A Scalable, Point-Of-Care, Microfluidic Approach For Assessing Thrombosis And Hemostasis

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Abstract
Coagulation testing is an important diagnostic tool for the detection of excessive bleeding risk or obstructive clot formation (thrombosis), using blood samples from patients. Microfluidic flow devices have been well established to provide insights on the impacts of shear rate, drug action, and disease state on coagulation and platelet biology. The bulk of the microfluidic devices and assays used in the past have relied upon manual assembly using poly(dimethylsiloxane) (PDMS), a material and construction method not well suited to use in a clinical setting. This thesis describes the design and testing of a single-use, storage stable evolution of previous PDMS microfluidic designs, manufactured via injection molding and pressure-sensitive adhesive bonding. Using this device, we demonstrate the ability to make internally consistent and repeatable measurements of platelet and fibrin fluorescence intensity in clots forming under venous shear rate, using a bench-top LED microscope, and physiologically representative constant-pressure driven flow. We also demonstrate the ability to detect a strong, dose-dependent reduction in the fibrin fluorescence intensity signal in response to in vitro spiking of direct oral anticoagulants (DOACs). Further, we showed the ability to reverse this inhibition, through the addition of small quantities of drug reversal agents. In a separate study, the presence of DOACs in the blood of patients on the medications was clearly detected. Utilizing the previously obtained data from in vitro spiking, the relative response of DOAC patient blood with and without reversal agent was used to generate a quantitative prediction for the current concentration of drug in their system. The field of coagulation testing has lacked a definitive candidate for a fast, reliable means of accurately assessing patient anticoagulation status. Taken together, the ability to both identify the presence and predict the quantity of DOAC in a patient’s blood using a single-use microfluidic chip approach, as described in this thesis, represents a potential promising new direction for coagulation testing at the bed-side.

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A SCALABLE, POINT-OF-CARE, MICROFLUIDIC APPROACH
FOR ASSESSING THROMBOSIS AND HEMOSTASIS

Jason M. Rossi

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A SCALABLE, POINT-OF-CARE, MICROFLUIDIC APPROACH FOR ASSESSING THROMBOSIS AND HEMOSTASIS

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ABSTRACT

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Jason M. Rossi

Scott L. Diamond

Coagulation testing is an important diagnostic tool for the detection of excessive bleeding risk or obstructive clot formation (thrombosis), using blood samples from patients. Microfluidic flow devices have been well established to provide insights on the impacts of shear rate, drug action, and disease state on coagulation and platelet biology. The bulk of the microfluidic devices and assays used in the past have relied upon manual assembly using poly(dimethylsiloxane) (PDMS), a material and construction method not well suited to use in a clinical setting. This thesis describes the design and testing of a single-use, storage stable evolution of previous PDMS microfluidic designs, manufactured via injection molding and pressure-sensitive adhesive bonding. Using this device, we demonstrate the ability to make internally consistent and repeatable measurements of platelet and fibrin fluorescence intensity in clots forming under venous shear rate, using a bench-top LED microscope, and physiologically representative constant-pressure driven flow. We also demonstrate the ability to detect a strong, dose-dependent reduction in the fibrin fluorescence intensity signal in response to \textit{in vitro} spiking of direct oral anticoagulants (DOACs). Further, we showed the ability to reverse this inhibition, through the addition of small quantities of drug reversal agents. In a
separate study, the presence of DOACs in the blood of patients on the medications was clearly detected. Utilizing the previously obtained data from *in vitro* spiking, the relative response of DOAC patient blood with and without reversal agent was used to generate a quantitative prediction for the current concentration of drug in their system. The field of coagulation testing has lacked a definitive candidate for a fast, reliable means of accurately assessing patient anticoagulation status. Taken together, the ability to both identify the presence and predict the quantity of DOAC in a patient’s blood using a single-use microfluidic chip approach, as described in this thesis, represents a potential promising new direction for coagulation testing at the bed-side.
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CHAPTER 1 : INTRODUCTION

1.1 Hemostasis

1.1.1. Platelets

Platelets are anuclear, discoid cell fragments found in blood which have multiple functions contributing to the process of hemostasis – the arresting of blood leakage from damaged vasculature. Platelets, 2-4 microns in diameter, are formed from megakaryocytes in the bone marrow via thrombopoiesis and remain in circulation for approximately 7-10 days after their formation [1]. When stimulated with any of several agonists, platelets can transform into an activated state which promotes their adhesion to both one another and to exposed subendothelial tissue; a process that is also marked by shape change and granule release [2]. To form a clot in vivo, platelets must aggregate under flow in response to an injury. To achieve this, platelets first adhere to the exposed collagen and von Willebrand factor (vWf) which are revealed by damage to the endothelial cells lining a vessel wall. Collagen binding occurs via the GPVI receptor and $\alpha2\beta1$ integrin complex, and vWf engagement happens through the GPIb-V-IX receptor, although this interaction is considered more important at arterial shear rates [3]. Activation of platelets via collagen binding is significant; fibrillar collagens are considered to be among the most potent platelet activating substances [1]. After this primary aggregation phase, subsequent secondary aggregation occurs via positive feedback release of ADP and TxA2 from activated platelets. Activated platelets bind one another through bridging of fibrinogen molecules between the GPIIb/IIIa integrin complex on separate platelets [1].

In addition to aggregating to form a hemostatic plug in their own right, platelets also serve to support a series of enzymatic reactions known as the coagulation cascade.
Through the activation process, platelet membranes become charged and phospholipids from the inner leaflet are exposed, which can act as a scaffold on which the coagulation reactions can occur [4]. Thrombin, a reactive product of the coagulation cascade, can also act as a platelet agonist and further activate platelets via interaction with protease activated receptors PAR1 and PAR4 on platelet surfaces [1].

1.1.2. Coagulation Cascade

Apart from the action of platelets, the physiological process of coagulation is a series of enzymatic reactions culminating in the formation of polymerized fibrin which can form a mesh network to help stabilize clots. Upon initial vessel damage during an injury, Tissue Factor (TF), is exposed along with the collagen in the subendothelium. Tissue factor subsequently complexes with Factor VIIa from the circulating plasma to form the TF:VIIa complex, also known as extrinsic tenase [5,6]. This complex is the root of what is typically referred to as the extrinsic pathway, which ultimately activates Factor X to Xa (Figure 1.1). This is generally referred to as the initiation phase of coagulation.

The extrinsic pathway is considered to be the primary pathway by which factor Xa is generated in vivo, but a second pathway also exists, known as the intrinsic pathway. Intrinsic pathway activation is initiated by plasma interaction with negatively charged surfaces, and the subsequent activation of factor XII to XIIa. The intrinsic pathway then proceeds through the reactions of factors XI, IX, and VIII, and culminates in the formation of the VIIIa:IXa complex, or intrinsic tenase. Whether Xa is generated via the intrinsic or extrinsic pathway, it will subsequently complex with activated Factor V to form Xa:Va, also know as the prothrombinase complex. This complex and the subsequent reactions leading to Factor IIa (thrombin) and fibrin are referred to as the common
Although initiation through the intrinsic pathway is considered less relevant in vivo, parts of the pathway become important even when coagulation is initiated by TF, as thrombin can activate Factor V to Va, VIII to VIIIa and XI to XIa, enabling formation of intrinsic tenase in the absence of factor XIIa [7]. This is referred to as the amplification phase of coagulation.

**Figure 1.1: The primary reactions of the coagulation cascade**

The main reactions of both the intrinsic and extrinsic coagulation pathways, and the shared common pathway [6].

One of the most significant results of the common pathway of coagulation is the formation of large amounts of fibrin polymer from the plasma protein fibrinogen. This
polymerized fibrin forms a porous network and a loose clot structure which is highly elastic and deformable [8]. Bleeding disorders resulting from deficiencies in coagulation factors such as hemophilia A and B, and reduced life expectancy in fibrinogen knockout mice indicate that fibrin is necessary for maintenance of hemostasis [8]. The phase in which the significant amounts of thrombin formed in the amplification phase have precipitated the formation of physiologically relevant amounts of fibrin is known as the propagation phase [6].

1.1.3. **Thrombosis**

While the processes of platelet aggregation and thrombin formation through the coagulation cascade are important physiological responses to tissue damage, excessive or unneeded clotting can cause extreme deleterious effects. Contemporaneous with platelet and coagulation activating processes, down-regulating mechanisms are continuously at work in blood to prevent excessive accumulation of activated platelets and thrombin. Antithrombin, heparin cofactor II and activated Protein C help to inhibit thrombin and its reactivity, while prostaglandins released from the endothelium can stimulate inhibitory internal signaling pathways in platelets [9,10]. In addition, fibrinolytic processes begin through the activation of plasminogen to plasmin upon contact with tissue plasminogen activator and work to dissolve and break apart fibrin clots [9]. When either these down-regulating mechanisms become underactive, or the procoagulant mechanisms become overactive, excessive clotting can occur causing occlusive thrombi to form which can stop blood flow in vessels. Both venous thromboembolism (VTE), caused by thrombus formation in the veins and subsequent embolization posing risk of pulmonary embolism, and Myocardial Infarction (heart attack) are potential life
threatening thrombotic complications which can result from disregulated hemostatic pathways.

1.2 Standard Laboratory Coagulation Testing Methods

1.2.1. Prothrombin Time

The prothrombin time (PT) is a blood plasma assay used to measure the time required for fibrin to be generated after the activation of the coagulation cascade by addition of TF – typically referred to as clot time [11]. Since coagulation in this assay is initiated by TF, as shown in Figure 1.1, this is considered an extrinsic pathway dependent test. PT is run using plasma anticoagulated with sodium citrate, which chelates and sequesters calcium ions which are required for platelet activation and most of the coagulation reactions to proceed. Typically, TF is added in these tests as thromboplastinin – a mixture of TF protein and phospholipid material which can act as a scaffold for the coagulation reactions in the absence of activated platelets (Figure 1.2). This thromboplastin is generally added in a calcium containing buffer in order to restore the plasma’s ability to coagulate by replacing the chelated calcium ions. The endpoint of the assay is indicated by the formation of fibrin as detected either optically or mechanically, and typically occurs 10-15 seconds after initiation [11]. PT data is sometimes presented as an internationally normalized ratio (INR) defined as the ratio of patient’s clot time to a reference clot time which has been defined as “normal”, raised to the power of the international sensitivity index (ISI) (Equation 1.1) [12]. This approach enables comparison of PT values gathered across different equipment and calibrations.
Despite clot times being relatively short for PT/INR, the full cycle time of the test can be more than 60 minutes [13]. Further, since the INR was originally developed for patients on Vitamin K antagonists and includes data from such patients in the calculation of the ISI, the application of the approach to patients not on oral anticoagulants is not necessarily rigorous [13]. The test also cannot differentiate the independent contributions to coagulation, and provides no information on platelets or clot stability.

1.2.2. *Activated Partial Thromboplastin Time*

Activated partial thromboplastin time (aPTT) is another plasma assay that measures the time to fibrin formation like PT, but initiates coagulation via the intrinsic
pathway – through activation of factor XII – rather than the extrinsic pathway (Figure 1.1). Like PT, the assay is run using citrated plasma, and calcium ions and phospholipid are added to the sample prior to initiation. Typically, a charged inorganic material such as kaolin or silica is added to initiate activation of factor XII. Clot times are longer for aPTT than PT, at around 25-35 seconds [11]. Activated partial thromboplastin time shares many of the major limitations of prothrombin time, most notably the excessively long turnaround time [13]. In addition, both tests are performed in the absence of platelets and red blood cells, limiting the biology they are able to interrogate. Inhibitors present in physiological coagulation such as protein C are also not necessary represented completely in either of the assays due to the lack of thrombomodulin, meaning inferences about the total amount of thrombin being generated in these assays can be misleading.

1.2.3. Viscoelastic Measurements

Viscoelastic measurement of clots has been in use at the lab scale since the late 1940’s and relies on the detection of changes in the rheology of blood from more viscous to more elastic as clotting occurs and fibrin forms [14]. The main outputs of this measurement are the timing of the transition from primarily viscous to elastic, and the stiffness of the clot once that transition occurs, as defined by the shear elastic modulus [10]. The most common implementation of this approach is the TEG-5000s device, wherein the deflection of a torsion wire in response to increased clot elasticity is monitored as a temperature controlled cup containing whole blood is oscillated [14]. The use of whole blood, unlike PT/INR or aPTT means that platelet influence in the clotting process can be captured by thrombelastography (TEG).
The deflection of the pin in Figure 1.3 can be detected by an electromechanical proximity sensor plot amplitude versus time, yielding a characteristic curve as shown in Figure 1.4. Initially, prior to significant thrombin/fibrin formation or platelet aggregation, there is minimal deflection and the amplitude remains constant. The onset significant of deflection is marked by an ‘R’ parameter which is functionally similar to the clot time from the plasma assays, ranges normally from 4-8 minutes. Other typically used parameters include the maximal deflection denoted as the maximum amplitude (MA) and the slope of the amplitude with time at the midpoint between onset and the MA (α), which captures rate of clot formation. Unlike the previously described plasma assays, TEG can produce data on the stability of clots by maintaining the experiment through the onset of clot lysis mediated by plasmin, and the restoration of viscous behavior. TEG data is impacted by the processes of coagulation and platelet aggregation, although the contributions of
each cannot be completely decoupled. The specific values of R and α generally reflect the rate of coagulation, while the time of maximum amplitude is generally more reflective of platelet activation status and quantity [14].

![Diagram of coagulation process]

**Figure 1.4: TEG example output data**

Example results for TEG experiment based on pin deflection as described in Figure 1.3. Reaction time (R), α angle and maximum amplitude are the main values derived from these plots [14].

Rotational thromboelastography (ROTEM) is a largely similar approach to the TEG system, with the primary difference being the way in which the cup and pin and move about one another. In the ROTEM system, the cup is held stationary, while in TEG the pin is held stationary and the cup is rotated. First generation devices for both technologies were marked by significant variance from lab to lab and instrument to instrument [14]. Although neither of the viscoelastometry methods has been considered an international standard in coagulation testing like the PT/INR and aPTT tests, the technology has been in use for several decades now, for research purposes in
indications such as trauma and anticoagulation therapy detection. Still, the reliability and accuracy of results generated by these methods is still somewhat controversial, and their adoption has not been universal [15,16].

1.3 Microfluidic Platelet and Coagulation Testing methods

1.3.1. Flow Hemodynamics and Coagulation

One common deficiency in each of the previously described laboratory tests is that none of them capture full physiological hemodynamics by including the impacts of flow on the coagulation process. The specific properties of the flow field through a vessel govern the convective mass transport that can occur for coagulation factors and other reactive species into and out of the growing thrombus. In addition, the flow regime can impact both cell to cell interactions and cell interactions with the boundaries of the particular flow path, either the physiological vessel walls or the walls of a flow chamber [17]. At very low shear rates, cell to cell interactions, particularly between red blood cells can substantially modify the rheology of the fluid, owing to rouleaux formations that can occur as the flat side of the erythrocytes adhere to one another [17]. In this way, blood viscosity depends strongly on local shear rate, and therefore the particular hemodynamics the environment being monitored, with lower shear rates yielding higher viscosity [2]. On the opposite end, high shear rate environments have been demonstrated to activate platelets and alter both the manner in which TF is distributed throughout a clot and the rate of coagulation factor activation [17,18]. There has also been evidence that TF originating in the blood is a major source of coagulation initiation in vivo, and therefore the convective transport of this blood-borne tissue factor will also be highly flow dependent [19]. Shear rate and flow directionality has also been well
demonstrated to have a major impact on the endothelial cells lining vessel walls, affecting both their morphology and alignment, and the relative amounts of receptors expressed on their surfaces [20].

