The use of microfluidics in hemostasis: clinical diagnostics and biomimetic models of vascular injury

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Purpose of review
This article reviews the application of microfluidic technologies in hemostasis. The emphasis is on promising developments in devices for clinical applications and novel approaches to modeling complex hemodynamics.

Recent findings
Microfluidics combined with micropatterning of prothrombotic substrates provides devices for measuring platelet function and coagulation with low blood volumes (~100 μl) over a wide range of shear stresses. This technology has been applied to the diagnosis of bleeding and thrombotic disorders, as well as to dosing and monitoring of anticoagulation and antiplatelet agents. Microfluidic devices that mimic vascular geometries such as bifurcations, stenosis, and complex interconnected networks yield complex flow fields that have revealed new mechanisms of platelet adhesion and aggregation. Applying techniques from tissue engineering by endothelializing these networks is beginning to close the gap between in-vitro and in-vivo models of vascular injury.

Summary
Microfluidic technology enables researchers to create in-vitro models of vascular disease with unprecedented control of the biochemical and biophysical conditions. Two promising directions are flow-dependent clinical assays and biomimetic vascular networks. These approaches are particularly well suited for modeling the microvasculature. However, caution should be used when extrapolating results from microfluidic channels to the pathophysiology of thrombosis in large arteries and veins.

Keywords
biorheology, coagulation, endothelium, microfluidics, platelets

INTRODUCTION

This review covers the application of microfluidic technology to the study of flow-dependent thrombus formation. Our focus here is on clinical translation of microfluidic devices and novel devices that mimic geometries and flow conditions that are not possible in conventional flow assays. The use of microfluidics in hemostasis is based upon a large body of work performed in what are generically called flow assays [1–3]. Flow assays include annular and parallel plate chambers. These have been indispensible tools for measuring shear stress-dependent receptor–ligand dynamics and shear rate-dependent coagulation and fibrin formation. The shear stress [τ (force/area)] represents the force experienced by the endothelial wall and adherent blood cells because of flowing blood. The shear rate [γ (1/time)] represents the velocity gradient near the wall and is proportional to the flow rate through a channel. The shear stress is related to the shear rate through the viscosity [$\tau = \mu \times \gamma$], which in the case of blood depends both on the fluid velocity and the size of the vessel. The shear rate is lowest in veins (20–200 s^{-1}) and higher in arterioles (500–1600 s^{-1}) and large arteries (300–800 s^{-1}).

There are limitations to applying conventional flow assays beyond the research setting. First is the

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Curr Opin Hematol 2013, 20:417–423
DOI:10.1097/MOH.0b013e3283642186

KEY POINTS

- Microfluidics offers low volume, high-throughput testing of thrombus formation under flow.
- Microfluidic devices have shown potential for clinical diagnostics and drug testing/monitoring.
- The latest advances are moving toward a vessel-on-a-chip that integrates endothelial function with platelet function, coagulation, and hemodynamics within microfluidic formats.

relatively large blood volume requirement (10–100 ml). Second, the throughput is low with typically only one condition (shear rate and substrate) per chamber. Third, the geometries of these chambers are usually straight channels that result in a unidirectional laminar flow. As such, these chambers do not mimic the complex fluid dynamics that give rise to important biophysical mechanisms of platelet function and coagulation. Microfluidic technology addresses each of these limitations and has opened new lines of research in shear-dependent phenomena related to thrombus growth.

MICROFLUIDIC DEVICES FOR MEASURING PLATELET FUNCTION AND COAGULATION

A review of a few key studies of microfluidic applications in hemostasis demonstrates the advantages of this approach compared with conventional flow assay. For a broader background on microfluidic device fabrication and applications in cell adhesion, we point the reader to two excellent review articles by Prabhakarpandian *et al.* [4] and Westein *et al.* [5].

One feature of microfluidic devices is that it is easy to incorporate many channels, and thus observe many shear rates simultaneously using a small volume of blood (Fig. 1a). An elegant example of this parallelization is an eight-channel microfluidic device designed by Gutierrez *et al.* [7]. The salient feature of this device is that blood flows through each channel (24 μm × 200 μm) at a different shear rate, providing a 100-fold range of shear rates using just 15 μl of whole blood in a 4 min assay. The low volume requirement lends itself to studies with genetically engineered animal models without having to sacrifice the animal.

