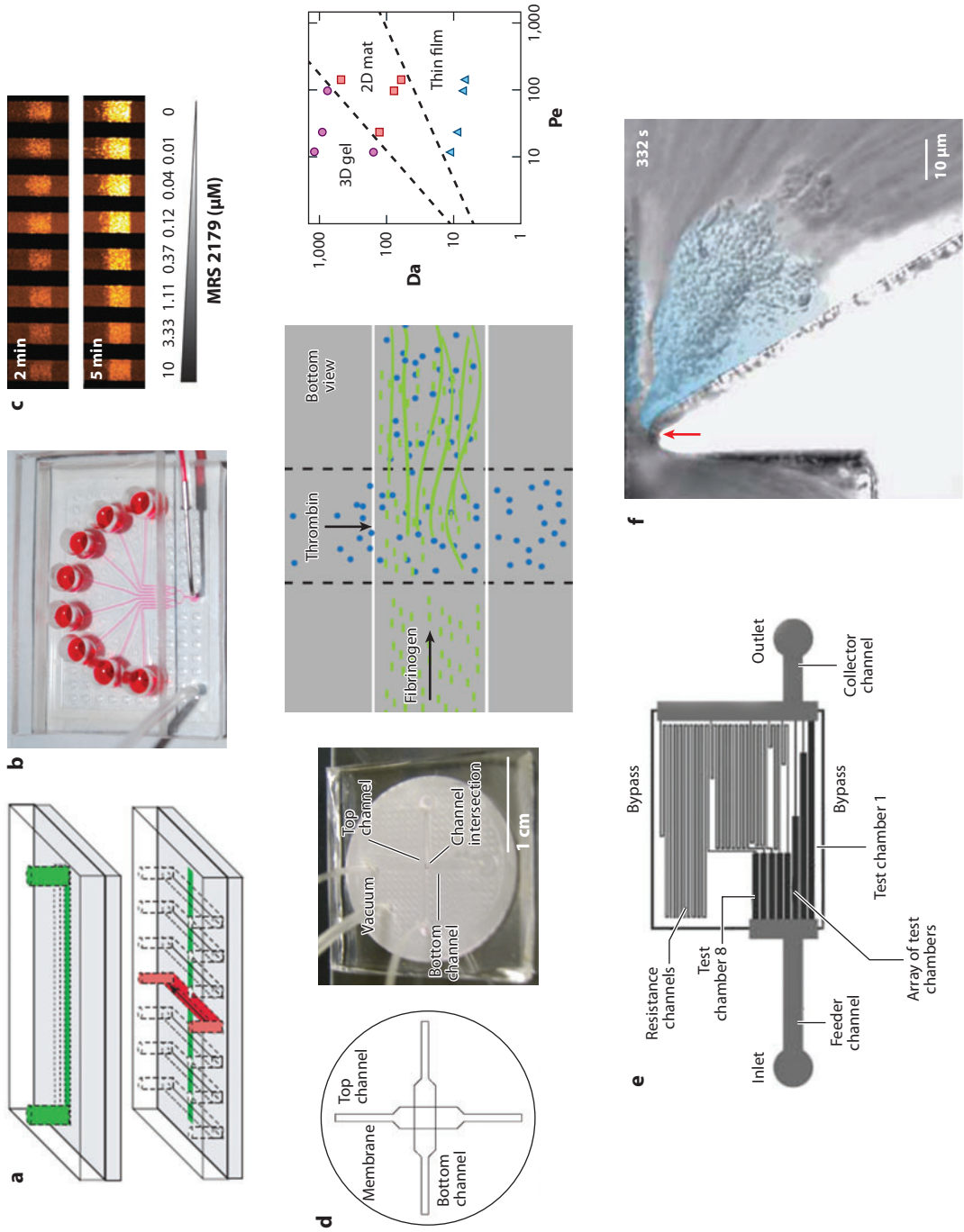
$$u(y, z) = \frac{48Q}{\pi^3 b w} \times \frac{\sum_{n=1,3,5,\dots}^{\infty} \frac{1}{n^3} \left[ 1 - \frac{\cosh\left(n\pi \frac{y}{b}\right)}{\cosh\left(n\pi \frac{w}{2b}\right)} \right] \sin\left(n\pi \frac{z}{b}\right)}{1 - \sum_{n=1,3,5,\dots}^{\infty} \frac{192b}{n^3 \pi^3 w} \left[ \tanh\left(n\pi \frac{w}{2b}\right) \right]}. \quad (1)$$