Initial experimental attempts at addressing the impact of flow phenomena on coagulation in vitro primarily utilized large parallel plate flow chambers or glass capillary devices. Flow chamber devices consist of parallel plates with variable spacing to achieve different shear rates, and typically utilize some form of collagen fibril on one of the plate surfaces to initiate platelet adhesion, and lipidated tissue factor to induce coagulation, and have been largely successful in recapitulating the processes which regulate arterial thrombosis [17,21]. A major drawback, however, is that these flow chambers require large reservoirs of blood and lengths of tubing in order to pump blood quickly enough to achieve the physiological shear rates of interest. Device gap dimensions are typically 100-200 microns and chambers can require up to 10 mL per assay. Miniaturized perfusion chambers and glass capillary based devices were developed to help mitigate this issue and have been used successfully as well for several studies relating to critical concentrations of TF required for coagulation and leukocyte adhesion [21–23]. Still, these smaller flow chamber and capillary devices remain relatively restrictive in terms of the parallelization and geometric freedom which they allow.

1.3.2. PDMS Microfluidics

The application of photolithography and soft lithography techniques to platelet and coagulation assays has enabled the development of poly(dimethylsiloxane) (PDMS) devices which largely address the limitations of the previous technologies. Micron scale resolution and freedom in the relative placement of independent flow channels in
proximity to one another enable high degrees of parallelization and repetition of experimental conditions, while utilizing very small volumes of blood (<0.5 mL per experiment). Flow paths in devices manufactured by PDMS soft lithography typically have rectangular channel geometries in the 10-500 micron range, yielding a laminar velocity profile of the form:

\[
\begin{align*}
    u(y, z) &= \frac{48 Q}{\pi^3 hw} \times \\
    &\sum_{n=1,3,5, \ldots}^{\infty} \frac{1}{n^3} \left[ 1 - \frac{\cosh(n \pi \frac{y}{h})}{\cosh(n \pi \frac{w}{2h})} \right] \sin(n \pi \frac{z}{h}) \\
    &\sum_{n=1,3,5, \ldots}^{\infty} \frac{192 h}{n^5 \frac{w}{2h}} \tanh(n \pi \frac{w}{2h})
\end{align*}
\]

for \(-w/2<y<w/2\) and \(z<=h\). With rectangular channels of high enough aspect ratio (w/h), this flow profile provides an approximation of two-dimensional infinite parallel plate flow within the middle portion of the channel, away from the side walls. Rectangular geometry is both a necessity of the manufacturing method, and an aid in visualization; since the platelet accumulation and coagulation reactions are localized on a plane perpendicular to the field of view of an epifluorescence microscope, the entire clot can be image simultaneously.

The development of multiplexed PDMS devices for running multiple experimental conditions on a single chip has enabled studies on the effects of drugs, disease states, and thrombogenic surfaces [24–28]. The ability to customize the hemodynamic conditions has also allowed for specific device and study design for high shear vWf capture and transthrombus transport and pressure gradients [29–32]. The majority of PDMS device studies have utilized optical detection via immunofluorescence to transduce signal from the clot data, but other approaches including electrical impedance and mechanical responses have been used as well [33,34]. Similar microfluidic devices
can also be manufactured in poly(methyl methacrylate) (PMMA), cyclic-olefin-copolymer (COC) and other materials by alternative manufacturing techniques such as laser micromachining, injection molding, and lamination techniques [35]. Despite the scope of materials and geometries available, and although microfluidic devices have been used to study blood from patients from clinical visits, no single chip technology has presented itself as a replacement for the gold standard coagulation and platelet function tests available. A device which utilizes the unique advantages of microfluidic technology, but which has been adapted to be storage-stable and operable by untrained personal therefore represents an opportunity to offer unique advantages in hemostasis testing.

1.4 Point-of-Care Testing Methods

1.4.1. Unmet needs in Coagulation Testing

Although routine coagulation testing by the standard laboratory methods described earlier can provide valuable insight to physicians, an emerging need for faster, easier to use approaches has presented itself over the last decade. Direct oral anticoagulants (DOACs) have, during this period, increased rapidly in usage for indications such as atrial fibrillation (a-fib) and deep vein thrombosis (DVT), owing to their increased safety due to reduced risk adverse events such as intracranial hemorrhage, and their lack of need for routine blood tests to monitor drug levels as required by previously used anticoagulants such as warfarin [36]. As a result of this, more than 4 million patients were currently on DOAC therapy as of 2016 [37]. Although DOACs have shown increased safety, as with any anticoagulant, they carry with them an increased risk of adverse bleeding events, particularly in vulnerable populations such as the elderly and those on certain other medications [38]. In addition, patients who
experience traumatic injury or who must undergo urgent intervention surgery (perioperative management) while on DOAC therapy are similarly at-risk, and the ability to detect their current anti-coagulation status has presented itself as a more and more pressing need. Tests such PT and aPTT have been shown to yield variable responses to DOAC drugs depending on the reagents used, with their use being largely limited to screening assays, and no standarized test to detect and quantify DOACs has emerged in their place [38]. Although drug reversal strategies have been developed to reduce bleeding risk for patients on DOACs in specific scenarios, their prohibitively high cost (up to $50000/dose) means that methods for detection of DOAC levels in both a cost and time efficient manner to assess the need for those reversal agents is critically important.

Similarly, the need to assess bleeding risk in proximity to surgical procedures regardless of the presence or absence of anti-coagulation therapy is an emerging unmet need. Cardiac surgery has been associated with a high risk of coagulopathic bleeding, with as many as 20% of patients undergoing procedures experiencing significant bleeding events [39]. In cases such as these, the timing associated with obtaining the results is critically important, and so increasingly point of care (POC) coagulation and platelet assays have been utilized to inform decision making in acute situations. The ability to detect specific coagulation disorders in short time periods, then, is a major cause of the rapid increase in number and type of point of care diagnostic methods.
1.4.2. Current Point-of-Care Technologies

1.4.2.1 Platelet function Assays

Many of the POC testing methods currently available focus on either platelet function or coagulation function exclusively. The VerifyNow (Accumetrics) is one such assay focusing on detection of platelet response to specific drugs. The assay detects changes in turbidity optically upon activation of platelets in proximity to fibrinogen coated beads, with either adenosine diphosphate (ADP) or arachadonic acid (AA). As platelets are activated, Gp(IIb)/IIIa binds to the fibrinogen on the beads, forming bridges, causing aggregation and changes in light transmittance [40]. There are specific editions of the assay for P2Y12 inhibitors (such as clopidogrel) and for Aspirin, but in either case the assay requires ~2 mL of blood and has provides results in <20 minutes [41,42]. The assay is widely used for undergoing coronary interventions and has been FDA approved for platelet function testing, but is considered quite expensive.

The platelet function analyzer (PFA-100/200) (Siemens) is a similar platelet focused assay. Unlike the VerifyNow, the PFA utilizes an open system flow assay where whole blood is delivered via vacuum pressure across a collagen membrane coated with an additional variable agonist such as ADP. As platelets are activated, they accumulate in the membrane and eventually cease the flow – yielding a ‘clot time’ as a result [40]. The assay is simple and uses less than 1 mL of blood, but can be highly dependent on the vWF level in a particular sample, and on the erythrocyte concentration as well [42].

Both TEG (Haemonetics) and ROTEM (Pentapharm) have iterations of their viscoelastic assays designed specifically for the detection of platelet aggregation via specific activation via ADP, and which can be used in bedside operation as a POC diagnostic [42]. In addition, the TEG-6s is newer iteration of thrombelastography
technology utilizing microfluidic cartridges to handle small volumes of blood and oscillate the fluid via a resonance method rather than cup and pin motion [43]. The TEG-6s can be used to provide information on platelet-fibrin clot formation by utilizing specific agonists as with the previous versions of the technology [42]. Although the TEG-6s reduces the volume of blood required and the complexity of the experiment, the assay still requires 45 minutes for full results and has been shown to have limited correlation with patient outcomes in clinical settings [43].

1.4.2.2 Coagulation function assays

Just as some POC assays have focused on platelet function detection, others have focused on detection of thrombin and fibrin formation and the coagulation cascade. POC iterations of the previously described PT/INR and aPTT are increasingly becoming available. The Coumatrak (Biotrack) assay has been available for use in bedside operation since the 1990’s and uses of a battery-powered laser photometer to measure the clot time previously described. Whole blood is obtained from standard lancet finger-stick, and added to a cartridge containing thromboplastin [39]. The device shows significant time and cost savings over lab based PT/INR but has issues with incomplete correlation with laboratory results and concerns about the accuracy and precision of INR values [44]. The CoaguChek system from Roche is a more recent miniaturized version of the technology which similarly utilizes whole blood from finger-stick, but with an electrochemical detection method and a much smaller form factor. Results from the device have shown generally good correlation with laboratory INR analysis, but some results have been shown to differ >15%, and in some cases would cause different clinical decision making up to 33% of the time [45,46]. Similar devices have been
released by CoaguSense and Abbott Laboratories, but all are generally subject to incomplete agreement with laboratory PT/INR and the fundamental limitations of the PT/INR assay.

1.4.2.3 Complete Hemostasis Assays

Several technologies exist which have the capability to detect both platelet function and coagulation simultaneously. The T2 Magnetic Resonance assay is one such technology which detects changes in the transverse relaxation time of nuclear magnetic resonance as clotting proceeds upon the addition of stimuli. As water’s molecular motion becomes more restricted as clotting proceeds, changes are caused in their spin-spin relaxation times \[47\]. Applying algorithms to T2 relaxation curves allows independent attribution of behavior to red blood cells, plasma proteins and platelets. Experiments are run in cuvettes on a portable benchtop reader. The assay requires very small volumes of blood (~.04 mL) and provides results in approximately 15 minutes, but does not capture any contributions of flow to the hemostasis process \[48\].

As described earlier, viscoelastic methods such as TEG and ROTEM can be utilized in POC operation and can be made to provide insight on platelet function as it pertains to specific receptors such as P2Y\(_{12}\), through specific modifications of the assay (ex. TEG PlateletMapping). In normal mode of operation, however, both TEG and ROTEM generally capture the contributions of thrombin, fibrin formation and coagulation to blood rheology far more significantly. In this way, the methods can provide full insight on hemostasis, typically not within the same experiment. Still, the TEG-6s POC device can generate the same parameters as and has shown good agreement with the cup and
needle iterations such as the TEG5000, with no reagent handling and minimal fluid handling [43].

Table 1: Selection of POC hemostasis assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Detection</th>
<th>Sample</th>
<th>Platelet Detection</th>
<th>Coagulation Detection</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEG-6s</td>
<td>Optical detection of Viscoelastic Changes</td>
<td>Citrated Whole blood</td>
<td>Yes (with modified assay)</td>
<td>yes</td>
<td>Variable results, relatively slow, No flow</td>
</tr>
<tr>
<td>VerifyNow</td>
<td>Optical Detection of bead aggregation</td>
<td>Whole blood</td>
<td>yes</td>
<td>no</td>
<td>Relatively expensive, No flow</td>
</tr>
<tr>
<td>PFA-100/200</td>
<td>Detection of Pressure Drop</td>
<td>Whole blood</td>
<td>yes</td>
<td>no</td>
<td>Wide normal range</td>
</tr>
<tr>
<td>T2 Resonance</td>
<td>T2MR of water</td>
<td>Whole blood</td>
<td>yes</td>
<td>yes</td>
<td>Unintuitive output data, No flow</td>
</tr>
<tr>
<td>Miniaturized PT/INR</td>
<td>Variable</td>
<td>Whole blood</td>
<td>no</td>
<td>no</td>
<td>No platelet contribution, No flow</td>
</tr>
<tr>
<td>PDMS Microfluidic Approaches</td>
<td>Variable, typically immunofluorescence</td>
<td>Whole blood (CTI)</td>
<td>yes</td>
<td>yes</td>
<td>Labor intensive, not designed for POC</td>
</tr>
</tbody>
</table>

1.4.2.4 DOAC Detection Assays

In addition to the general hemostasis and platelet function assays described above, a subset of assays have been developed with the specific aim of detecting the presence of DOACs in blood samples. ClotChip (Xatech) is a dielectric spectroscopy assay that measures changes in the electrical permittivity (via impedance) of whole blood in response to coagulation proceeding, and corresponding changes red blood cell interfacial polarization, using a small volume microfluidic device [49]. For a fixed frequency, the presence of thrombin (or factor Xa) inhibiting drugs shifts the timing of a
characteristic peak in electrical permittivity to later timepoints, and in this way DOAC presence can be detected. The chip has also been adapted to be read in a hand-held reader facilitating application to POC settings. In clinical work with patients on DOAC drugs, however, the AUC for receiver operating curve (ROC) analysis for the presence of DOAC was found to be as low as 0.82 for apixaban, indicating a somewhat limited detection sensitivity for certain DOACs [49]. Further, the general assay does not capture the contributions of flow to hemostasis, and utilizes a somewhat unintuitive metric in permittivity. Another DOAC specific POC assay that has been developed is a microfluidic fluorescence assay by CoaguloMed. Fluorescence intensity is monitored as a sample of blood plasma labeled with fluorescent-conjugated fibrinogen undergoes coagulation in a custom designed microfluidic device under quiescent conditions. As fibrin polymer is formed, the optical signal changes until the process is complete and a clot time is specified [50]. The optical detection of fluorescence signal is carried out in a handheld reader device, making use in clinical application easy. In work with clinical patients on DOACs, ROC analysis yielded and AUC of .984 for detection of the presence of a DOAC, indicating reasonably good sensitivity for the drugs. In addition, coagulation in the assay is initiated with 2 different agonists (A and B), one upstream of Xa and one downstream of Xa, yielding the ability to differentiate between IIa and Xa inhibitors. As with Xatech, however, the assay is carried out under static conditions, and additionally can only be run with plasma, rather than whole blood. Perosphere Inc. have also developed an assay for DOAC detection, utilizing infrared spectroscopy to detect the formation of fibrin polymer in whole blood samples, which can be run on whole blood with no anticoagulant [51]. Thus far, however, DOAC detection has only be
demonstrated in rats. Table 2 shows a qualitative comparison of the difference DOAC assays.

Table 2: DOAC specific POC assays

<table>
<thead>
<tr>
<th></th>
<th>CoaguloMed</th>
<th>PeroSphere</th>
<th>XaTech</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Detection Technology</td>
<td>Fluorescence Microscopy</td>
<td>IR Spectroscopy</td>
<td>Dielectric Spectroscopy</td>
</tr>
<tr>
<td>Test Time</td>
<td>10 min</td>
<td>&lt;5 min</td>
<td>15-30 min</td>
</tr>
<tr>
<td>Advanced Analytics/Data Processing</td>
<td>Multivariate Regression Modeling</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Reagents Requirement</td>
<td>Fluorescent Labels, ‘Agonist A’, ‘Agonist B’, Calcium</td>
<td>Calcium or No additives</td>
<td>Calcium</td>
</tr>
<tr>
<td>Differentiation of Xa and IIa inhibitors</td>
<td>w/ upstream and downstream activators (agonist ‘A’ and ‘B’)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Blood product compatibility</td>
<td>Whole blood or Plasma</td>
<td>Whole blood or Plasma</td>
<td>Whole Blood</td>
</tr>
<tr>
<td>Platelet Diagnostic</td>
<td>No, no detection</td>
<td>No, No detection</td>
<td>Partial, inferential detection</td>
</tr>
<tr>
<td>Anti-coagulant</td>
<td>Citrate</td>
<td>Citrate or None</td>
<td>Citrate</td>
</tr>
<tr>
<td>Form Factor</td>
<td>Handheld</td>
<td>Handheld</td>
<td>Handheld</td>
</tr>
<tr>
<td>Extensibility to new analytes</td>
<td>Extensible, but no platelet receptors</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Clarity of metrics</td>
<td>FI scales with fibrin clot size, Clot time is intuitive, Clot score is highly manipulated</td>
<td>Clot time intuitive, IR absorbance peak and decay behavior abstract.</td>
<td>Permittivity behavior abstract. Platelet behavior unintuitive.</td>
</tr>
<tr>
<td>Quantification or Presence only</td>
<td>Presence</td>
<td>Presence</td>
<td>Presence</td>
</tr>
</tbody>
</table>
CHAPTER 2 : ADAPTING PDMS MICROFLUIDIC DEVICES FOR USE IN POINT OF CARE APPLICATIONS

2.1 Introduction

The monitoring of blood coagulation, anti-platelet therapy, anticoagulation therapy, hemophilia therapy, surgical bleeding, trauma bleeding, and platelet function form the basis of a very large (> $1 billion) diagnostics market. Few devices or systems exist on the market for monitoring blood under flow conditions analogous to those that exist within the human body and those that do have limited utility and only operate in the absence of full coagulation that includes thrombin and fibrin generation. Microfluidic assays based on PDMS soft lithography have offered solutions in each of these fields while retaining physiological flow and full coagulation biology, but suffer from several limitations which preclude their straightforward application clinical monitoring [25–27,52–54].