A second feature of microfluidic devices is the ability to combine them with micropatterning techniques to define small, focal patches of prothrombotic substrates. Neeves *et al.* [8] described a device consisting of a 100 μm strip of collagen patterned on a glass substrate perpendicular to an array of 13 microfluidic channels (80 μm × 100 μm) [8]. This collagen 'injury' mimics the focal nature of animal models like the laser injury model, eliminates issues regarding upstream activation of platelets, and simplifies image quantification. Additionally, unlike animal models, the shear stress in flow assays is a user-defined parameter. This feature has been used to subject thrombi to high shear stress challenges as a measure of stability. For example, in a dyslipidemia mouse model ($LDLR^{-/-}$), mice deficient in the contact-dependent platelet receptor sema4D produced thrombi that were mechanically less stable at high shear rates (10 000 s^{-1}) compared to mice with the receptor [9]. Devices based on this model have been applied in several studies of genetically modified mice as a complementary measurement of platelet function to in-vivo injury models [9–11].

With a growing appreciation of the profound role that flow plays in coagulation, anticoagulation, and fibrin formation [12–14], there is a mounting need to integrate these pathways into in-vitro flow assays. One approach is flowing plasma or whole blood over procoagulant and anticoagulant proteins in immobilized lipid bilayers [15–17]. This approach was used in a series of studies to control the spatial presentation of lipidated tissue factor (TF) to static and flowing plasma [16,18–20]. An important outcome of these studies is the description of the critical size and surface concentration of TF necessary to initiate thrombin generation under flow. An alternative approach is introducing platelet agonists and/or coagulation zymogens/enzymes at a known rate from one wall of a channel [21,22]. For example, a membrane microfluidic device was used to define the conditions under which fibrin fibers can form under flow in a purified system of thrombin and fibrinogen [22].

In a whole blood system, Okorie *et al.* [23] used a microarray tool to copattern collagen and TF in a parallel plate flow chamber. They reported a distinct threshold surface concentration necessary to induce fibrin formation at shear rates of 100–1000 s^{-1}. This collagen–TF copatterning approach has since been modified to microfluidic formats [24,25▪▪,26▪]. In that approach, rather then relying on physical adsorption, liposomal TF is conjugated to fibrillar type 1 collagen using a streptavidin–biotin linkage [24]. This surface was used to measure the role of fibrin in stabilizing thrombi under normal and pathological shear rates [25▪▪]. In another model, a side channel filled with collagen–TF was used to measure the permeability and retraction of thrombi (Fig. 1b) [26▪]. From this study, a novel biophysical mechanism was reported in which the cessation of flow results in enhanced platelet retraction. Under flow, soluble platelet agonists are diluted, whereas