Rectangular capillaries with high aspect ratio are ideal for low-volume and high-quality visualization and create a quasi-two-dimensional flow in the central region away from the side walls. Cylindrical microcapillaries, though more physiological (no corners), present challenges for microscopy-based imaging. The advent of polydimethylsiloxane (PDMS) has allowed the creation of rectangular flow channels with feature sizes down to about  $3\text{ }\mu\text{m}$ , and the development of microstamp and cantilever technologies has allowed microprinting of proteins or lipids on substrates with spatial resolution below  $1\text{ }\mu\text{m}$ .

PDMS and microprinting approaches are well reviewed elsewhere (87). Generally, microfluidic devices made of PDMS are cured over master molds fabricated using photolithographic techniques. To summarize briefly, a photoresistive substrate, such as SU-8 or KMPR-1050, is spin coated onto a silicon wafer to a height that will represent the channel depth. The coated wafer is then brought into close contact with a high-resolution transparency of the desired channel design using a mask aligner. The sample is exposed to UV light, which initiates cross-linking in the photoresistive substrate. Unexposed photoresist is then removed with a development solution. The resulting master mold can produce hundreds of PDMS casts.

## 2.3. Microfluidic Devices: Focal Thrombosis and High Replicate Number

In 2008, Neeves et al. (88) demonstrated the use of a micropatterned surface of collagen in a PDMS microchannel. Protein was patterned onto a glass slide using a single channel device that was removed to allow the placement of the flow device, which had 13 channels running perpendicular to the collagen stripe (**Figure 2a**; note that the illustrated device is not identical to the one used in the experiment). The resulting device consisted of 13 individual flow paths, each with a well-defined injury site where collagen was presented to the flow. Murine platelets adhered only to this focal collagen region and were visualized in real time using epifluorescence microscopy. This design proved particularly useful in the *ex vivo* study of mouse blood because of its low blood-volume-per-channel requirement ( $<50\text{ }\mu\text{L}$ ). Mouse platelets deficient in integrin  $\alpha_2$  adhered very poorly in this assay. Activation of PAR4 receptor with a peptide agonist enhanced platelet deposit stability when challenged with a step change in shear rate to  $8,000\text{ s}^{-1}$ . Human blood samples behaved in a manner similar to murine samples in that they formed platelet aggregates only on the collagen surfaces. Furthermore, these aggregates grew in response to the local hemodynamic conditions. Platelet aggregates formed  $10\text{--}50\text{-}\mu\text{m}$  mound- or dune-like structures that had a circular shape



at venous shear and an ellipsoidal shape at higher shear rates, approximately twofold elongated in the direction of flow (89).

In a more recent generation of the focal thrombosis model, Maloney et al. (90) developed an eight-channel design (**Figure 2b**), which was perfused by withdrawal through a single outlet. Using less than 0.5 mL of blood in this device, the authors reported on eight-point dose-response curves for multiple antiplatelet agents (**Figure 2c**). Small molecular inhibitors of P2Y<sub>1</sub> and P2Y<sub>12</sub> had similar potency under flow as measured by equilibrium binding; however, apyrase potentiated platelet deposition under flow, owing to its rapid conversion of released ATP to ADP. This eight-channel device was recently used to phenotype individual donors and their responses to COX-1 inhibitors (aspirin and indomethacin), a P2Y<sub>1</sub> inhibitor (MRS-2179), and an IP activator (iloprost) (12), leading to the discovery of a novel V241G mutation in the thromboxane receptor. In this study, a full simulation of platelet deposition on collagen (no thrombin) was possible using a patient-specific model of platelet response to combinatorial stimulation (14, 76).