Generally, microfluidic devices are designed for functionality and ease of fabrication, rather than ease of use – e.g., inlet wells being arranged in a semicircular fashion, rather than being aligned linearly for use with a multichannel pipette. Further, these devices typically need to be passivated in some way to prevent off-target platelet and coagulation activation, typically with bovine serum albumin (BSA). In some devices, this process can be quite inefficient and require multiple steps: 1) inlet wells are filled with buffer one at a time, 2) buffer is drawn through the device through the outlet, and 3) excess buffer from each inlet wells is removed manually prior to blood addition – a process which scales with the multiplexing of the experiment. In addition, fluid delivery for these assays is often performed by either gravitational pressure head or withdrawal
through a syringe pre-filled with water using a syringe pump, with both methods requiring significant set-up time and some training to execute correctly [26,32,55]. A final consideration is that for many parallelization strategies in microfluidics, several distinct inlet conditions are combined at the outlet after the area of detection for streamlined fluid handling [26,32]. This allows the coagulation processes to occur semi-independently for each experimental condition, without requiring dedicated fluid driving for each one. This approach has inherent limitations, however, as there can be significant cross-talk from one channel to another as clots approach occlusion and their fluidic resistance increase sharply. Flow can then be diverted to certain experimental conditions (the least occluded), changing the shear rate and altering the nature of the experiment. In this way, if parallelization of channels can be achieved while avoiding this cross-talk, experiments could be run longer and with complete independence of the experimental conditions. The opportunity exists, therefore, for a microfluidic device specifically designed for use by untrained personnel to provide unique insight on platelet function and coagulation biology in clinical settings.

2.2 Materials and Methods

2.2.1. Device design and flow simulation

A previously published eight channel microfluidic device was used as a basis for channel dimensions (250 μM x 60 μM) and focal imaging region design, while the footprint was modified substantially [26]. Inlet wells were re-arranged to a linear orientation with 96-well plate standardized spacing (9 mm) such that a multichannel pipette can fill each one simultaneously. For the original, design the semi-circle orientation was selected such that the length of channel between the wells and the focal
region, and therefore fluidic resistance, for each of the 8-channels was identical. This allows uniform flow in each channel for fluid delivery mediated by a single pressure source at the outlet, as verified by relative bead populations measured by flow cytometry [26]. To recapitulate this uniform flow with the linearly arranged inlet wells, the microfluidic resistance (Eqn. 2.1) was again held constant between the inlet well and the converged focal regions, where the channels are oriented in parallel and have a spacing of 125 μm.

Eqn. 2.1

\[
R = \frac{12 \mu L}{wh^3 \left[ 1 - \frac{192}{\pi^5} \sum_{n=1,3,5,...}^{\infty} \tanh\left( \frac{n \pi w}{2h} \right) \frac{n^5}{n^5} \right]}
\]

In order to prevent variations in the platelet margination or red blood cell concentration via Farhaeus effect, and avoid excessive shears that may activate platelets, the cross-section of each channel retained as close to constant as obtainable, such that wherever possible, additional length was added for the interior channels via circuitous paths, and altering of channel width was done sparingly.

Previously designed microfluidic devices have been structured with all inlet wells converging to a single outlet well, for ease of fluid handling. With this arrangement, however the channels of interest act as parallel flow paths, meaning that the flow rate in one of the channels is affected by changes in resistance to the other channels, for a fixed total flow rate as shown in Equation 2.2 where, \( R \) is the hydraulic resistance of a channel and \( Q \) is volumetric flow rate [56]. To reduce the potential for fluidic cross-talk between channels via changes in resistance as clots reach occlusion, the single outlet of the previous design was replaced with individual outlets, each emptying into a single collection reservoir open to atmosphere on the device’s surface. In addition, a PDMS
manifold was built around each of the inlet wells, allowing positive pressure from a single source to address each simultaneously.

\[
Q_n = \frac{1}{\frac{1}{R_n} + \frac{1}{R_1} + \cdots + \frac{1}{R_N}} Q_{\text{total}}
\]

Eqn. 2.2

To address the need for more streamlined process for device passivization by pre-filling with a protein solution (priming), an additional inlet port was added to the device flow path, in fluidic communication with both the inlets and outlets. Also, for each channel, a small region connecting the end of the converged focal region to the outlet reservoir was added, with a smaller channel width than the rest of the device such that the resistance (Eqn 2.1) for this small segment was equal to the resistance of the full width (250 μM) up to this point. The flow path from the additional priming inlet splits and branches to each channel at the point where the full width channel meets the condensed width channel; in this way fluid injected through the priming inlet reaches the outlet reservoir and in the inlet well at the same time without accumulating an excess of priming fluid in either location. BSA (0.5% in HEPES-buffered saline pH=7.4) was used as priming buffer for all experiments. Steady state flow simulations (COMSOL 5.3a) were performed to verify uniformity of flow rates in each of the channels and to verify wall shear rates in the focal region. Aqueous Food-grade dye was used for visualization and confirmation of priming procedures.

2.2.2. Blood Collection and labeling

Whole blood was obtained from healthy individuals via venipuncture into direct thrombin inhibitor PPACK to a final concentration of 100 μM. Informed consent was
obtained for each volunteer and all procedures were performed in accordance with IRB approval from the University of Pennsylvania’s Office of Regulatory Affairs and the Declaration of Helsinki. Platelets were labeled with PE-conjugated mouse anti-human CD61 (BD). For in vitro addition of acetylsalicylic acid (aspirin/ASA), crystalline solid was dissolved in DMSO at 500 mM, diluted in HBS, and then finally further diluted into blood for final concentrations of 50 and 500 μM.

2.2.3. Device Fabrication and Imaging

Microfluidic devices were fabricated using poly(dimethylsiloxane) (Ellsworth Adhesives) from silicon masters, made using KMPR (MicroChem Corp.) negative photoresist, spincoated to 60 μm on 100 mm wafers (Pure Wafer). Inlet and outlet ports were made using 0.5 mm ID biopsy punches. Additional PDMS retaining walls were added to both the inlet region and out region to act as reservoirs, made by cutting a rectangular hole within a slab of PDMS (Figure 2.1). For the inlet side, a single slab of PDMS was then placed on top of the retaining walls with to make a pressure seal such that a positive pressure regulator (Fluigent), hooked up to a diaphragm pump could apply a single pressure to all inlet wells simultaneously by being inserted into a biopsy punch through-hole through the slab (Figure 2.1). Pressure was calibrated such that an initial flow rate of 1 μL/min (γ= 100 s⁻¹) was obtained (7.5 mmHg). For the outlet side, the reservoir was used to collected blood as exits from the outlets of each channel. A luer-lock check valve (Qosina) was attached to the device at the priming inlet using a 23 gauge blunt needle with plastic hub. Channels were spaced 125 μm apart for the focal region such that two at a time could fit within the field of view of 10x objective for an inverted epifluorescence microscope (IX81, Olympus). Type-1 equine fibrillar collagen
(Chronolog) was applied in a 250 μm strip using a single channel PDMS patterning device on a glass slide treated with SigmaCote (Sigma Aldrich). Channels were pre-filled prior to experiments with a 0.5 w% BSA in HBS solution in order to passivate all surfaces.

2.3 Results

2.3.1. Device Design and Validation

A re-oriented 8-channel PDMS device was successfully designed with inlet wells arranged linearly with the spacing of a 96 well plate (9 mm) (Figure 2.2). Some reductions in channel width were necessary in order to keep the entire footprint of the device as small as possible; the specific dimensions used for each channel segment are shown in Figure 2.2B, where only four channels are displayed due to device symmetry. A dedicated priming port was added such that priming fluid can be injected through a

Figure 2.1: Pressure control and fluid perfusion in 8 by 1 PDMS device

Top View (A) and Side View (B) of fully assembled PDMS device. A sealed air head above the wells is pressurized using a diaphragm pump and Fluigent pressure controller, connected to the device with a 23 gauge needle slotted into a biopsy punched thru-hole in the PDMS slab.
check valve to fill the device with BSA solution, where the priming fluid conduit thereafter behaves a ‘dead end’ for the experiment (Figure 2.3) Flow rate uniformity was verified by COMSOL steady-state 2D laminar flow simulations, yielding equivalent velocity profiles in each of the 8 channels for a fixed pressure at each inlet of 15 mmHg, confirming that the fluidic resistance in each channel was identical (Figure 2.4).

Figure 2.2: 8 by 1 PDMS device channel schematic
Flow path scheme and back prime approach (A), Fluid is injected into the devices through the back-priming port which fills the channels both to the inlet and outlet (red). Blood is added to the inlet wells and displaces the priming solution to the outlet, but not towards the check valve. (B) Dimensions of the each channel (channels are symmetric about center line), where fluidic resistance was kept constant to keep flow rates and shear rates constant over the reaction zone identical.
Figure 2.3: 8 by 1 PDMS device priming
Visualization of priming process. An empty device (A) is filled with priming fluid (blue food coloring) through the priming port with check valve using luer lock plastic syringe. The filled device has fluid advance up to the inlets and outlets simultaneously to prevent waste (B).

Figure 2.4: COMSOL Simulation for velocity in 8 by 1 PDMS device
Steady state laminar flow COMSOL simulation results for velocity profile, given 15 mmHg pressure at all inlets and 0 mmHg pressure at all outlets. Uniform coloring for each channel indicates equivalent flow and equivalent shear rates, suggesting hemodynamic conditions for each channel are identical. Check valve was modeled as a no-transport wall condition.
2.3.2. Whole Blood Experiments

As a proof of concept, clotting behavior of healthy donor blood was verified for venous shear rates. Perfusion was driven through all $n = 8$ channels simultaneously by application of positive pressure (7.5 mmHg) at the inlet wells. Uniform behavior was observed over all 8 channels, with channel occlusion occurring at approximately 600 seconds in each Figure 2.5. No obvious differences in clot morphology were observed compared to clots formed under flow driven by fluid withdrawal via syringe pump. Consistent morphology across this chip suggested to major differences in shear rate in each channel.

![Figure 2.5: Whole blood clotting using in-line PDMS device](image)

Montage of fluorescence images for PE-anti-human CD61 stained platelets, where three time points are shown for each channel for N=1 donor (A). Average fluorescence intensity across device versus time (B), shows characteristic behavior for PPACK anticoagulated blood.

To investigate the ability of the newly designed device to detect drug action, two different concentrations of aspirin were evaluated; 50 and 500 μM. A dose-dependent reduction in platelet fluorescence intensity was observed for an initial wall shear rate of 100 s$^{-1}$ 7.5 mmHg Figure 2.6. This suggests the after primary aggregation via exposure to the glass-bound collagen fibrils, platelets were more insensitive to the secondary
agonist TXA₂ reducing to the total accumulation of platelets. Results are qualitatively consistent with dose response data collected using the previous 8-channel device design with syringe pump withdrawal for fluid delivery [27].

Figure 2.6: Aspirin Effect on platelets in PPACK blood
Dose response for N=1 donor for acetylsalicylic acid (Aspirin) added from DMSO solution to blood ex vivo, perfused at 7.5 mmHg, or approximately 100 s⁻¹ initial. Results consistent with previously published results from original 8-channel device with perfusion performed by syringe pump.
2.4 Discussion

The development of in vitro microfluidic assays has enabled studies on various aspects of hemostasis and thrombosis, including evaluation of disease states, geometric pathologies, drug action, and many others [27,30,31,57]. In cases where samples of interest must be obtained outside the lab, as in the case of patient studies, the need to either transport pre-prepared microfluidic devices or the blood itself causes logistical difficulties in terms of timing and stability. Devices that are amenable to use on-site in any location and by untrained personnel would therefore present a significant improvement in this regard. The device described in this work addresses several of the major difficulties in operation of PDMS microfluidic devices, while retaining their general utility.

A critical step in the operation of PDMS microfluidic devices for hemostasis testing is the pre-filling of the devices with buffer designed to both passivate the wetted materials against platelet adhesion and contact activation, and to ensure smooth perfusion of blood through the devices without the pinning of air bubbles. In some devices, this is done by manually pipetting a BSA buffer into inlet wells, drawing through the outlet with a syringe, and then removing the excess from the inlet wells prior to blood addition. This is both labor intensive and prone to significant error, as PDMS in relatively hydrophobic and will tend to pin large bubbles in the corners of inlet wells. Further, the number of steps in the procedure scales with the number of channels, making a translation of this paradigm to next generation devices more and more problematic. The newly designed, in-line 8-channel PDMS device addresses this issue by addition of a dedicated priming line, making the process facile and one-step. Volume of priming buffer can be allocated such that no excess is accumulated in the inlet wells, eliminating the
need for removal of excess fluid from the inlet wells. Another area of improvement for the new design is liquid handling for transferring blood into the device. Rather than requiring independent pipette actions for each of the channels, arrangement of the inlet wells along a single line with 9 mm spacing enables the use of a multi-channel pipette to fill all 8 wells simultaneously. This both streamlines the process and reduces the likelihood of bubble entrapment.

Several aspects of the device described in this work are novel, and their properties are captured in patent application US62/645,525, included in its entirety in Appendix II.
3.1 Introduction

One of the key functions of blood in the human body is to maintain hemostasis—that is, to maintain circulation and arrest leakage at the site of an injury when it occurs. An injury event exposes tissue factor and collagen from the vessel that stimulate clot formation over the damaged area. This is achieved by the activation and accumulation of platelets into a plug, and the formation of a mesh of fibrin generated from the polymerization of the circulating plasma protein fibrinogen, which is carried out by the coagulation cascade. In order to monitor and assess this function of blood, various assays exist that detect either the presence or action of either platelets or fibrin or both.

Over the last decade, microfluidic assays have proven to be an effective diagnostic tool for assessing platelet function and coagulation, creating relevant flow hemodynamics, while utilizing a minimal volume of blood [24–27,29,31,58–63]. Polydimethylsiloxane (PDMS) is a popular choice for devices in these assays due to the speed and ease of fabrication, re-usability, and fidelity to the design geometry [64,65]. PDMS enables the replication of flow channel geometries on the scale of tens of micrometers, allowing physiological shear rates while utilizing only microliters of blood. Flow parallelization, as demonstrated in the previously developed eight-channel microfluidic device, allows for testing varied experimental conditions simultaneously, such as dose–response testing of platelet or coagulation inhibitors in a single donor blood sample [26,27,53,66]. More specialized single-thrombus devices have also been
demonstrated, which incorporate trans-thrombus transport into the assay [29,31,61]. The versatility of the microfluidic platform allows for variations in the particular thrombogenic surfaces and flow delivery methods used in each assay, providing more complete recreation of thrombotic and hemostatic processes [24,59,61,62,67,68].