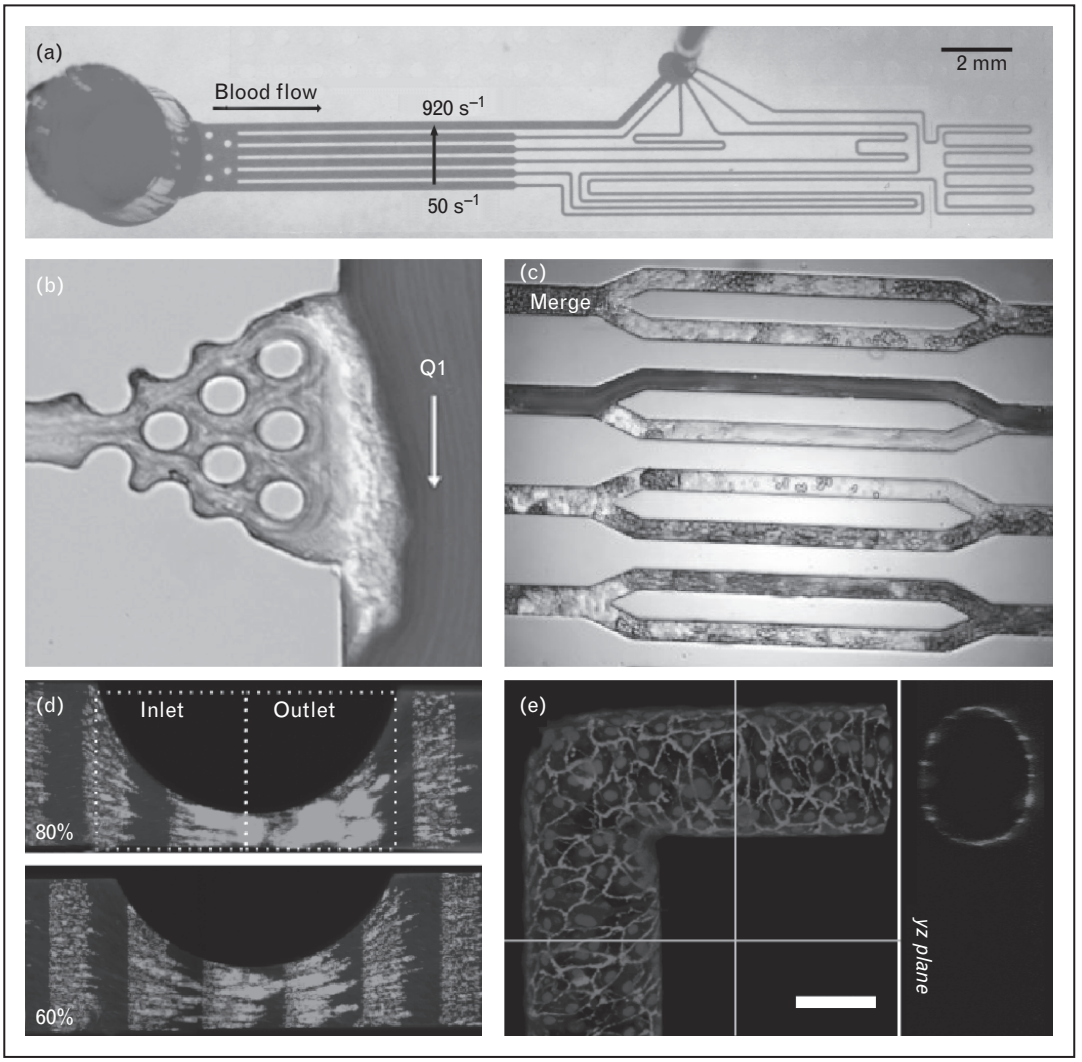

FIGURE 1. Microfluidic devices for measuring hemostasis and thrombosis under physiological and pathological flow conditions. (a) High-throughput device for measuring platelet function over a range of shear rates on ~300 micropatterned collagen spots [6▪]. Channels are 250 μm wide and 50 μm high. (b) Side-channel design for measuring intersitial flows through thrombi at controlled pressure gradients [26▪]. The side channel is filled with collagen and TF. Blood flow runs perpendicular to the side channel at a known flow rate (Q1). The thrombus consists of a fibrin-rich zone within and adjacent to the collagen–TF and platelet-rich outer shell. (c) Endothelialized microvascular mimic for measuring thrombotic occlusions [44]. Endothelial cells were treated with the toxin STX to simulate HUS resulting in VWF secretion and subsequent accumulation of platelet and leukocytes. Channels are ~30 μm in diameter. (d) Platelets preferentially aggregate downstream of the stenotic region in this microfluidic model of atherosclerotic geometry [49▪]. Platelet accumulation increases with increasing occlusion as seen in the difference between an 80% occlusion and 60% occlusion of a 300 μm-wide channel. VWF/fibrinogen strips are used to differentiate the spatial dependence of platelet accumulation in different areas of the stenosis. (e) Endothelialized microfluidic vessel networks embedded in a collagen gel [46▪▪]. Endothelial cells are stained for CD31 and nuclei. Note that the cross-section of these channels is semicircular, which avoids low-flow regions inherent in rectangular channels. TF, tissue factor; HUS, hemolytic–uremic syndrome; STX, shiga toxin type 2; VWF, von Willebrand factor.

under stasis these accumulate and promote outside-in signaling through adenosine diphosphate (ADP) and thromboxane receptors, ultimately leading to retraction.