Gutierrez et al. (91) also considered the multiple channel/single outlet design but used length-varying flowpaths for each channel to create different shear rates on the same device (**Figure 2e**). This allowed the simultaneous investigation of platelet adhesion under multiple shear rate environments via a single withdrawal. The design also required small blood volumes (<100  $\mu$ L) per test, which proved very useful in the study of genetically modified mouse blood.

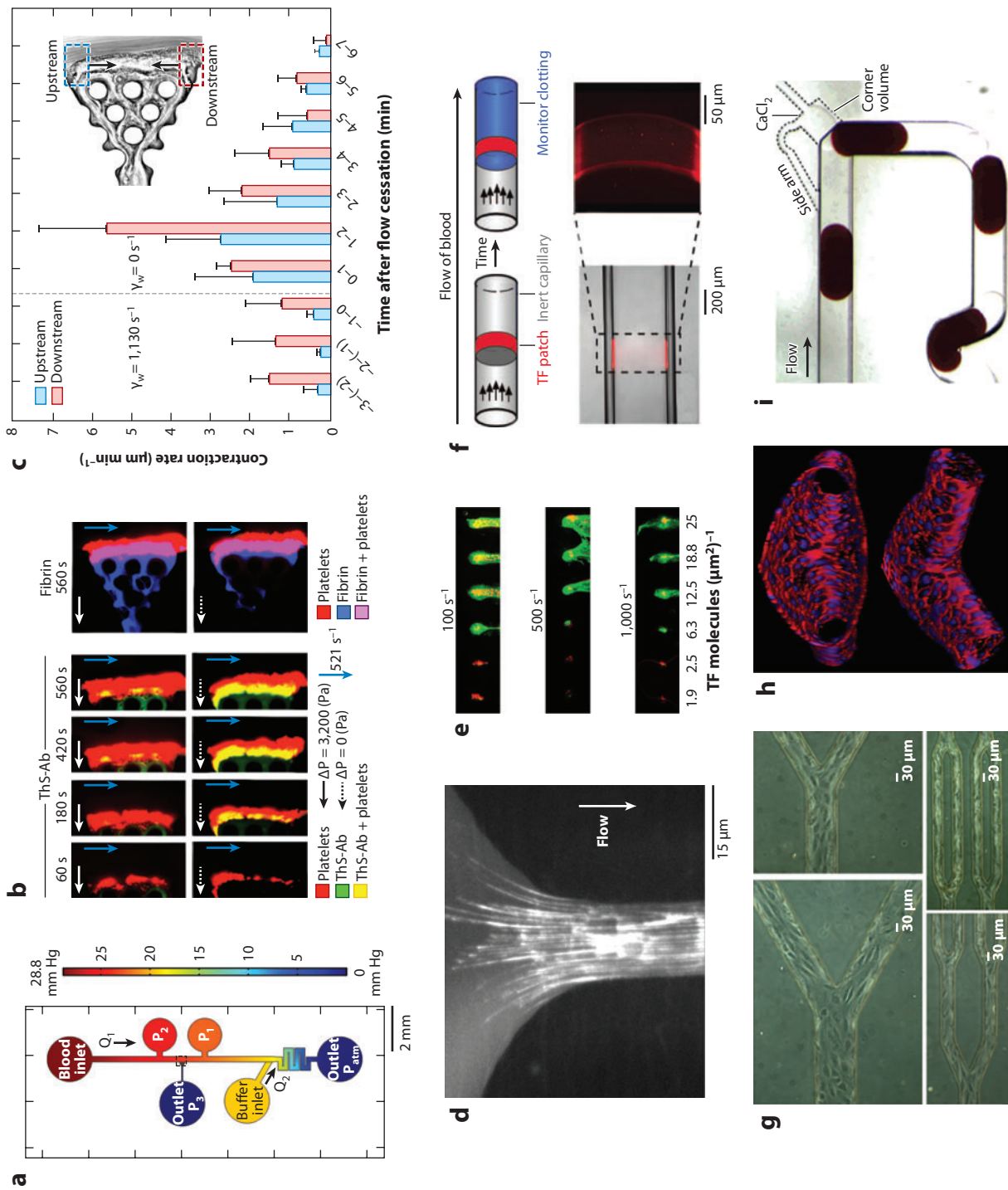
Microfluidic devices have also been used for high-throughput studies of coagulation. The plug-based system developed by Song et al. (92) was designed such that a train of separated blood/reagent droplets could be formed, mixed, and analyzed in a serpentine channel (**Figure 3i**). This technique has been used to measure APTT in high replicate (92) as well as to study the role of mixing in coagulation (93).