Although PDMS devices are reliable and easy to construct, they are typically only transiently sealed, often with vacuum or clamping, and need to be assembled prior to each use. Several studies have been performed with blood samples from clinical cohorts, but these experiments require a highly trained individual to perform them. Challenges associated with preparing devices ahead of time complicates experimental timing and typically necessitates the use of strong anti-coagulants [25,27,63,69]. Furthermore, traditional soft lithography methods for producing PDMS devices from silicon masters do not have an obvious method of production scale-up. Although PDMS microfluidic devices offer significant design freedom and enable novel observations in thrombosis and hemostasis research, their application to the clinical environment remains an existing challenge.

Several point-of-care (POC) diagnostic techniques exist for assessing patient anti-coagulation status and platelet function, but many have very specific readouts, and none have demonstrated themselves as the clinical standard for hemostasis diagnostics. Thromboelastography and rotational thromboelastometry (TEG/ROTEM) are both POC viscoelastic tests that rapidly produce data from whole blood samples over a 30–60 min test [70]. Viscoelastic methods emerged over 50 years ago and are beginning to find use in the field of trauma surgery. Viscoelastic methods monitor a whole blood clot but do not capture the impact of flow in a physiological way, due to their closed-system nature. Furthermore, viscoelastic measurements do not detect specific molecular entities
(platelet receptors, fibrin) and only recently entered the stage of POC use [15,70]. The Platelet Function Analyzer 100 (PFA-100) is a microfluidic assay that uses pressure sensors to detect the occlusion of an aperture coated with collagen and adenosine diphosphate (ADP), with the time to reach occlusion as the primary metric. This test is limited to the platelet function, however, and operates at very high shear rates (>5000 s\(^{-1}\)), limiting its ability to capture physiological flow conditions and making the assay quite sensitive to variations in the von Willebrand factor (VWF) [71]. A second microfluidic technique that has been under recent investigation is the total thrombus formation analysis system (T-TAS). This assay has the advantage of operating at physiological shear rates and in an open system, but generates only a single clot per chip, has shown a poor correlation with direct oral anti-coagulant (DOAC) doses, and has poor agreement with the more established thromboelastographic methods [71,72].

This study used validated flow experiments and reagents from PDMS microfluidic techniques and deployed an injection molded chip to create a versatile technology for medical professionals at the bedside [24–26,60,69]. A POC microfluidic device needs to (1) utilize small volumes (<1 ml) of whole blood, (2) have a scalable production method, (3) be simple to prime and operate by non-expert users, (4) retain uniform flow behavior across all test conditions, (5) be storage stable in a prepared state, and (6) have a total experimental footprint suitable for benchtop use. Thus, by retaining the critical geometric parameters of prior PDMS devices, but manufacturing the parts via injection molding, and streamlining the operational procedures, a new microfluidic device serves clinical studies in thrombosis and hemostasis testing (1). Stable sealing and stable reagents are also required [73,74]. We demonstrate a storage stable device that can be rapidly used
with a fresh whole blood sample in a multiplexed eight-channel readout of platelet and fibrin function.

### 3.2 Materials and Methods

#### 3.2.1. Device design and flow simulation

A previously published eight-channel microfluidic design deployed in PDMS devices was translated to fit on a 75.5 mm by 25.5 mm standard microscope slide footprint, to facilitate translation to an injection molding platform Figure 3.1A. SolidWorks 2018 (Dassault Systèmes) was used to generate a full 3D model of the device flow paths. Steady state flow simulations (COMSOL 5.3a) were performed to verify uniformity of flow rates in each of the channels and to verify wall shear rates (Newtonian viscosity = 4 cP). To experimentally verify flow rate uniformity, solutions of FITC-conjugated flow cytometry beads (Spherotech) with four distinct peaks were added symmetrically to each of the four wells on both sides of the chip. The bead solutions were perfused through the device for 10 min and the effluent was collected and analyzed on an Accuri C6 flow cytometer (BD Biosciences). These results were compared with a uniformly pre-mixed control sample of all bead populations. Additional simulations were performed to monitor flow rate, pressure drop, and wall shear rate as a simulated clot formed and reduced the channel cross section.
3.2.2. Device preparation and flow control

Experimental chips were assembled from pre-made, injection-molded cyclic-olefin-copolymer plastic components (ChipShop) with flow-paths molded into them as rectangular troughs. Type I equine fibrillar collagen (Chrono-Log) and lipidated tissue factor (TF) (Siemens) were coated as a 250 μm wide strip to a sheet of an optically clear substrate with an adhesive (ThorLabs), using a PDMS patterning device as previously...
described [26]. The adhesive sheet was then used to form the fourth wall of the rectangular cross section (Supplemental Figure 1). Blood was perfused through the completed chips by applying a negative pressure at the outlet using a KPV14A miniature diaphragm vacuum pump (Koge Electronics) with a USB-controlled pressure controller (Fluigent). The pressure was calibrated to yield a centerline velocity of 0.004 m/s and an initial flow rate of 14 μl/min, corresponding to an initial wall shear rate of 200 s$^{-1}$. The effluent blood from the device was captured in a liquid trap with a custom tubing adapter (Fluigent).

3.2.3. **Blood collection**

Blood was collected from consenting healthy donors self-reporting as free of any medications for at least 10 days. Blood was drawn via venipuncture into the corn trypsin inhibitor (CTI, Haematologic Technologies) to a final concentration of 4 mg/dl (40 μg/ml), in order to prevent Factor XIIa activation prior to the experiment, while allowing tissue factor-initiated thrombin generation in the surface test region of coated collagen/TF. All procedures were conducted in accordance with protocols approved by the University of Pennsylvania’s Institutional Review Board and the Declaration of Helsinki.

3.2.4. **Platelet and fibrin detection during clotting**

Platelets were labeled in whole blood by the addition of a non-function blocking, AlexaFluor 488-conjugated anti-CD61 molecule (Bio-Rad Laboratories), while fibrin was quantified by the addition of AlexaFluor 594-conjugated human fibrinogen (Life Technologies). Chips were mounted on a three-color, LED-based Lumascope 620 (Etaluma) benchtop epifluorescence microscope with a 2.5× objective and primed with
150 $\mu l$ of a solution 0.5% bovine serum albumin (BSA) in HEPES-buffered saline (HBS). A volume of 35 $\mu l$ of blood was added to each of the eight inlet wells, with a negative pressure applied to the single outlet to initiate flow at $t = 0$. Fluorescence images were captured at 10 s intervals for green and red LED channels to obtain kinetic data for platelet and fibrin accumulation. Image sequences were analyzed with ImageJ to calculate the average pixel brightness of a 200 $\mu m$ by 200 $\mu m$ region center within each clot zone, in order to exclude sidewall effects of the rectangular channels.

3.2.5. Stability testing

In order to assess the ability of collagen and tissue factor reagents to retain activity over time, chips were prepared as described and then stored in a 4 °C refrigerator in an air-tight chamber with a desiccant. After 6 months, chips were removed from the chamber and tested in the microfluidic assay. For comparison, a second device prepared immediately before use was also tested with the same blood sample.

3.2.6. DOAC testing

To test the effects of DOACs added ex vivo, dry rivaroxaban or apixaban (SelleckChem) were dissolved in DMSO (20 mM stock solution) and diluted to 100 $\mu M$ in HBS, then further diluted for final concentrations of 0.05, 0.2, 0.5, and 5 $\mu M$ after the addition of 90 $\mu l$ of whole blood in separate microcentrifuge tubes. HBS was added to each tube such that the total volume of drug solution and additional buffer was 10 $\mu l$ in each, to ensure the hematocrit remained consistent across all conditions and whole blood composed 90% of the final volume.
To compare the Xa inhibitors with a direct thrombin inhibitor, Dabigatran (active form) (SelleckChem), dry powder was dissolved in a solution of 10% trifluoroacetic acid in de-ionized water, to an initial concentration of 40 mM. This stock was then diluted to 100 μM in HBS and again then further diluted for final concentrations of 0.05, 0.2, 0.5, and 5 μM after the addition of 90 μl of whole blood in separate microcentrifuge tubes. The platelet and fibrin accumulation assay described above was then performed for each DOAC, with one well of the control condition (no drug), one well of 5 μM drug, and two wells each for the remaining concentrations. Chips were run immediately after DOAC dosing of blood.

To evaluate the extent that DOAC reversal can be observed using this assay, Andexanet Alfa (Portola Pharmaceuticals) was added to whole blood containing Apixaban. Samples were prepared for 0.02 μM Apixaban, 0.02 μM Apixaban with a 1:1 molar ratio of Andexanet Alfa, and 0.02 μM Apixaban with a 1:5 molar ratio. For each chip, two wells each were allocated for the control condition (no drug, no reversal agent), and two each of the aforementioned drug combinations. Chips were run immediately after dosing of blood.

3.2.7. Statistical analysis

Statistical significance was calculated for an unpaired t-test, using GraphPad Prism (GraphPad Software). Shaded error regions on plots represent the standard deviation from the mean. Coefficients of variation (CV) are reported for the 400 s time points in each experiment. Concentrations for 50% inhibition (IC\textsubscript{50}) were calculated using the t = 400 s time point for each concentration, with the “log(inhibitor) vs response” 3-
3.3 Results

3.3.1. Injection-molded, disposable chip

A previously described eight-channel PDMS device was successfully converted to an injection molded 1 × 3 in.² microscope slide footprint. The 60 μm height × 250 μm width of each channel was retained from the original device. The inlet well locations were altered to be linearly oriented, with 96-well spacing for convenient pipetting. Serpentine elements were added to the flow-paths such that the fluidic resistance of each channel was identical, ensuring that the flow rate through each channel was identical (Figure 3.1A). The final chip (up to 5 chips at a time) was bonded with manual pressure (single pass with moderate pressure) applied by “ink” roller, a processing step highly amenable to roller automation. Failure of bonding indicated by fluid leakage was never observed in manufacturing runs up to 100 chips. An additional port was added to the design for back-priming of the device with 0.5% BSA for the passivation of the wetted surfaces to minimize FXIIa generation and platelet adhesion (Figure 3.1B). Extra flow resistance was added to the converged outlet channel such that priming fluid was evenly split toward the inlet channels and the single outlet port. Priming was achieved by injecting 150 μl priming fluid with a 1 ml plastic syringe through a luer check valve (Supplemental Figure 1).
3.3.2. Flow simulations

Uniformity of flow was verified by both simulation (Supplemental Figure 2) and experiment (Supplemental Figure 3), ensuring identical hemodynamics in each lane. In order to characterize the changes to the flow as experiments progress for a new fluid delivery method, COMSOL simulations were performed for an inlet pressure of 16 000 Pa (120 mmHg) over each inlet and an outlet pressure of 0 Pa (atmosphere) in order to match the total ΔP to the negative pressure typically applied to the outlet in experiments (160 mbar vacuum). Pressure was maintained as constant, as the hydrostatic pressure is a negligible contribution (~20 Pa) and, therefore, changes in inlet well height do not alter the value meaningfully. Furthermore, the reservoir hydrostatic head never drove blood flow spontaneously in the primed chip prior to operation. Blood was modeled as a Newtonian fluid with a density of 1060 kg/m$^3$ and a viscosity of 4 cP, as blood's non-Newtonian properties are considered unimportant for shear rates in excess of 100 s$^{-1}$[75]. The impact of thrombus growth on the overall flow was modeled by enforcing a reduction in the lumen area of the channel over the 250 μM × 250 μM collagen-intersection region in discrete steps and running a separate flow simulation for each thrombus height (Figure 3.2A). This height restriction was represented as an occlusion fraction between 0 and 1. The flow rate, pressure drop across the clot region, and wall shear rate at the top surface of the thrombus were calculated for occlusion fraction (Figure 3.2B and C). In all experiments, clot height changes during the experiment if platelets are functional, causing the fluidics to evolve. When full occlusion is reached, this corresponds with platelet signal reaching its maximal value and, in this way, the instantaneous clot height relative to the channel height can be interpreted by the fluorescence intensity in proportion to the maximal value [61]. Although experiments for
this study were not run to full occlusion, this value has been observed as \( \sim 60 \) A.U. for the lighting conditions and label concentrations used in this work.

For experiments with <70% occlusion (\( \sim 70\% \) maximal platelet FI), the pressure drop is essentially constant, as is the flow rate, however, the wall shear rate increases as shown in Figure 3.2. As the clotting event approaches full occlusion, the wall shear rate on the exposed surface of the clot becomes pathological reaching 20 000 s\(^{-1}\), a shear rate sufficient to extend and deposit von Willebrand factor (VWF) and mediate shear induced platelet activation (Figure 3.2D) [61,76].

**Figure 3.2: Flow Simulations for injection molded chip**

(A) 2D side view of the focal region of a single channel from a 3D model of chip. (B) Volumetric flow rate as a function of occlusion fraction \((h/60)\), for a constant outlet vacuum pressure. (C) Pressure drop across the clot as a function of occlusion fraction. (D) Shear rate at the top surface of the clot as a function of clot height.
3.3.3. Healthy whole blood clotting on collagen and tissue factor

In order to establish the baseline performance of the newly designed device, whole blood from N = 10 healthy donors was perfused through devices prepared as described at 200 s$^{-1}$, with all n = 8 channels running simultaneously (Figure 3.3A and B). For the 10 healthy donors (8 male), the platelet signal measured by image analysis of immunofluorescence showed an average interdonor CV of 30%, with the fibrin signal displaying a CV of 22%. No differences were observed between the platelet or fibrin signals and the specific inlet well from which the blood originated, verifying that the flow in each channel was identical.

![Diagram](image)

**Figure 3.3: Healthy Donor for injection molded chip**
Average platelet intensity (a) and fibrin intensity (b) vs time for N = 10 donors, and n = 8 clots per donor for a constant outlet pressure yielding an initial wall shear rate of 200 s$^{-1}$. (c) Fluorescence microscopy images for platelets (AF488 CD-61) and fibrin (AF594-conjugated human fibrinogen) at t = 400 s.
3.3.4. Chip stability

Prefabricated microfluidic chips retained activity when stored at 4 °C for 6 months in dry conditions when tested at the same 200 s⁻¹ venous condition as previously described. Both platelet and fibrin signal showed similar response when compared to a chip prepared immediately prior to use (NS, p > 0.05), indicating minimal degradation of the collagen or tissue factor reagents during the storage period (Figure 3.4). Interestingly, chips stored at room temperature showed significant reduction in the platelet signal after less than one week, likely indicating loss activity in the GPVI binding domains of the collagen.

![Figure 3.4: Chip storage stability at 4 °C](image)

Comparison of a chip used immediately after preparation (day 1) and a chip used 6 months after preparation (stored at 4 degrees Celsius in a desiccated chamber) for platelet (a) and fibrin (b) intensity. Data for N = 1 donor, and n = 8 clots per chip, where blood is perfused with constant outlet pressure yielding an initial wall shear rate of 200 s⁻¹. No significance was detected in the fresh vs stored chips for either platelets or fibrin (p > 0.05)
3.3.5. **DOAC detection**

To assess the ability of our assay to determine the anti-coagulation status of blood sample, the platelet and fibrin accumulation assay was run at four concentrations of rivaroxaban. For \( N = 3 \) healthy donors, a strong dose dependent response in fibrin signal was observed for each DOAC added to blood *ex vivo*, yielding an IC\(_{50}\) of 120 nM for rivaroxaban and apixaban, and 60 nM for dabigatran [Figure 3.5C,F, Figure 3.6C]. The lowest concentrations of rivaroxaban, apixaban, and dabigatran (50 nM) all showed a statistically significant difference in fibrin signal at 500 s (\( p < 0.01 \)). The platelet deposition signal had minimal correlation with drug concentration, indicating that in this assay, collagen activation of platelet GPVI dominated the platelet deposition.