Since the publication of these studies, there has been an explosion of devices using microfluidic technology to study platelet and endothelial function [4,5,27]. There are now several commercial turn-key microfluidic systems available for studying cell adhesion under flow that make this technology available to any laboratory with an inverted microscope [28–31].

DIAGNOSTIC DEVICES: MOVING MICROFLUIDICS INTO THE CLINIC

Bleeding and thrombotic disorders are flow-dependent disorders. Platelet adhesion and aggregation is shear stress dependent; coagulation, fibrin formation, and fibrin lysis are shear rate dependent. However, there are few clinical assays that measure these phenomena under physiological flow conditions. Microfluidics has the potential to be a transformative technology with respect to improving the sensitivity and specificity of platelet and coagulation disorders.

One barrier to adopting flow assays in the clinic has been their low throughput. As described above, micropatterning techniques, when combined with microfluidics, can result in a low volume focal injury model. Prothrombotic substrates [collagen, von Willebrand factor (VWF), and TF] have been micropatterned in features with sizes of 1–250 μm using microarray pin tools [23,32], microcontact printing [6▪,33], and microfluidic patterning [8]. Taken a step further, several microscale injury sites can be integrated within a single device. For example, Hansen *et al.* [6▪] described a high-throughput device for measuring shear stress-dependent platelet function (Fig. 1b). Microcontact printing, which is a technique that uses an elastomer 'stamp' to print a protein 'ink' on a substrate, was used to define arrays of 20, 50, and 100 μm spots of collagen. A multishear device, similar to that of Gutierrez mentioned above, is placed over an array of collagen spots giving 50 spots per shear rate, or 300 individual test sites per flow assay. There is a threshold size of collagen necessary to support platelet aggregation. For instance, at 300 s^{-1} collagen spots less than 50 μm were unable to promote firm platelet adhesion [6▪]. There is also a limit on the density of spots because of the transport of platelet agonists and coagulation factors from a downstream spot to an upstream spot. Flow assays over collagen–TF spots showed a clear accumulation of fibrin between adjacent spots [23].

The dosing and monitoring of antiplatelet and anticoagulant drugs is another potential application for microfluidics. Maloney *et al.* [34] described a multichannel device for measuring the half-maximal inhibitory concentration (IC_{50}) of ADP receptor inhibitors. An interesting finding from this study underscores the importance of measuring platelet function under flow. Apyrase, an enzyme that degrades ADP that is commonly used to inhibit ADP signaling in static systems, was found to have no effect under flow because the kinetics of degradation are slow compared with dilution by flow. Hosokawa *et al.* [35] conducted dose–response experiments for heparin, agratroban, abciximab, and OS-1 (GP1bα antagonist) over collagen–TF in a microfluidic device at venous and arterial shear rates. They found that metrics of clot formation in their microfluidic device (changes in pressure) were more sensitive to drug concentration than thromboelastography. The same device was used to show difference in the dynamics of thrombus formation in FVIII-deficient mice and simulated hemophilia B using an anti-FIXa aptamer [36▪]. FVIII and FIX deficiencies diminished thrombus growth at 100 s^{-1}, but had no effect at 1100 s^{-1}, thus demonstrating the shear rate dependence of the pathophysiology of hemophilia. Taken together, these studies show that microfluidic devices are potentially more sensitive than static assays in assessing the efficacy of anticoagulant and antiplatelet agents.

Li *et al.* [37▪] describe a point-of-care device based on stenotic geometries in which a label-free optical measurement of platelet aggregation is used to determine occlusion volume and time. Here, the channels are allowed to come to full occlusion, providing a more relevant metric for thrombosis potential. The optical measurement of clot formation is based on light scattering from a laser diode rather than microscopy. This approach gives a label-free measurement that is easily and economically scalable to hundreds of simultaneous measurements.