In addition to being used to study surface chemistry, microfluidic constructs have been used to localize the release of soluble agonists (ADP and thrombin) into flowing samples, a technique described by Neeves and colleagues (94, 95). To summarize briefly, a porous membrane was used to separate two stacked PDMS channels oriented in a perpendicular cross formation (**Figure 2d**).

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## Figure 2

Microfluidic devices for blood biology. (*a, top*) A schematic drawing of a microfluidic channel cased in PDMS and intended for patterning procoagulant proteins onto the surface of glass slides. (*bottom*) A flow device, also in PDMS, affixed onto the protein stripe; samples are perfused via syringe pump. (Reproduced with permission from Reference 88, ©International Society on Thrombosis and Hemostasis.) (*b*) An eight-channel microfluidic device, which consists of eight inlets perfused via syringe pump from one outlet. The device is adhered reversibly to a glass substrate via vacuum (tube at *bottom left*) with a protein stripe patterned perpendicular to the region in which the eight channels run parallel. (Reproduced with permission from Reference 98, ©American Heart Association.) (*c*) Platelet adhesion monitored in the eight-channel microfluidic device using epifluorescence microscopy. Here, fluorescently labeled platelets adhere to a collagen surface at venous shear rate in the presence of eight different concentrations of the P2Y<sub>1</sub> antagonist, MRS 2179. (Reproduced from Reference 90 with permission from the Royal Society of Chemistry.) (*d, left*) A schematic representation of a 3D microfluidic device that allows for the localized release of soluble agonists from the bottom channel to the top channel through a porous membrane. (*center left*) An image of the device illustrated to the left. (*center right*) A drawing of the site of release of thrombin into a channel of flowing fibrinogen. Fibrin is generated and adheres to the membrane. (*right*) In a thrombin release experiment, the fibrin deposit formed had a morphology that was dependent on the ratios of reaction rate to convection (Da) and convection to diffusion (Pe). (Reprinted from Reference 95 with permission from the Biophysical Society.) (*e*) A schematic representation of a microfluidic device designed to study the role of shear stress in platelet adhesion. Each of the test chambers is connected to a downstream channel of varying length, which provides varying resistance and defines the flow rate (and therefore shear rate) in each of the chambers. (Reproduced from Reference 91 with permission from the Royal Society of Chemistry.) (*f*) A microfluidic model in which a severe stenosis has been generated. Blood flow, from left to right, experiences extreme accelerating and decelerating flows as it traverses the stenosis apex (*red arrow*). As the platelets exit the high-shear-stress region, they form a thrombus in the deceleration region (*light blue*). (Reprinted from Reference 99 with permission from Macmillan Publishers ©2009.) Abbreviations: 2D, two-dimensional; 3D, three-dimensional; PDMS, polydimethylsiloxane.



Agonists were perfused in the bottom channel, and their flux into the top channel was controlled by their flow rate relative to the flow rate of the sample. Depending on device operation, solute flux into blood flow stream could be diffusive, convective, or both. This technique led to the discovery of critical thrombin concentrations at which polymerization of fibrin occurs under flow (95). Recently, a thrombin sensor was developed to show in real time the formation of thrombin in thrombi formed over collagen and lipidated TF (96). In addition, permeability of formed thrombi is an area of active research. The development of a device to measure intraluminal clot retraction showed that this process occurred in the absence of flow only when platelet releasates were allowed to collect (**Figure 3a,b**). This collection of releasates permits platelet aggregates to contract centrally and conveys a flow sensing ability to formed aggregates (**Figure 3c**) (97).