![Figure 3.5: Xa inhibitor Dose Responses in healthy donors](image)

Fluorescence data for whole blood treated with Factor Xa inhibitors rivaroxaban (a)–(c) and apixaban (d)–(f), showing limited platelet response (a) but a strong dose-dependent response for fibrin signal (b). (c) and (f) Log[inhibitor] vs fibrin intensity indicating an IC\(_{50}\) of 120 nM for both inhibitors in this assay. A statistically significant difference in fibrin signal was observed at 500 s for both drugs (*\( p < 0.01 \)).
Dabigatran was significantly more potent than either of the factor Xa inhibitors in these assays for fibrin signal (Figure 3.6). Controls run with TFA alone confirmed that this difference was not due to the action of the vehicle solvent but rather the drug itself.

Platelet signal was similarly limited in response to drug concentration for each DOAC.

Figure 3.6: Dabigatran Dose response
Fluorescence data for whole blood treated with Factor IIa inhibitor Dabigatran, showing limited platelet response (a) but a strong dose-dependent response for fibrin signal (b), and micrographs of the same at t = 400 s (d). (c) Log[inhibitor] vs fibrin intensity indicating an IC50 of 60 nM in this assay. A statistically significant difference in fibrin signal was observed at 500 s (*p < 0.01).
3.3.6. DOAC reversal

To evaluate the ability of the assay to detect reversal of DOAC drug action in whole blood, platelet and fibrin accumulation assays were run for three combinations of apixaban and the mutant inactive factor Xa molecule Andexanet Alfa. For N = 3 healthy donors, the anticipated ~50% inhibition of fibrin signal was observed for 0.2 μM apixaban alone, which was completely reversed to control behavior by an equimolar ratio of Andexanet Alfa [Figure 3.7B]. A 1:5 ratio of Andexanet Alfa to apixaban yielded partial restoration of fibrin signal. For all combinations of drugs, no clear trend was observed in the platelet signal [Figure 3.7A].

Figure 3.7: Reversal Data for Xa-inhibitors added in vitro with Andexanet Alfa

Fluorescence data for whole blood treated with 200 nM Factor Xa inhibitor apixaban with and without mutant inactive factor Xa molecule Andexanet Alfa (Andexxa) as a reversal agent. Platelets (a) show no dependence on either drug or reversal agent, whereas fibrin (b) is partially inhibited by apixaban, but is recovered completely by an equimolar ratio of Andexanet Alfa, and partially recovered by 1:5 deficit of Andexanet Alfa.
3.4 Discussion

Custom microfluidic devices are widely used in thrombosis/hemostasis research laboratories for the interrogation of small blood samples under diverse hemodynamic flows and real time multi-color imaging. PDMS devices have allowed the study of hemophiliac blood, neonatal blood, and trauma patient blood [25,69,77]. Additionally drugs such as COX inhibitors, P2Y12 inhibitors, reFVIIa, and polyphosphate inhibitors have been studied using microfluidics [25–27,53,60,66]. However, such PDMS devices are difficult to use in the emergency room (ER) or bedside point-of-care (POC) diagnostics due to obstacles in (a) manufacturing scale-up and (b) ease of operation.

Using only 250 $\mu$l of whole blood, this single use and disposable chip allowed eight independent clotting events to proceed simultaneously under venous shear rates, while monitoring platelet deposition and fibrin production over time. A chip design was developed to demonstrate (1) the ability to injection mold small features into plastic; (2) successful collagen/TF microprinting; (3) successful mechanical bonding of the chip layers; (4) successful priming fluidics; (5) collagen/TF integrity during bonding; (6) retention of collagen and tissue factor activity in a dry state at 4 °C; and (7) blood compatibility. Operation of the chip required eight-channel pipetting of blood into reagent wells (detectors, inhibitors, etc.) and then onto the chip, suitable for manual or automated pipetting.

Injection molding enables this chip design to be manufactured at scales of thousands per run, rather than the tens per day possible by PDMS replica molding, while maintaining a low cost per chip. By altering the flow delivery to a constant negative pressure applied through a dry tubing line, the assay preparation process has been simplified by removing the need for a prefilled syringe to mount in a syringe pump.
Furthermore, clots formed under a constant outlet pressure show reduced ballistic embolization of near-occlusive clots, compared to clots formed under constant flow rate perfusion.

Several alternative microfluidic sealing methods exist to create multilayer devices, such as ultrasonic welding, solvent bonding, and thermal bonding [78–80]. The need to retain the activity of biological reagents within the chip through sealing limits options, however, and was verified in this study. The use of collagen pre-patterned in a localized, ready-to-use dried state in this format offers a unique advantage to this new device. In other assays, alternative agonists such as adenosine diphosphate are used (TEG-6S) or collagen is typically added from a solution either stored refrigerated or frozen (aggregometry) [81]. The pressure sensitive adhesive used in this study provided a robust seal that held for >6 months and showed no observable off-target platelet adhesion or fibrin formation, when used with a passivating BSA priming solution. The new device retained uniform behavior channel to channel, as indicated by the 10% intrachip CV. In addition, manufacturing is scalable to mass manufacturing levels of $10^2–10^3$ chips per run.

More than $4 \times 10^6$ U.S. patients are currently prescribed DOACs for diseases such as atrial fibrillation (A-fib) and deep vein thrombosis (DVT) [37]. With increasing use of DOACs comes a corresponding increase in patients at risk of bleeding, making monitoring patients for bleeding risk an ever more critical task. There does not exist, however, a reliable, standardize laboratory method of monitoring the anticoagulation status for DOAC patients in clinical settings, a need that the assay described in this work may help address. Expected values for drug concentration ($C_{\text{max}}$ and $C_{\text{trough}}$) levels for patients on dabigatran (150 mg twice daily), rivaroxaban (20 mg once daily), and
Apixaban (5 mg twice daily) are known from plasma measurements, providing a range of interest for detection [82]. Typical rivaroxaban plasma concentration ranges between approximately 115 and 320 nM, depending on the dosage, a range that is within the detectable range of the described assay, based on the measured 120 nM IC$_{50}$ [83]. The IC$_{50}$ for rivaroxaban has previously been reported at lower values (21 nM) in other assays, by directly quantifying Factor Xa activity rather than correlating with fibrin signal, in assays carried out in plasma under static conditions [84]. The ISTH recommended maximum rivaroxaban concentration prior to urgent intervention is 30 ng/ml (~70 nM) for patients at risk of bleeding, where Factor Xa inhibitor reversal agents are recommended at all higher concentrations [85]. With 50 nM rivaroxaban detectable in the assay reported (corresponding to ~22 ng/ml), the device presented may be useful in quantifying DOACs to help assess patient risk relative to ISTH guidelines. Comparable detection ranges have been reported for the TEG-6s (30 ng/ml), but without the ability to run multiplexed assays to the same extent, the ability to independently monitor platelets and fibrin, and without physiological flow conditions [86]. With the high cost of Factor Xa reversal agents (>50 000 per full dose), the ability to provide fast, point-of-care measurements of a patient's current inhibition levels could potentially improve decision making for management of bleeding patients [87]. In addition, since the assay is quantifying fibrin rather than Xa activity, IIa inhibitors can be detected without modifying the assay, as shown in Figure 3.6, unlike in TEG assays.

In addition to patients on DOACs, traumatic injury patients present another case where monitoring current anticoagulation status is critical. Trauma is the leading cause of death in the U.S. and accounted for approximately $1.7 \times 10^8$ hospital visits a year for the period of 2000–2011 and can lead to platelet dysfunction and bleeding risk from
trauma induced coagulopathy (TIC) [88]. The ability to independently monitor platelet and fibrin signal may also help differentiate DOAC anticoagulation and trauma induced coagulopathy (TIC), as TIC is typically associated with platelet hypofunction as well as coagulation dysfunction, which has been previously observed in previous microfluidic assays [52,69]. Direct inhibition of Factor Xa, contrastingly, does not show a significant impact on platelet signal in this assay, except the highest concentrations—far in excess of therapeutic doses [Figure 3.5A and D]. Other currently utilized methods such as prothrombin time (PT) and activated partial-thromboplastin time (aPTT) are not able to differentiate DOAC anticoagulation and dysfunction due to TIC [87]. Although sensitivity to DOACs has been demonstrated in TEG, the single “amplitude” output signal similarly makes distinguishing between platelet and coagulation dysfunction more ambiguous without performing additional comparative experiments in the presence of platelet α₂β₃ inhibitors [89].

Detection of the action of a reversal agent in a DOAC measurement assay is critically important to distinguish drug-related anticoagulant behavior from disease or trauma associated coagulopathy. The ability of the microfluidic assay described in this work to detect the action of reversal agent on therapeutic dosages of a DOAC using less than 0.5 ml of blood, and ~1 μg of reversal agent could provide utility in bedside applications for bleeding risk assessment.

The device defined in this study allows rapid POC use for the multiplexed assay design (8 channels ×2 or 3 colors), ideal for blood phenotyping, drug dose–response, and risk assessment.
CHAPTER 4 : RAPID DETECTION OF DOACs IN DVT PATIENTS USING A SINGLE-USE MICROFLUIDIC CHIP UNDER WHOLE BLOOD FLOW

4.1 Introduction

Over the last decade since their inception, utilization of Direct Oral Anticoagulants (DOACs) targeting either thrombin (dabigatran) or factor Xa (apixaban, rivaroxaban, edoxaban) has grown significantly for indications such as deep vein thrombosis (DVT) and atrial fibrillation (Afib) [36,90,91]. Compared to the previously ubiquitous Vitamin K antagonists, DOACs have been shown to have a more rapid onset of action and reduced risk of adverse bleeding events such as intracranial hemorrhage, while having superior or equivalent endpoint efficacy, although in some cases an increased risk of less severe gastrointestinal bleeding has been reported [36,92–94]. Owing to these advantages and the lack of requirement for routine monitoring, DOAC prescription claims for diseases like Afib have increased significantly at the expense of vitamin K antagonists (VKA) such as warfarin [93,95,96].

Although routine monitoring is not required for DOAC use, particular clinical scenarios may require diagnostic information on DOAC action, such as traumatic injury or bleeding, perioperative management, possible drug interactions or overdose, or suspicion of patient non-adherence to dosing regimes [93,97]. In situations such as these, standard laboratory coagulation tests such as PT/INR and aPTT provide variable information based on the specific reagents and instruments used and are not suitable to make quantitative measurements; at trough plasma concentration levels, these tests can produce results which appear as ‘normal’ blood samples with no drug present [96,98,99]. Further, these assays cannot distinguish between thrombin and Xa inhibitors. Assays
specifically designed for these DOACs, such as calibrated anti-Xa assays, can provide greater insight and even quantitative information on drug concentrations, but are slow and not widely available. To date, only one test has been authorized by the FDA for measurement of DOACs, the HemosIL Liquid Anti-Xa test kit, for apixaban.

Recent years have seen an influx of new potential POC devices being developed, although none has shown clear superiority for detection of drug presence, or demonstrated quantitation of drug concentrations [49–51,100,101]. Therefore, the need for fast, cost effective methods to detect and quantify DOACs, particularly in emergency situations remains. To address these needs we have developed a whole blood microfluidic assay for detection of factor Xa inhibitors in DVT patients utilizing small amounts of Xa-inhibitor reversal agent (~0.5 μg). We evaluated the assay for detecting the presence of DOAC action, and also for quantitation of drug concentration and compared against LC/MS measurements of plasma samples from the same patients.

4.2 Materials and Methods

4.2.1. Device design

A previously published eight-channel design used for injection molded devices was reoriented such at all 8-channels were in a single line [102]. In order to maintain equivalent volumetric flow rates for fluid withdrawal through a single outlet, the fluidic resistance (Equation 4.1) was held constant for each of the 8 channels.

\[
R = \frac{12 \mu L}{wh^3 \left[ 1 - \frac{192}{\pi^5} \sum_{n=1,3,5,\ldots}^\infty \left( \frac{\tanh \left( \frac{n \pi w}{2h} \right)}{n^5} \right) \right]}
\]
In order to maintain the height and width of each channel as constants to avoid causing potential differences in platelet margination, hematocrit or shear rate rate in each of the channels, length was required to remain constant from each inlet well to the focal imaging region. Serpentine flow paths were added for the wells closest to the imaging region in order to maintain equivalent resistance (Supplemental Figure 4). 2D steady state flow simulations (COMSOL 5.3a) were performed to verify uniformity of flow rates in each of the channels.

4.2.2. Device preparation and flow control

Microfluidic chips were assembled from injection-molded cyclic-olefin-copolymer plastic components (ChipShop) as described previously [102]. Briefly, type I equine fibrillar collagen (Chrono-Log) and tissue factor (TF) (Siemens) were coated as a 250 μm strip to an acrylic adhesive substrate (ThorLabs). The adhesive sheet was then laminated to the injection molded part to complete the device. Blood was perfused through the completed chips by negative pressure at the outlet from a KPV14A miniature diaphragm vacuum pump (Koge Electronics), maintaining constant pressure with a controller (Fluiqent) operated via usb communication with a laptop. The pressure was calibrated with EDTA anticoagulated blood to yield an initial flow rate of 14 μl/min, corresponding to an initial wall shear rate of 200 s⁻¹. The effluent blood from the device was captured in a 15 mL centrifuge tube liquid trap with a custom tubing adapter (Fluiqent).
4.2.3. Blood collection

Blood was collected from either consenting healthy adult donors or DVT patients on either apixaban or rivaroxaban, in accordance with protocols approved by the University of Pennsylvania's Institutional Review Board and the Declaration of Helsinki. For patients, blood was drawn via venipuncture into untreated ‘no additive’ vacutainers (BD) and promptly transferred to 15 mL centrifuge tubes containing corn trypsin inhibitor, to a final concentration of 4 mg/dL (40 μg/mL). All patient blood was collected on site at the Hospital of University of Pennsylvania, and then transported prior to analysis, with approximately 10-20 minutes between collection and experimentation. For healthy donors, blood was collected into 3 mL luer-lock syringes containing CTI to a final concentration of 40 μg/mL.

4.2.4. Platelet and fibrin detection during clotting

Platelets were labeled in whole blood by the addition of a non-function blocking, AlexaFluor 488-conjugated anti-CD61 molecule (Bio-Rad Laboratories), while fibrin was quantified via addition of AlexaFluor 594-conjugated human fibrinogen (Life Technologies). Chips were mounted on a three-color, LED-based Lumascope 620 (Etaluma) benchtop epifluorescence microscope with a 2.5× objective, and primed with 150 μl of a solution 0.5% bovine serum albumin (BSA) in HEPES-buffered saline (HBS, 20 mM HEPES, 160 mM NaCl, pH 7.4). A volume of 40 μl of blood was added to each of the eight inlet wells, with a negative pressure applied to the single outlet to initiate flow at t = 0. Fluorescence images were captured at 10 s intervals for green and red LED channels to obtain kinetic data for platelet and fibrin accumulation for a total of 15 minutes. Image sequences were analyzed with ImageJ to calculate the average pixel
brightness of a 200 μm by 200 μm region center within each clot zone, in order to exclude sidewall effects of the rectangular channels.

4.2.5. DOAC reversal and detection

For N=3 healthy donors, Andexanet Alfa (AA, Portola pharmaceuticals) was added to a final concentration of either 50 or 300 nM, in order to assess the impact on blood without inhibitor present. For N=8 patients, in order to simulate patient blood without the presence of the anticoagulant, small amounts of AA were added to four of the eight wells for each patient: two wells with a final concentration of 300 μM and two wells with a final concentration of 50 μM (Supplemental Figure 5). All AA was stored as a 10 mg/mL solution in buffer at -80 °C and thawed and diluted as needed. To provide a ‘fully inhibited’ response, additional Xa inhibitor, either rivaroxaban or apixaban, to the match the drug already in the patient blood, was added to two of the channels for each patient. Rivaroxaban or apixaban (SelleckChem) were dissolved in DMSO (20 mM stock solution) and diluted to 100 μM in HBS, then added to a final concentration of 5000 nM to two of the wells. Finally, the remaining two wells were run as collected from the patient. For all wells, make-up HBS volume was added such that whole blood comprised exactly 90% of the final volume of the mixture, in order to ensure the hematocrit remained constant for each condition.