Many challenges remain in order for microfluidic assays to make an impact in the clinic. First and foremost, in order to quantify pathological platelet function, it is necessary to quantify the normal platelet function. The variability in platelet function on collagen in a microfluidic flow assay was recently described in a cohort of 104 normal donors [38]. Plasma VWF levels were found to be the biggest source variability between donors, followed by the genotype of *GP6*. These results complement similar studies in parallel plate flow chambers in which VWF levels and $\alpha_2\beta_1$ density were found to be sources of variability [39].

The greatest source intrinsic variability within flow assays is the source, composition, and concentration of the prothrombotic substrate [40]. The most accepted substrates are type I fibrillar collagen from animal sources because these give the most potent signaling response through the GPVI receptor and are thought to be more physiological than reconstituted collagens [41]. However, recent studies using collagen-related peptides and collagen thin films show that these substrates are capable of supporting platelet adhesion, signaling, and aggregation [42,43]. A distinction should be made here in the different purposes of flow assays for basic research and those for diagnostic applications. For basic research, recreating physiology or probing a

certain signaling pathway is a worthwhile endeavor. For diagnostics, the primary goal is to define an assay that can discriminate between normal and pathological, and ideally, describe the degree of severity in the case of disorder. To that end, a homogeneous and repeatable surface trumps recreating the precise physiology of the subendothelium.

ENDOTHELIALIZED MICROFLUIDIC MODELS OF THE MICROVASCULATURE

Turning to models that seek to capture the fluid dynamics and biology of real vessels, we start by considering two fascinating studies of the microvasculature. At the venule–capillary–arteriole level, the vasculature is a complex network of bifurcating channels. One result of these bifurcations and other branching structures is that nonuniform flow fields exist that can have a profound effect on thrombus formation. Another feature of blood flow in the microvasculature is that thrombus formation in one vessel causes immediate rerouting of flow in neighboring channels. This is important because it maintains the pressure within these vessels to a fairly small window. Finally, the role of endothelium in thrombus formation is usually neglected in flow assays. The studies described below demonstrate that endothelial cells can be integrated into microfluidic devices and stimulated to induce thrombus formation.

Tsai *et al.* [44] developed a microfluidic network of bifurcating channels in which endothelial cells were cultured to confluence on the channel walls (Fig. 1c). They applied this model to study the biophysical mechanisms of microvascular occlusion in sickle cell disease (SCD) and hemolytic–uremic syndrome (HUS). Perfusion of whole blood from SCD patients led to the occlusion of ~50% of the smallest channels (30 μm), whereas perfusion of blood from SCD patients receiving hydroxyurea led to less than 10% occlusion. HUS was simulated by treating endothelial cells with shiga toxin type 2 (STX2), a toxin produced by *E. coli*, which induces the secretion of large VWF multimers. Platelet adhesion and microchannel occlusion formation were reported on STX2 stimulated endothelial cells in a shear stress-dependent manner. Even more complex networks based on actual images of the microvasculature have also been transferred into a microfluidic device and endothelialized [45].

Zheng *et al.* [46▪▪] used a tissue engineering approach to define a three-dimensional microvascular network. The novelty of the approach is that rather than using a rigid material for the channel walls, microfluidic channels are molded into collagen hydrogels (Fig. 1d). Endothelial cells are cultured within the channels and pervasicular cells such as smooth muscle cells can be cultured within the gel. A notable feature of this system is that the endothelial cells deform the collagen gel so that the channels have circular cross-sections compared with the rectangular cross-section in most microfluidic devices. This feature has important implications for the distribution of shear stresses along the walls of the channels at channel intersections. Thrombus formation was initiated by stimulating endothelial cells with phorbol-12-myristate-13-acetate (PMA), which induces the secretion of VWF. Webs of VWF and VWF–platelet strings were found to transverse the lumen, leading to rapidly formed occlusive thrombi. Platelet accumulation was highest near bifurcations and vessel junctions.