## 2.4. Control of Hemodynamic Conditions

In contrast to in vivo mouse models of thrombosis, the flow environment in microchannels is well defined and can be controlled through the use of syringe pumps or pressure heads to generate a wide range of shear conditions that mimic normal physiologic or pathologic conditions. It is important to note that the use of syringe pumps generates a constant flow rate environment in which a growing thrombus serves only to develop large pressure drops rather than to perturb the flow rate. With this in mind, Colace et al. (98) used the eight-channel microfluidic device described above (eight inlets, one outlet) to create a constant pressure drop setting in which growing thrombi in four of the eight channels diverted flow to four channels feeding from EDTA-treated samples. Combining this technique with computational fluid dynamics, the authors demonstrated that a growing clot experiences a wide range of shear rate conditions ( $100\text{--}100,000\text{ s}^{-1}$ ) as it progresses to

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### Figure 3

Microfluidic devices for coagulation research. (a) A microfluidic model of bleeding designed for the study of thrombus permeability under flow. The device has integrated ports for localization of collagen and TF to the channel wall, pressure sensors, and buffer flow for precise pressure control. Blood flow (from top to bottom) will seep through the collagen scaffold connected to Outlet P<sub>3</sub> until a platelet plug is formed. (Reproduced with permission from Reference 97, ©American Heart Association.) (b) Epifluorescence microscopy performed at the collagen/TF scaffold of the device pictured in panel a. This analysis reveals a large platelet aggregate (red) formed during perfusion, which also stains positive for thrombin using a novel thrombin biosensor. Comparing when blood flow is allowed (top) with when it is not allowed (bottom) to seep through Outlet P<sub>3</sub> reveals that thrombin permeation into the clot is enhanced when it is not convected away. These clots also stain positive for fibrin (right). (Reproduced with permission from Reference 96, ©International Society on Thrombosis and Hemostasis.) (c) Thrombus contraction in a microfluidic bleeding model. Approximately 1 min after flow cessation, the rate of clot retraction, measured by tracking the movement of trapped fluorescent beads, is enhanced threefold. (Reproduced with permission from Reference 97, ©American Heart Association.) (d) A microfluidic model of stenosis revealing long fiber bundles of von Willebrand factor forming on a collagen type 1 surface under plasma flow at shear rates  $>30,000\text{ s}^{-1}$ . (Reproduced with permission from Reference 101, ©American Heart Association.) (e) Microcontact printing of collagen and varying amounts of TF reveals a steep threshold for fibrin generation (green) under whole blood flow at varying shear rates in a parallel plate flow chamber construct. Platelets are labeled in red. (Reproduced with permission from Reference 56, ©American Society of Hematology.) (f) TF patterned into microcapillary flow models using photolithographic techniques. A soluble fluorescent reporter of thrombin will be activated downstream of the procoagulant patch. (Reproduced with permission from Reference 55, ©Wolters Kluwer Health.) (g) Culture of confluent monolayers of endothelium achieved in a PDMS microfluidic device (reproduced from Reference 27 with permission from the American Society for Clinical Investigation) and on three-dimensional collagen scaffolds. (h) The in vitro vessels pictured were designed for studies involving permeability and angiogenesis, as well as thrombus formation after endothelial activation. (Reproduced with permission from Reference 29.) (i) Microfluidic droplet reactor designed to study clotting time in the presence of various inhibitors. Plugs of citrated whole blood, to which CaCl<sub>2</sub> is added, enter at the top. The plug at the bottom left is entering the mixing region, and clotting will be assessed in a downstream region of the channel. (Reprinted with permission from Reference 92, ©2006 American Chemical Society.) Abbreviations:  $\gamma_w$ , wall shear rate; P<sub>1</sub>, pressure 1; P<sub>2</sub>, pressure 2; P<sub>3</sub>, pressure 3; P<sub>atm</sub>, atmospheric pressure; PDMS, polydimethylsiloxane; Q<sub>1</sub>, blood flow rate; Q<sub>2</sub>, buffer flow rate; TF, tissue factor; Ths-Ab, thrombin sensor.



full occlusion or embolus in a microfluidic channel. They used this platform to study the strength of clots under flow and described a 28-fold increase in clot shear resistance attributable to the fibrin network.