4.2.6. DOAC concentration prediction

In order to predict the concentration of DOAC in the patients systems, the relative fluorescence intensity of the patient’s unmodified blood and the high reversal agent condition were compared. These were then scaled to a previously measured dose.
response of the fibrin fluorescence intensity to apixaban and rivaroxaban added in vitro to healthy donor whole blood (Figure 3.5), according to Equation 4.2, where any signal in the 5000 nM anticoagulant condition is considered as purely background signal.

Eqn. 4.2

\[
FI_{\text{measured}} = \left( \frac{FI_{\text{patient}} - FI_{\text{HighAC}}}{FI_{300 \text{nM}} - FI_{\text{HighAC}}} \right) \times \left( FI_{\text{Dose Response max}} - FI_{\text{Dose Response Min}} \right) + FI_{\text{Dose Responsee Min}}
\]

The resulting \( FI_{\text{measured}} \) from eqn. 4.2 was set as the output of the 3 parameter Sigmoid fit of the IC50 curve from the in vitro dose response model of the relevant Xa inhibitor (Figure 3.5), of the form

Eqn. 4.3

\[
FI_{\text{measured}} = FI_{\text{Dose Response Min}} + \frac{\left( FI_{\text{Dose Response Max}} - FI_{\text{Dose Response Min}} \right)}{1 + \left( \frac{X}{IC\ 50} \right)}
\]

where a Hill slope of 1 is assumed, and solving for \( X \) yields the concentration that would give the \( FI_{\text{measured}} \) obtained for each patient, mapped to the in vitro scale.

4.2.7. Statistical analysis

Statistical significance was calculated by two-tailed t-test, using GraphPad Prism (GraphPad Software). Shaded error regions on plots represent the standard deviation from the mean. For evaluation of coefficients of variation, fluorescence intensity at 400 s was selected. Receiver operator characteristic curves were calculated using the ROC analysis tool in GraphPad Prism for a >95 confidence interval with the Wilson/Brown method. For each scatter dot plot, fluorescence intensity are reported for a single time-point from the 15 minute experiment; either the 500 second time-point, or at the time where the average of the 300 nM reversal agent condition was at 83% of its maximal
value over the full 15 minutes. Where indicated, data for each patient is normalized by the maximum value among the lanes where the 300 nM AA is used.

4.3 Results

4.3.1. Healthy whole blood clotting on collagen and tissue factor for new device design

To confirm the performance of the newly designed 8x1 chip, whole blood from $N = 5$ healthy donors was perfused through devices prepared as described above at 200 s$^{-1}$, where perfusion occurs through all $n = 8$ channels simultaneously by application of negative pressure at the outlet (Figure 4.1, A and B). Across all 5 healthy donors, the platelet signal measured by image analysis of immunofluorescence showed an average interdonor CV of 25%, with the fibrin signal displaying a CV of 20%. No differences were observed between the platelet or fibrin signals and the specific inlet well from which the blood originated, verifying that the flow in each channel was identical. For healthy donors with Andexanet Alfa added ex vivo, no effect on fibrin signal was observed at either concentration, suggesting there are no major off-target effects at the concentrations utilized in this work (Figure 4.2).
Figure 4.1: Healthy adult whole blood using 8 by 1 COC chip design

Average platelet intensity (A) and fibrin intensity (B) vs time for N = 5 donors, and n = 8 clots per donor for a constant outlet pressure yielding an initial wall shear rate of 200 s⁻¹. (C) Fluorescence microscopy images for platelets (AF488 CD-61) and fibrin (AF594-conjugated human fibrinogen) at t = 400 s.
To assess the ability of our assay to detect the presence and activity of DOACs in the blood of DVT patients on apixaban or rivaroxaban, an assay was designed to utilize small volumes of reversal agent to provide a 'control' condition for each patient to compare their unmodified blood with. For N=8 patients, consistent significant restoration of the fibrin signal was observed for the addition of 300 nM of reversal agent (Figure 4.3.2).

Figure 4.2: Healthy donor blood with Andexanet Alfa alone
Average fibrin signal for N=3 healthy donors with either 0 nM, 50 nM, or 300 nM of Xa-inhibitor reversal agent added. No statistical significance (p>.05) was observed between 0 nM and either of the other concentrations.

4.3.2. DOAC patient data

To assess the ability of our assay to detect the presence and activity of DOACs in the blood of DVT patients on apixaban or rivaroxaban, an assay was designed to utilize small volumes of reversal agent to provide a 'control' condition for each patient to compare their unmodified blood with. For N=8 patients, consistent significant restoration of the fibrin signal was observed for the addition of 300 nM of reversal agent (Figure
4.3B,C). For 50 nM of reversal agent, an intermediate restoration of fibrin fluorescence intensity was observed as well. As anticipated, a high concentration (5000 nM) of matching DOAC drug (either rivaroxaban or apixaban) added to the patient blood resulted in near abolishing of the fibrin signal. Previous studies using in vitro spiked whole blood from healthy donors showed maximal differentiation between DOAC-inhibited and unmodified (control channels) channels at around 500 seconds (Figure 3.5). With the greater level of variance both in patient characteristics (age, health history, etc) and in timing between blood collection and assay execution for these patients, however, the time scale of clotting was found to vary more for the DVT patients than healthy donors, even with curves that were otherwise substantially the same. For this reason, fluorescence intensities were evaluated for each patient at the time where the high reversal agent condition FI was 83% of its maximum value; the same percentage the healthy donors were at for 500 seconds (Figure 3.5). This ensures that each patient curve is being evaluated at generally the same region of the characteristic sigmoid curve in fibrin signal (Figure 4.3A), rather than a single fixed time across all patients. For fibrin FI evaluated this way, a statistically significant difference (p<.0001) was observed between the unmodified blood condition and high reversal condition. When, for each patient, the fibrin signal at the time point where the high reversal agent condition is used to normalize the rest of the conditions, the behavior becomes more consistent patient to patient, and this significance is maintained (Figure 4.3C).
Contrary to the fibrin signal, platelet fluorescent intensity was largely agnostic to the presence of reversal agent. Regardless of the time where fluorescence intensity is evaluated, no statistically significant difference was found between the control and the reversed lanes [Figure 4.4A]. Limited significance was found between the patient blood and high anticoagulant condition (p=.0045), indicating that platelets are only impacted when thrombin is completely abolished and cannot act as a platelet antagonist. This is also apparent in the temporal curves for the individual patients, where the +5000 nM condition is typically visually separated from the rest of the curves after around 200 seconds. Platelet behavior is also homogenized somewhat by the normalization to fluorescent intensity of the fully reversed channel, but not to the same extent as fibrin.
4.3.3. DOAC presence prediction

To evaluate the ability of our essay to differentiate blood that has reversal agent and unmodified blood from patients, a receiver operating characteristic analysis was performed. For normalized fibrin fluorescence intensity, complete differentiation between patient blood and fully reversed blood was possible with an AUC of 1, and sensitivity and specificity both equal to 100% for a cutoff threshold of normalized FI > .85 [Figure 4.5A]. Using the lower reversal agent concentration, these results were .8163, 87.5% and 75% respectively [Figure 4.5C]. Using the raw fluorescence intensity to compare patient blood and the 300 nM reversal agent condition, the AUC was reduced to .9063, and the

Figure 4.4: DVT Patient Platelet Data
Platelet fluorescence intensity curves for a single patient (A), where no significant restoration of signal is observed for the addition of 300 nM Andexanet Alfa, but consistent reduction in signal is observed for high concentrations of ex vivo inhibitor. (B) Combined data for all N=8 patients, where only the fluorescence at 500 s is reported. (C) Data from B where each patient’s data is normalized by the channel with the maximum fluorescence intensity for that experiment.
sensitivity and specificity were 87.5% and 100% respectively, for a cutoff threshold of FL>39.46.

4.3.4. DOAC concentration prediction

In order to predict concentrations of drug present in patient blood, differences between the fluorescence intensity for neat patient blood and patient blood treated with high doses of reversal agent and excess doses of inhibitor were compared against dose response data collected for whole blood from healthy, donors spiked with DOACs in vitro (Figure 3.5). For each patient, the equivalent fluorescence intensity on the healthy donor curve was identified according to the approach outlined in Figure 4.6, and the corresponding concentration associated with the curve was reported (Table 3), with the majority of patients predicted to be around 100 nM concentration, or near typical trough.
concentrations. Missing values are for patients for whom data sheets have not been made available [103].

Figure 4.6: DOAC Concentration prediction schematic
Methodology for mapping DVT patient results to ex vivo drug addition for healthy donor results. It is assumed here the fundamental metric of importance is the ratio between the unmodified blood and the fully reversed (300 nM treated) blood, with the high anticoagulant condition treated exclusively as background noise.
4.4 Discussion

Although initially considered an advantage of DOACs, the lack of a required routine testing strategy has meant that standardized methods for detection of presence and quantification of drug concentrations has become a more and more urgent need as adoption has increased [95,104,105]. Testing may be particularly relevant in two types of specific scenarios: (1) acute event risk assessment and (2) individual patient dose control. Potential acute scenarios include testing in proximity to major surgical or invasive procedures, cases of traumatic injury and major bleeding, and prior of fibrinolytic treatment for cases such as ischemic stroke [91,106]. Threshold recommendations have been made by the ISTH for drug concentrations of <30 ng/mL (∼70 nM) for concentrations of apixaban, rivaroxaban and dabigatran for perioperative situations, with 50 ng/mL (∼120 nM) considered to represent moderate bleeding risk [107]. These recommendations set the requirements for a point of care testing strategy.

<table>
<thead>
<tr>
<th>Subject</th>
<th>001</th>
<th>002</th>
<th>003</th>
<th>004</th>
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<td>F</td>
<td>M</td>
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<td></td>
</tr>
<tr>
<td>Drug</td>
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<td>5 mg A, 2x</td>
<td>2.5 mg A, 2x</td>
<td>5 mg A, 2x</td>
<td>20 mg 1 x R</td>
<td>2.5 mg A, 2x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Last Dose</td>
<td>9.5 h</td>
<td>2 h</td>
<td>4.5 h</td>
<td>3 h</td>
<td>2 h</td>
<td>11 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Meds</td>
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<td>Tranexamic acid, fumerate-FA, foxic acid</td>
<td>Pravastatin</td>
<td>None</td>
<td>None</td>
<td>Statin 20 mg daily</td>
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<td>Concentration model @ 500 s</td>
<td>122 nM</td>
<td>104 nM</td>
<td>100 nM</td>
<td>90 nM</td>
<td>40 nM</td>
<td>13 nM</td>
<td>125 nM</td>
<td>117 nM</td>
<td></td>
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Table 3: Patient information and concentration predictions
to provide utility to clinicians in risk management for patients on DOACs. The
development of reversal agents for DOACs such as Andexanet Alfa for Xa inhibitors and
Idarucizumab for dabigtran have enabled clinicians to respond to situations where
bleeding is suspected, however, these reversal are prohibitively expensive at up to
$50000 per 800 mg dose, making improved information for clinicians as to their need
critical to efficient patient care [108,109].

Patient dose optimization testing may be relevant during initiation of treatment, in
cases where patients are either over or underweight, in situations where non adherence
to dosing is suspected, and in cases where possible interference from other drugs is
suspected [105,106]. Since the half-life of DOACs is significantly shorter than that of the
previously used VKA drugs, inconsistent adherence to dosage recommendations
represent a significantly greater risk of reduced protection from thromboembolism [105].
DOAC concentrations have also been demonstrated to increase dramatically in
response to antiviral agents used for COVID-19, suggesting particular disease states
and treatments may present a compelling need for DOAC monitoring [110].

Current testing methods have proved inadequate for either testing application, as
tests such as PT/INR and aPTT have been shown to be highly dependent on the
particular reagents (thromboplastins) used. ‘Normal’ readings can be produced for
concentrations of drug that are considered clinically risky for scenarios such as surgery
or bleeding events, and PT has even been reported to be insensitive to the effects of
apixaban [91,111]. In either case, no specific normalization strategy such as the INR
exists for DOACs, making results highly dependent on the particular lab they are carried
out in [106]. In more acute testing scenarios, more precise but slow or uncommon
methods such as chromatography or chromogenic anti-Xa assays may be impracticable, highlighting the need for fast, accurate, point of care assays [38].

The assay described in this work performs assessment of platelet and fibrin activity under whole blood flow at physiological venous shear rates using very small amounts of blood (<400 μL). With eight independent experimental conditions, patient blood can be reversed on chip with extremely small amounts (~1 μg) of reversal agent, providing an internal control for each patient and a reference point for drug action. This concentration (300 nM andexanet alfa) was chosen such that approximately an equimolar ratio of reversal agent to DOAC would be present based on anticipated plasma concentrations, allowing for complete reversal of any DOAC present [103]. Using this approach, our assay was able to detect the presence of DOAC drug in every (N=8) patient tested, with 100% specificity and sensitivity and AUC of 1, within a test time of less than 15 minutes, for patients on both rivaroxaban and apixaban taken together. These results represent a significant improvement on both common laboratory methods such as PT and aPTT where sensitivities and specificity can be in the range of 50% depending on the thromboplastin used, and on shows an improvement on or general parity with previously published POC diagnostic devices, albeit for a smaller sample size [49,50,100,112].

There are some important limits to this study, including a relatively small number of patients and data heavily weighted towards apixaban rather than rivaroxaban. Additionally, although there is clear path forward, the assay described in this work is inefficient for differentiating IIa and Xa inhibitors, as certain other assays have demonstrated. If required, a second reversal agent (i.e. Idarucizumab) could be added and by comparison between the fibrin response for each reversal agent, classification
should be straightforward. In addition, this assay was not executed truly point of care due to rotating clinical space in response to COVID requirements. In reality, however, one may reasonably anticipate that the assay’s performance would only improve if testing was performed immediately after phlebotomy for CTI anticoagulated whole blood, as would be possible if the experimental apparatus was transferred to the hospital setting.
CHAPTER 5 : OTHER STUDIES

5.1 Pulsatile flow in Microfluidic Assays

5.1.1. Introduction

Rhythmic contractions of the heart muscle throughout each stage of cardiac cycle causes blood flow to vary in magnitude and even direction at different points in the cycle, and to different extents depending on the blood vessel of interest. Time-varying, or pulsatile flows, have been shown to have significant impacts on endothelial form and function compared with steady, unidirectional flow, with changes having been observed in protein expression and phosphorylation status of cellular adhesion molecules [20,113,114]. Similarly, reversing flows around venous valves due to venous reflux in the legs have been shown to be correlated with deep vein thrombosis (DVT) [115]. While the impacts of pulsatile flow on endothelial cells have been well studied, the effects of these flows on platelet accumulation and coagulation has not. In vitro studies of blood flow and hemostasis modeling are typically carried out in microfluidic devices at either constant flow rates or at a constant pressure drop, with flow only varying significantly in the area of interest when a clot is very close to vessel occlusion [116].

It is well known that clots formed under differing flow rates and shear rates have important differences in their composition and engagement of plasma proteins such as von Willebrand Factor (vWF). Venous clots are typically considered “red”, or fibrin and red blood cell rich, and arterial clots are considered “white”, or platelet rich [117]. For arterial shear rates, vWF becomes particularly important, participating significantly in platelet capture from flow at shear rates above 1000 s⁻¹ through GP1b mediated binding [118]. This capture then allows activation of the platelets via collagen binding of
GPIIb/IIIa [119]. In microfluidic devices, morphological differences can be observed between more regular clots formed under venous shear rates and more amphorphous clots formed under arterial shear rates. Given the differences in behavior observed for clots form under different steady state conditions, it is reasonable to assume that flow pulsatility and thus variations in the shear rate over time may cause differences in clot formation as well. Studies on this matter have been limited, however, and there have been conflicting reports on the impact of pulsatility on platelet adhesion [119–121].