MICROFLUIDIC MODELS OF VESSEL STENOSIS

A major advantage of microfluidic technology is that channels and networks of channels can be fabricated in almost any geometry. One relevant geometry for thrombosis studies is found in models of vessel stenosis [37▪,47,48,49▪,50]. Tovar-Lopez *et al.* [48] presented an analysis of how the size, shape, and rate of contraction–expansion of a model stenosis affects platelet aggregation in the absence of soluble agonists. This model has been used, in conjunction with an analogous animal model, to propose a mechanism in which the deceleration of platelets on the downstream side of stenosis promotes the aggregation of discoid platelet via membrane tethers [47]. Westein *et al.* [49▪] recently extended this model to show that downstream of a stenosis, both plasma VWF and endothelial-cell-secreted VWF promote increased platelet aggregation via GP1bα signaling (Fig. 1e).

Microfluidic stenosis models have also been used for testing drug delivery and monitoring strategies and as a diagnostics measuring thrombotic risk. The contribution by Li *et al.* [37▪] was discussed above. Korin *et al.* [50] report the formulation of shear-activated microparticles containing tissue plasminogen activator (tPA), which in response to pathological shear stresses break apart nanoparticles and release high concentrations of tPA near the periphery of a thrombus. In a microfluidic model of stenosis, the tPA loaded nanoparticles preferentially accumulate downstream of the stenosis following shear stresses of ~1000 dyn/cm^2.

LIMITATIONS OF MICROFLUIDIC MODELS

Despite the features described above, there are important limitations of microfluidic approaches

that should be accounted for when studying hemostasis and thrombosis in microfluidic devices. One issue is that most fabrication techniques give a rectangular cross-section. This geometry results in a shear rate of zero in the corners, and very slow flow adjacent to the corners. The consequence of this flow profile is that platelets and coagulation products tend to accumulate in the corners. It also means that the shear rate is not constant across the entire width of the channel, which can confound the data analysis. There are few approaches to mitigate this effect. One strategy is to use high-aspect ratio channels in which the width is at least ten times the height [51]. This geometry results in a constant shear rate profile across the majority of the channel. Another strategy is the use of hydrodynamic focusing to confine the blood to the center portion of the channel [52]. Alternatively, if micropatterned prothrombotic features are smaller than the channel width, then one can select only those features within the middle of the channel [6▪].

An important limitation of microfluidic devices is that these are intrinsically poor models of thrombosis in large vessels. Simply matching the wall shear stress of a large artery in a smaller channel does not provide similar fluid dynamics because the characteristic of the flow field (laminar, vortices, recirculation, and turbulence) is a function of the relative magnitudes of inertial and viscous forces. Inertial forces are related to the fluid momentum. Viscous forces are related to the force of the fluid on the vessel wall and adjacent fluid layers. The ratio of these forces is called the Reynolds number, and it is directly proportional to channel size. The Reynolds number in the human carotid and coronary arteries is 100–300 [53], so inertial forces dominate. The Reynolds number in a 100 μm microfluidic channel at a shear rate of 1500 s^{-1} is less than 1, so viscous forces dominate. As a consequence, it is impossible to get the same flow behavior and wall shear stress of an artery in a microfluidic channel and care should be taken in extrapolating mechanisms from these devices to thrombosis in larger arteries and veins.

CONCLUSION

Almost every event in the formation of a thrombus is influenced by blood flow. This flow field imparts forces on the vessel wall and blood cells and transports coagulation factors, anticoagulation molecules, and platelet agonists to and from the thrombus. Consequently, researchers and clinicians need methods that probe these mechanisms in a flow-dependent manner. Microfluidic devices are the latest entry onto the ever-growing list of assays that measure thrombus formation under some sort of simulated flow. Whether these will suffer the same fate of other promising, but ultimately disappointing technologies is unknown. However, the ability to recreate the biochemical and biophysical environment of vascular injuries and disorders as reviewed here is an encouraging sign that microfluidic technologies may succeed where others have failed.

Acknowledgements

This work was supported by a Scientist Development Grant from the American Heart Association, the National Heart, Lung, and Blood Institute (HL100333), the Colorado Office of Economic Development and International Trade, the Bayer Hemophilia Awards Program and the Boettcher Foundation's Webb-Waring Biomedical Research Award.

Funding received for some of this work was supported by the National Institutes of Health (NIH).

Conflicts of interest

There are no conflicts of interest.

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- ▪▪ of outstanding interest

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