Although defining the flow conditions around a thrombus is important for understanding its growth and stability, understanding its dissolution, both by physiologic and by therapeutic mechanisms, requires characterization of transport within the clot. Recently, Muthard & Diamond (97) described a microfluidic bleeding model that allows for the loss of flowing sample through a permeable collagen scaffold to be measured via epifluorescence microscopy (**Figure 3a**). If the sample was whole blood, then clot formed on the scaffold perturbed sample loss, offering the first measure of clot permeability under flow (**Figure 3b**). The microfluidic design allowed for precise measurement and control of pressure drop across the collagen network as the thrombus evolved under flow. Incorporating TF-bearing liposomes into the collagen scaffold led to a dense fibrin network that further perturbed transport across the thrombus.

Microfluidic platforms have also been used to study the role of pathologic shear, such as that through a severe stenosis ( $>10,000\text{ s}^{-1}$ ). Although the turbulent flow that can be generated as a result of these environments is not captured in these models, the low flow rates necessary to create these large shear rates make them valuable. In 2006, Ruggeri et al. (48) demonstrated that platelet rolling and platelet-platelet adhesion in microfluidic flow chambers could be achieved in high-shear-rate environments ( $>15,000\text{ s}^{-1}$ ) and that this interaction was dependent on soluble vWF. More recently, Nesbitt et al. (99) reported exacerbated thrombosis of discoid platelets in the outlet regions of severe stenosis ( $>20,000\text{ s}^{-1}$ ), when strong decelerating flows are present. A microfluidic model of this geometry was generated to test their hypothesis and allowed for high-resolution imaging of the process (**Figure 2f**) (100). A novel stenosis microfluidic channel has also been described by Colace & Diamond (101), who used a device to demonstrate the formation of long vWF fibers ( $>100\text{ }\mu\text{m}$ ) composed of many stretched molecules (**Figure 3d**) (101). In the future, microfluidics will be instrumental in defining a role for pathological shear in diseases such as thrombosis, acquired vWF syndrome, and thrombotic thrombocytopenic purpura (TTP).

## 2.5. Patterned Surfaces—Techniques

Micropatterned surfaces of hemostatically active proteins such as fibrinogen, collagen, vWF, and lipidated TF have been described in the literature using a variety of techniques and geometries. Often, microchannels are filled with a protein of interest and rinsed before sample perfusion, but this presents a variety of issues. More sophisticated techniques can prevent clogging by ensuring that samples and patterned proteins do not come into contact in regions of the channel with undefined flows. Microcontact printing, a practice that uses a small pin to dispense samples into discrete arrays on a glass substrate, was used by Okorie et al. (56) to generate a surface of collagen and TF to support both platelet aggregation and coagulation in a flow chamber (**Figure 3e**). This technique helped to define a threshold level of TF to support coagulation under flow. Ismagilov and colleagues (102) generated arrays of TF patches using photolithographic techniques to study the role of diffusion in coagulation under static conditions. They extended this work to coagulation under flow using a technique to localize TF into a lipid membrane inside a cylindrical microcapillary (103). Using a fluorescent reporter of thrombin generation, this system elegantly demonstrated the shear rate-dependent nature of coagulation over well-defined TF coatings (**Figure 3f**). Microchannel-patterned stripes of protein, which generate distinct regions of thrombosis in microfluidic channels, ensure that samples do not come into contact with hemostatically active surfaces prior to the viewing window. Colace et al. (104) used this technique to generate surfaces of collagen and immobilized TF in PDMS microchannels. This technique



is important in the study of coagulation, as TF patch size has been described as a very sensitive variable. Corum et al. (105) have used PDMS as a means to stamp fibrinogen onto glass substrates to create well-defined patterns of variable surface coverage; these surfaces were then used to study platelet adhesion and spreading dynamics.