Microfluidic devices have enabled a wide range of assays on hemostasis owing to their low requirements for blood volume and flexible geometries. This work describes an approach for carrying out similar microfluidic assays with fluid delivered in time varying manner, in order to examine directly the impact of variations in shear rate at physiological time scales (~1 Hz).

5.1.2. Materials and Methods

5.1.2.1 Device Design, Fabrication and Preparation

A previously designed 8-channel microfluidic device was adapted to a 4-channel design in order to reduce the total volumetric flow of blood required to achieve high shear rates. This new 4-channel design is otherwise identical to the previously described device in order to maintain the flow characteristics (Figure 5.1) [26]. Devices were prepared using poly(dimethylsiloxane) (PDMS) Sylgard 184 (Ellsworth Adhesive). Thrombogenic surfaces were prepared by coating glass slides (75x38 mm) with SigmaCote (Sigma Aldrich) and subsequently patterning a 250 μm strip of type I equine fibrillar collagen (ChronoLog) using a dedicated PDMS patterning device as previously described [61]. The collagen was subsequently coated with either tissue factor (Dade
Innovin, Siemens), or vWF (Haematological Technologies). A solution of 1% bovine serum albumin (BSA) (Sigma Aldrich) in HEPES buffered saline (HBS) was used to prime each of the devices prior to use.

### 5.1.2.2 Waveform generation and verification

Flow was delivered by withdrawal through the single outlet of the device with a syringe pump (Harvard PHD ultra) connected to a 1 mL glass syringe with a fixed blunt 22G needle (Hamilton), pre-filled with de-ionized water, with all air carefully eliminated in order to prevent compressibility in this line. In order to achieve time-varying flow, a triangle wave was programmed into the device using the onboard firmware to define “ramp steps” to build a triangle wave. For arterial shear rate studies, ramps were programmed for 20 μL/min to 160 μL/min in 1 s, followed immediately by 160 μL/min to 60 μL/min in 1 s, yielding a total cycle time of 2 seconds, or frequency of 0.5 Hz and a shear rate of 2200 s⁻¹ +/- 75%. For venous studies, an average of 200 s⁻¹ +/- 75% was used. Waveforms were verified by flow rate measurement using a thermal mass flow-through sensor and a solution of methyl cellulose in water to a viscosity of 4 cP (Flui gent Inc).
5.1.2.3 Blood Collection

Blood was obtained from consenting healthy adult donors via venipuncture into corn trypsin inhibitor (CTI, Haematologic Technologies) or H-D-Phe-Pro-Arg-chloromethylketone (PPACK, Bachem) to final concentrations of either 40 μg mL⁻¹ or 100 μM to prevent contact (XIIa) activation or thrombin generation respectively. All procedures were performed in accordance with protocols outline and approved by the University of Pennsylvania’s Institutional Review Board.

Figure 5.1: Pulsatile Flow Experimental Design
Experimental design where a 4-channel device is prepared using previously described methods, and then blood is perfused using a syringe pump programmed with a time-varying flow profile [23]. For high frequency imaging, a 90 fps camera is utilized.
5.1.2.4 Platelet and Fibrin Imaging

Platelets were labeled with PE-mouse anti-human CD61 (BD) and fibrinogen was quantified using alexafluor-647 conjugated human fibrinogen (Life Technologies). Devices were imaged on an inverted epifluorescence microscope (Olympus IX81) and images captured with either an ORCA-ER CCD camera (Hamamatsu) or a high framerate firewire-800 camera (Unibrain). Simultaneously experiments were run on the same equipment; one for a steady shear rate, and one for a pulsatile flow, using two different syringe pumps.

5.1.3 Results

In order to verify the function of the triangle waveform in delivering pulsatile flow at high flow rates, a thermal mass based flowrate sensor was used with a methyl cellulose solution in water to model blood viscosity. For a 0.5 Hz wave at 2200 s\(^{-1}\) +/- 75%, flowrates confirmed precisely to expectation, reaching a peak of 160 μL/min and dropping all the way to 20 μL/min within in 1 second, repeatably as shown in Figure 5.2. Sine waves were also demonstrated as feasible, but were not used in experiment due to requiring external communication with the PHD ultra pump to continuously vary the flow rate set point. It is important to note that without the use of a fused needle tip syringe, less consistent behavior was observed, likely due to the increased dead volume added by connecting a luer lock plastic hub needle to a luer syringe. Flow rate measurements for an average shear rate of 200 s\(^{-1}\) showed similar fidelity. Flow rate fidelity at arterial shear rates was further confirmed by high frame rate acquisition (90 fps). Inspection of the platelet fluorescence intensity for the triangle waveform showed changes in brightness of the same frequency as the waveform (0.5 Hz) (Figure 5.3). This
was confirmed by calculating the fast fourier transform of the platelet signal, yielding a peak in the power spectrum at 0.5 Hz (Figure 5.3B). In experiments where TF was present and fibrin was monitored, a corresponding response in the fibrin fluorescence was not observed, suggesting platelets may adhere and be removed within a single waveform cycle, but fibrin is protected from this process, possibly due to forming in close proximity to the glass slide at the tissue factor surface.

Figure 5.2: Pulsatile Waveform Verification
Flow rates detecting using the Fluigent FRP thermal mass flow rate detection system for 4 cP buffer containing methyl cellulose, for a sine wave input (A) or triangle wave input (B). Flow rates correspond to 2200 s⁻¹ +/- 75% in each lane of a 4-channel microfluidic device.
At an average shear rate of 200 s$^{-1}$ flow pulsatility had no observable effect on the platelet accumulation in PPACK whole blood when compared with a steady flow rate (Figure 5.4C). In contrast, significant differences were observed for PPACK blood perfused with a triangle wave, with greatly enhanced platelet accumulation occurring for the unsteady flow (Figure 5.4A). This may be due to the fact that during the low flow

**Figure 5.3: Frequency analysis of platelet and fibrin for pulsatile flow**
Platelet (A) and fibrin (B) fluorescence intensity for a single channel perfused with a triangle waveform, with images captured at 90 frames per second. Due to the frame rate, both channels cannot be image at once, so there is no fibrin data for the first 350 seconds. Panels (B) and (D) are the same data with the axes truncated to better visualize the time varying response. Plates (B) respond in time with the 0.5 Hz waveform used, as verified by fourier analysis (inset). Fibrin (D) does not shome the same time varying response.
rate regions of the pulse cycle, platelets are able to adhere preferentially, and remain adherent through the higher shear portion of the cycle. Minimal platelet adhesion was observed for the constant flow case at 2200 s\(^{-1}\), suggesting vWF from circulation did not adsorb to the collagen and facilitate adhesion to a significant extent in these experiments. When the same experiment was run for CTI blood with a collagen and tissue surface, differences between the two flow profiles largely disappeared, suggesting fibrin may help stabilize platelets against removal at high shear rates, or that platelets activated by thrombin may have strong adhesion forces (Figure 5.4B).

Figure 5.4: Platelet fluorescent intensity in constant versus pulsatile flow

Average platelet signal for N=5 donors, for PPACK whole blood perfused over collagen with either a triangle wave at 2200 s\(^{-1}\) +/- 75% or steady flow at 2200 s\(^{-1}\) (A), CTI whole blood perfused over collagen with tissue factor, with either a triangle wave at 2200 s\(^{-1}\) +/- 75% or steady flow at 2200 s\(^{-1}\) (B). (C) Average platelet signal for N=5 donors, for PPACK whole blood perfused over collagen with either a triangle wave at 200 s\(^{-1}\) +/- 75% or steady flow at 200 s\(^{-1}\).

When vWF was coated to the the collagen thrombogenic surface instead of TF, the comparative enhancement of platelet accumulation was reduced for pulsatile flow versus constant, in relation to the results with no vWF present. This is likely attributed to
vWF facilitating adhesion of platelets at high shear rates, reducing the relative impact of the lower shear excursions of the triangle waveform. Some enhancement is still observed, however, suggesting vWF alone does not render the platelets completely agnostic to flow profiles. It should be noted that there may have been a vast excess of vWF present on the collagen compared to true physiology, as the stock was coated to collagen at the full concentration from the supplier, and no quantification was performed for the amount deposited.

Figure 5.5: Platelet fluorescence intensity with CTI blood and a vWF/collagen surface
PPACK whole blood perfused over collagen pre-coated with vWF, with either a triangle wave at 2200 s\(^{-1}\) +/- 75% or constant flow at 2200 s\(^{-1}\).
5.1.4. Discussion

Platelet adhesion onto collagen alone was not impacted by pulsatility in flow at venous shear rates at an average of 200 s⁻¹. This is not unexpected, as at these shear rates platelets can likely adequately adhere to the collagen at all points in the flow rate cycle relatively equally, meaning the total flux of platelets is the critical determining factor for deposition. Since the average flow rate is the same for either steady or pulsatile flow, therefore, similar accumulation was observed. Conversely, at arterial shear rates, a substantial difference was observed for platelets adhering to collagen alone for pulsatile versus constant flow. It is well known that as shear rate increases above ~1000 s⁻¹ platelet adhesion without vWF is significantly impaired [122–124]. For platelet aggregation to occur at high shear rates, it is likely platelets first bind transiently to vWF via the GPIb receptor, although this interaction is weak and short-lived. During the platelet interaction with the vWF, where a brief period of rolling adjacent to the surface may occur, however, stronger bonds can form via the GpIIb/IIIa integrin and platelets can become firmly adherent [124]. It is possible that in this study, in the absence of significant amounts of vWF, the low shear rate portions of the pulse cycle function in a similar way. Platelets are allowed to associate more easily with the collagen surface at the troughs of the wave form, during which significant platelet activation and bonding via GpIIb/IIIa can occur which then protect the newly aggregated platelets from shearing off at the peaks of the waveform. This idea is supported by the appearance of Figure 5.3B, where a steep increase in platelet fluorescence intensity is observed with the same frequency as the waveform.

Enhancement of platelet accumulation during pulsatile flow was not observed under all cases at arterial shear rates. With a collagen-TF surface and CTI
anticoagulated blood, differences between steady and pulsatile flow were essentially eliminated. Previous studies have suggested that cleaving of the protease activated receptor-4 (PAR4) by peptides increases the aggregation strength of platelets and their resistance to shear stress [125]. It is possible, therefore, that the presence of thrombin in the collagen-TF experiments results in significant engagement of PAR4, stabilizing the platelet mass against shear stress based erosion. In the case of vWF coated onto collagen, reduced differentiation was observed between pulstile and steady flows at arterial shear rates. This is consistent with the idea that vWF-mediate periods of prolonged residence time near at the thrombogenic surface facilitate platelet adhesion. This process enables much more significant adhesion for steady flow at 2200 s$^{-1}$ than collagen alone, as evidenced by the relative platelet accumulation in Figure 5.4A compared with Figure 5.5. This process may be slightly more efficient at shear rates <1500 s$^{-1}$, allowing for a small increase in platelet accumulation to remain for pulsatile flow even with vWF present.

5.2 Measuring the intraclot diffusion using FRAP

5.2.1. Introduction

Consideration of mass transport through growing thrombi is of critical importance to full understanding of thrombosis. Upon initial endothelial damage upon injury at a vessel wall, coagulation factors from circulation have relatively open access tissue factor allowing coagulating to proceed. As a platelet and fibrin thrombus accumulates on top of the initial injury site, however, coagulation factors such as factor X would potentially need to diffuse through an increasingly thick and dense barrier to reach this tissue factor rich site, however [126]. Similarly, diffusion of thrombin formed adjacent to the the tissue
factor bearing surface must diffuse out through the clot to become available to cleave fibrinogen and platelet PAR receptors [127,128]. In either case, developing and accurate model of intra thrombus transport requires some degree of knowledge of the rate of diffusion through the clot. Previous measurements have suggested extremely low rates of diffusion for factor Xa, implying up to a 4 hour requirement for diffusion through 1 mm of platelet clot. For diffusion this limited, the fact that it is well known that clots can fully occlude vessels in less than 10 minutes requires a complete change in understanding of the fundamentals of thrombosis, for example requiring a secondary procoagulant source at the outer surface of a growing thrombus such as bloodborne tissue factor or platelet-derived factor XI for occlusive thrombi to form [126]. In this way, accurate information on transport parameters for relevant coagulation factors is critical to complete understanding of hemostasis and thrombosis.

Fluorescence recovery after photobleaching (FRAP) is a technique often used to measure the kinetics of diffusion on membranes and cell surfaces [129]. Briefly, a region of interest with fluorescent analyte present is exposed to high intensity light which bleaches all of the exposed area leaving a 'hole'. Subsequently, lower intensity light is used to observe a larger region around the bleaching region in order to observe the diffusion of unbleached molecules from the surrounding area into the 'hole' and diffusion of bleached molecules out of the 'hole', and thus a recovery in the brightness of the exposed area. Analysis of the rate of recovery after the bleaching process enables inference of the diffusion rate of the molecules of interest in the particular environment of the experiment. This method is typically applied at very small length scales, but has been demonstrated for inhomogeneous tissues [130]. In this way, application of FRAP
techniques to measure to the diffusion of analytes of interest through a thrombus may provide unique insight into transport parameters of thrombosis.

5.2.2.  *Materials and Methods*

Whole blood collected from a healthy donor and anti-coagulated with 100 µM PPACK was dosed with 1 mg/mL FITC-conjugated bovine serum albumin and perfused over a 250 µm strip of Type I fibrillar collagen (equine) patterned on a glass, using an 8-channel PDMS microfluidic device, with the flow rate maintained at a constant 8 µL/min by a syringe pump (Harvard PHD ULTRA). Upon channel occlusion, the device was decoupled from the syringe pump and moved to a Zeiss LSM 710 Laser-scanning confocal microscope for FRAP data acquisition. The clot was continuously illuminated in a 70 µm diameter circular region for 24 seconds at 100% laser power using a 488 nm wavelength in order to bleach the FITC-albumin located in the interstitial volume between platelets. Post-bleach frames were captured using a 20x objective at a rate of 1 frame every 3 seconds for a total of 140 seconds in order to capture the rate of brightness recovery due to the diffusion of unbleached albumin molecules. A modified bessel function fit of the recovery data was performed to determine a diffusion coefficient [131].

5.2.3.  *Results and Discussion*

Photobleaching of the FITC-albumin was incomplete even for a 24 second exposure. It is not entirely clear why this was the case, as FITC is a commonly used molecular probe for FRAP experiments, and should bleach readily [132]. Since an "immobile fraction" of albumin was likely adsorbed to the platelet surface, incomplete
recovery of fluorescence was also observed in the resulting time-lapse profiles – the absorbed albumin was not displaced with new albumin during the recovery period Figure 5.6B. To account for this, the fluorescence intensity of the bleach region was normalized assuming the final time point had recovered to 96% of the asymptotic value for the mobile fraction. The data was compared against a modified bessel function fit for diffusion limited recovery, suggesting an effective diffusion coefficient for albumin in a platelet clot generated under flow of $1.1 \times 10^{-7} \text{cm}^2/\text{s}$ – the typically reported value for albumin diffusion constant in water is $\sim 6 \times 10^{-7} \text{cm/s}$ [133]. It should be noted that the fit used to model the recovery data assumes 1) instantaneous bleaching, 2) complete bleaching in form a step function in brightness 3) no relevant diffusion in the z-axis 4) that the experimental media is isotropic. None of these assumptions holds completely, and thus there is likely some possibly significant error in the calculated diffusion coefficient, however, this data serves as a proof of concept.