In addition to surfaces of proteins, endothelial cell coatings have also been described in the literature. As described above, Nash and colleagues (71) have generated confluent monolayers in microcapillaries, and Tsai et al. (27) have had success culturing endothelial cells on fibronectin-coated surfaces in microfluidic chambers (**Figure 3g**). Recently, Stroock and colleagues (29) reported in vitro microvasculature consisting of endothelial monolayers grown on three-dimensional micropatterned collagen scaffolds (**Figure 3b**). The authors described the scaffolds' potential use in thrombosis models as well as in angiogenesis models.

The discovery of specific matrix protein–platelet receptor interactions, as well as coagulation initiation reactions, has demanded the development of novel surface coatings with which to characterize these interactions under flow. For platelet adhesion and aggregation studies, preparations of purified subendothelial matrix proteins are often used to coat glass or plastic surfaces. The adherence of these proteins is often nonspecific, making the preparation of these surfaces variable between protein preparations and between laboratories. To standardize these assays, specific fragments of the matrix proteins may be utilized, negating any confounding effects of protein structure in influencing platelet–matrix protein binding-site interactions. This approach has been used for collagen, the most abundant subendothelial matrix protein, with collagen-related peptide (CRP) (106). CRP has been shown to bind to platelet GPVI and subsequently lead to platelet activation. Further, platelet adhesion to CRP was found to potentiate GPVI signaling, as compared with platelets in suspension, outlining the importance for flow chambers in elucidating physiological roles of platelets in hemostasis. A novel approach to coating surfaces with thin films of soluble collagen demonstrated that a uniform coating of reconstituted fibers can be deposited on glass, and this approach may lead to better control of collagen surface preparations (107). Along these lines, the tripeptide arginine-glycine-aspartic acid (RGD) is present in many extracellular matrix proteins including collagen and fibronectin and can be utilized to simulate an adhesive cell surface for platelets (108).

## 2.6. Clinical Applications

Generation of microfluidic channels in PDMS requires access to a microfabrication facility. For interested users without this access, commercial products are available for microfluidic experiments. Specifically, the BioFlux system has been adapted to a well-plate format (109), offering high-throughput analysis. Clinically, a variety of diagnostic tests have been developed on microfluidic platforms because the small blood volumes required do not exclude neonatal patients. The Platelet Function Analyzer 100 (PFA-100), for example, perfuses small volumes of blood through a small collagen annulus coated with a platelet agonist such as ADP or epinephrine. Pressure sensors measure the time to annulus occlusion. The test results are strongly related to the traditional variables in platelet function testing, such as platelet count, hematocrit, antiplatelet therapy, and congenital platelet disorders (110). However, the high-shear-rate perfusion used by this device makes it acutely sensitive to disorders of vWF. Furthermore, its use of citrated whole blood excludes the device's use in coagulation testing and raises questions about  $\alpha_{IIb}\beta_3$  function. Other tests, such as the VerifyNow test, an aggregometry-based platelet function test, and TEG<sup>®</sup> 5000, a viscoelasticity-based coagulation test, are both closed-system designs, which, in all likelihood, do not reflect the physiologic kinetics of platelet aggregation and thrombin formation, despite proven utility (110–112). Each of the tests described here have niches in platelet function,

vWF function, coagulation, or fibrinolysis, which suggests the need for an integrated test of global hemostasis.

### 3. CONCLUSIONS

Microfluidic devices are finding increasing use in the study of blood function. These devices create controlled hemodynamic conditions that mimic in vivo flows while using a minimal volume of blood. When combined with micropatterning techniques, the experimentalist can precisely control the prevailing shear rate, duration of exposure, and prevailing pharmacological background. Such devices are fully compatible with multicolor imaging of in situ thrombus generation and embolism and proteomic analysis of the device effluent. Future work to create technologies for the clinical lab will require reliable manufacturing of stable devices and patterned surfaces, hands-free automated operation, and validation of meaningful diagnostic information to inform clinical decisions.

### DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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## Errata

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