![Figure 5.6: FRAP Data for Diffusion of FITC-albumin](image)

(A) Fluorescence image of a platelet clot formed in a PDMS microfluidic device, with FITC-conjugated albumin mixed with the blood prior to perfusion. (B) Brightness response to bleaching with a laser-scanning confocal microscope at maximum power for 24 seconds. Incomplete bleaching is observed and a substantial immobile fraction is present. (C) A sample of the curve from (B), post bleach, with the recovery profile fit to a modified bessel function described by Soumpassis (inset).
CHAPTER 6 : FUTURE WORK

6.1 Clot cytometry

Optical flow cytometers have been utilized for more than half a century to provide large-scale automated diagnostic information on cell populations, such as for diagnostic detection of markers of blood cancers [134]. A laser illuminates a stream of cells dispersed in solution, tapered down to a single cell at a time, and the scattering or absorption and emission of the incident light from the laser is thereafter detected by optical sensors. In addition to simple fluorescence, detectors can differentiate forward scattering of light in order to infer cell size and side-scattered light to indicate cellular granularity and therefore help determine cell type [135]. Their ability to process huge numbers of cells (over 70000 cells per second in some cases), in a multiplexed way has made optical flow cytometers an invaluable tool.

Clots formed in vitro have been an area of increased interesting over the last two decades owing to the development of microfluidic flow device technology. Microfluidics have allowed the study of numerous analytes, from the obvious such as platelets and fibrin, to others of more recent interest such as neutrophil extracellular traps (NETs) [136]. In reality, a plurality of potential analytes of interest exist that may localize in growing thrombi. Several analytes are directly related to hemostasis such as vWF, p-selectin, PAC1, CD61, CD63, CD151, phosphatidylserine (PS), factor XIIIa, and fibrin have been studied previously. Other analytes of interest, however, may also localize in thrombi to significant degrees, including markers of inflammation and cancer due to cross-talk between inflammatory and coagulation processes. HMGB1 has been implicated in deep vein thrombosis (DVT), elevated levels of C-reactive protein and Pentraxin 3 have been associated with increase risk of heart attack [137–139].
Similarly, complement proteins C5a and C3a have been shown to interact with thrombus formation via recruitment of neutrophils during myocardial infarction [140]. The serine protease Cathepsin G has also been connected with modulation of platelet clot formation through interactions with neutrophils [141]. Increased levels of plasminogen activator inhibitor-1 (PAI-1) and Carboxypeptidase CPB2 have also been associated with vascular thromboembolism [142,143]. Serum amyloid A has been found to bind to fibrinogen and promote amyloid formation [144]. Thrombospondin-1 can interact with vWF to modulate arterial thrombosis and impact platelet recruitment [145].

Using a multiplexed microfluidic device and a multicolor microscope to study clots formed under whole blood flow, the kind of diagnostic approaches which have been applied for flow cytometry could be taken advantage of to perform what is essentially “clot cytometry”. With a 3-color microscope and 8 channel microfluidic device, 24 analytes could be interrogated simultaneously. Growing thrombi may provide a locus in which certain more low concentration analytes may accumulate. For data collected in the chips described in Chapter 3, with imaging by an Olympus IX81 microscope, imaging of several analytes has already been demonstrated (Figure 6.1). Optimization of conditions to obtain quantifiable signals for the other analytes of interest remains to be done, particularly for the inflammation markers which may be at much lower concentrations.
Figure 6.1: Example Data for Clot Cytometry

Proof of concept images for clots formed simultaneously in an 8-channel microfluidic device with staining for 8 different analytes (pseudocolor). Contrast for each image was adjusted independently, as signal for TSP1, vWF and thrombin were notably weaker than the others.
Supplemental Figure 1. Components for final chip assembly, including the injection molded chip with channels (A), the adhesive used to form “bottom” surface (B), a check valve used for priming the device which is the closed (C), an Ink roller which is used to laminate the adhesive to the chip (D), and the final assembled chip filled with blue food coloring for visualization.
Supplemental Figure 2. Wall shear rate at focal region for channels 1-4 for an inlet pressure of 16000 Pa (channels 5-8 omitted as device is symmetric).

Supplemental Figure 3. (A) 4 individual fluorescence peaks from FITC calibration kit, mixed in equal proportion by pipette. (B) Bead solutions from peaks 1-4 added symmetrically to device such that channels mirrored about centerline of the chip have the same bead peak, perfused through device, and effluent measured. Bead counts from each peak reported as a fraction of count from manually pipetted mixture.
Supplemental Figure 4. (A) 8 by 1 channel orientation, re-designed from the 4 by 2 described in chapter 3. (B) Results from 3D laminar flow steady state COMSOL simulations, indicating equal shear rates in each channel.
Supplemental Figure 5. Assay design for DVT patients. Unmodified patient blood condition was split between ends of the device to help prevent too large an asymmetry in forming in the resistance caused by channel occlusion across the device as the experiment proceeded.
Title: MICROFLUIDIC DEVICES AND METHODS FOR MONITORING BLOOD BIOLOGY UNDER FLOW

Abstract: The present invention provides microfluidic devices and methods for measuring blood. The microfluidic devices of the present invention include an inlet port adapted and configured to receive a fluid sample, a microfluidic flow path in fluidic communication with the inlet port, an outlet in fluidic communication with the microfluidic flow path, the outlet having a smaller cross-sectional area than the microfluidic flow path, and adapted for communication with a pressure sink. The microfluidic devices further include a priming circuit in fluidic communication with the microfluidic flow path such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid will flow through the microfluidic flow path to the inlet port due to low resistance to laminar flow in the microfluidic flow path relative to the outlet.
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MICROFLUIDIC DEVICES AND METHODS FOR MONITORING BLOOD BIOLOGY UNDER FLOW

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 62/645,525, filed March 20, 2018. The entire content of this application is hereby incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant Nos. R01-HL-103419 and U01-HL-13 1053 awarded by National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The monitoring of blood coagulation, anti-platelet therapy, anticoagulation therapy, hemophilia therapy, surgical bleeding, trauma bleeding, and platelet function form the basis of a very large (> $1 billion) diagnostics market. Few devices or systems exist on the market for monitoring blood under flow conditions analogous to those that exist within the human body and those that do have limited utility and only operate in the absence of full coagulation that includes thrombin and fibrin generation.

SUMMARY OF THE INVENTION

Embodiments of the present invention provide a microfluidic device including: an inlet port adapted and configured to receive a fluid sample; a microfluidic flow path in fluidic communication with the inlet port; an outlet in fluidic communication with the microfluidic flow path, the outlet having a smaller cross-sectional area than the microfluidic flow path; and adapted for communication with a pressure sink; and a priming circuit in fluidic communication with the microfluidic flow path such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid will flow through the microfluidic flow path to the inlet port due to low resistance to laminar flow in the microfluidic flow path relative to the outlet.
In some embodiments, the outlet includes an outlet channel in fluidic communication with the microfluidic flow path; and an outlet port in fluidic communication with the outlet channel. In some embodiments, outlet channel has a cross-sectional area that is less than about 60% of a cross-sectional area of the microfluidic circuit.

In some embodiments, the microfluidic flow path, the outlet, and the priming circuit are coupled at a single location. In some embodiments, the priming circuit includes a check valve adapted and configured to resist flow from the microfluidic flow path into the priming circuit. In some embodiments, the check valve is a single check valve in fluidic communication with a plurality of microfluidic flow paths. In some embodiments, the microfluidics device of the present invention additionally includes one or more reagents dried within at least one of the inlet and the microfluidic flow path.

Another aspect of the invention provides a microfluidic device including a plurality of microfluidic circuits, each microfluidic circuits including: an inlet port adapted and configured to receive a fluid sample; a microfluidic flow path in fluidic communication with the inlet port; and an outlet in fluidic communication with the microfluidic flow path, the outlet: having a smaller cross-sectional area than the microfluidic flow path; and adapted for communication with a pressure sink; a priming circuit in fluidic communication with each of the microfluidic flow paths such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid will flow through the microfluidic flow paths to the inlet ports due to low resistance to laminar flow in the microfluidic flow path relative to the outlet.

In some embodiments, the microfluidic flow paths converge to a spatially compact sensing region.

In some embodiments, the microfluidic flow paths each have a substantially identical pressure drop between the inlet port and the spatially compact sensing region. In some embodiments, the microfluidic flow paths each have a substantially identical distance between the inlet port and the spatially compact sensing region.

In some embodiments, the plurality of inlet ports are arranged in a single line. In some embodiments, the plurality of inlet ports are spaced along the single line at an inter-port distance compatible with a multi-channel pipette.
In some embodiments, each of the microfluidic circuits further includes: one or more reagents dried within at least one of the inlet and the microfluidic flow path. In some embodiments, the one or more reagents differs amongst the plurality of microfluidic circuits.

Another aspect of the invention provides a method for measuring blood. The method includes: injecting a priming fluid through the priming circuit of the microfluidic devices as described herein; loading blood into one or more of the inlet ports; applying a pressure source to the one or more inlet ports to force the blood into the microfluidic flow paths; and imaging flow along the microfluidic paths.

In some embodiments, the blood is pre-mixed with one or more reagents.

Another aspect of the invention provides a microfluidic device including: a plurality of microfluidic circuits, each microfluidic circuit including: an inlet port adapted and configured to receive a fluid sample; and a microfluidic flow path in fluidic communication with the inlet port, wherein the microfluidic flow paths iteratively converge pairwise to form a converged single microfluidic flow path; an outlet channel in fluidic communication with the converged single microfluidic flow path; an outlet port in fluidic communication with the outlet channel, the outlet port adapted and configured to collect a fluid sample; and a priming circuit in fluidic communication with the converged microfluidic flow path and the outlet channel at a single location, such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid flows simultaneously through the microfluidic flow paths to the inlet ports and through the outlet channel to the outlet port. In some embodiments, the converged single microfluidic flow path and the outlet channel have cross-sectional dimensions within 10% of each other.

Another aspect of the invention provides: a microfluidic device including: a substrate having a surface; an adhesive coating deposited onto the surface of the substrate; a biochemical coating deposited in a pattern directly onto a portion of the adhesive coating; and a chip comprising: a plurality of microfluidic channels having an open boundary, each microfluidic channel comprising: an inlet port adapted and configured to receive a fluid sample; a microfluidic flow path in fluidic communication with the inlet port; an outlet channel having an open surface, the outlet channel in fluidic communication with the plurality of microfluidic channel; an outlet port in fluidic communication with the outlet channel, the outlet port adapted and configured to collect a fluid sample; a priming circuit having an open surface, the priming
circuit in fluidic communication with the microfluidic channels and the outlet channel, such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid flows simultaneously through the microfluidic flow paths to the inlet ports and through the outlet channel to the outlet port; wherein the chip is mounted onto the adhesive coating such that at least a portion of the microfluidic flow paths overlaps with the biochemical coating, and wherein the adhesive coating bonds the chip to the substrate, thereby fluidly sealing the open surface of the microfluidics channels, the outlet channel, and the priming circuit.

In some embodiments, the biochemical coating is deposited onto the adhesive coating by printing.

BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the nature and desired objects of the present invention, reference is made to the following detailed description taken in conjunction with the accompanying drawing figures wherein like reference characters denote corresponding parts throughout the several views.

FIGS. 1-5 depict a microfluidic device for monitoring blood biology under flow conditions according to an embodiment of the invention.

FIGS. 6A-6C depict a step-by-step method of priming a microfluidic device according to an embodiment of the invention (FIG. 6A), loading samples in wells of the microfluidic device (FIG. 6B) and pressurizing the microfluidic device, passing the samples through the sensing zone (FIG. 6C).

FIGS. 7A-7E depict a step-by-step method of preparing and priming a microfluidic device according to an embodiment of the invention.

FIG. 8 is a set of fluorescence images taken of 8 replicate channel locations showing fluorescent platelet deposition with time at a specific location where collagen was pre-patterned on one of the surfaces of each channel.

FIG. 9 is a graph reporting the average fluorescence intensity and standard deviation of platelet deposition with time for 8 replicate channels.

FIG. 10 depicts the assembly of an exemplary sandwich device of the present invention using biochemical constituents patterned directly on the adhesive coated on a substrate for adhesive bonding of the substrate to an additional component to form microfluidic channels.
FIG. 11 depicts an exemplary microfluidic device having a single priming channel for use in monitoring blood biology under flow conditions, according to an embodiment of the invention.

FIG. 12 depicts a zoomed-in view of a single location where the primer flow channel intersects with the inlet and outlet flow channels.

FIGS. 13A-13C depict a step-by-step method of preparing and priming an exemplary microfluidic device according to an embodiment of the invention.

FIG. 14 depicts an exemplary microfluidic device having eight sample inlet wells suitable for multi-channel pipetting, according to an embodiment of the invention.

FIG. 15 depicts an exemplary cartridge frame that can be used with the microfluidic device according to an embodiment of the invention.

DEFINITIONS

The instant invention is most clearly understood with reference to the following definitions:

As used herein, the singular form “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. “About” can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

As used in the specification and claims, the terms “comprises,” “comprising,” “containing,” “having,” and the like can have the meaning ascribed to them in U.S. patent law and can mean “includes,” “including,” and the like.

Unless specifically stated or obvious from context, the term “or,” as used herein, is understood to be inclusive.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41,
42, 43, 44, 45, 46, 47, 48, 49, or 50 (as well as fractions thereof unless the context clearly dictates otherwise).

Unless specifically stated or obvious from context, as used herein, two (or more) values can be understood to be “substantially identical” if the values are within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the other value(s).

**DETAILED DESCRIPTION OF THE INVENTION**

Aspects of the invention provide devices and methods for monitoring fluids (e.g., biological fluids such as blood) under flow conditions. Embodiments of the invention include novel microfluidics devices capable of generating biologically and hemodynamically relevant flow conditions. The devices and methods of the invention are suitable for monitoring platelet function, coagulation function, fibrinolysis, fibrinolytic resistance, and drug function in assays of bleeding risk, thrombosis risk or fibrinolysis risk. The devices and methods can also be used to monitor inflammation, immune systems activation, fibrinolytic resistance, and fibrinolysis. The invention can be applied in the fields of oncology, transplant therapy, trauma, cardiovascular disease management, hematological disease management, elective surgery, trauma surgery, and hemophilia management, and can aid in the treatment of diseases of the blood, blood cells, and blood plasma.

**Microfluidics Devices**

Referring now to FIG. 1, one embodiment of the invention provides a microfluidic device 100 including a microfluidic circuit 101 including an inlet port 102, a microfluidic flow path 104 in fluidic communication with the inlet port 102, an outlet 106 in fluidic communication with the microfluidic flow path 104, and a priming circuit 108 in fluidic communication with the microfluidic flow path 104 such that when a priming fluid is applied under pressure to the priming circuit 108, the priming fluid will flow through the microfluidic flow path 104 to the inlet port 102 due to low resistance to laminar flow in the microfluidic flow path 104 relative to the outlet 106.

The outlet 106 can have a smaller cross-sectional area than the microfluidic flow path and it can be adapted for communication with a pressure sink or atmospheric pressure. In certain embodiments, the outlet 106 includes an outlet channel 110 in fluidic communication with the -6-
microfluidic flow path 104 and an outlet port 112 in fluidic communication with the outlet channel 110. In a preferred embodiment, the outlet channel 110 can have a cross-sectional area that is less than about 60% of a cross-sectional area of the microfluidic flow path 104. The outlet channel 110 may have a cross-sectional area that is about equivalent to the cross-sectional area of the microfluidic flow path 104. Outlet channel 110 may have a circuitous or serpentine configuration, as shown in FIGS. 11 and 14.

The priming circuit 108 can, but need not, have the same cross-sectional area as the microfluidic flow path 104. The priming circuit 108 can have a larger or smaller cross-sectional area as the microfluidic flow path 104. In considering the delivery of liquids into empty and air-filled channels, Applicant believes that regardless of the cross-sectional area of the priming circuit 108, in some embodiments, for example, that shown in FIG. 3, the priming fluid will preferentially flow into a cross-sectionally larger inlet to microfluidic flow path 104 over the smaller cross-sectional entrance to the outlet 106. In another embodiment, such as that depicted in FIGS. 11 and 14, the fluid resistance in microfluidic flow path 104 and outlet 106 are approximately equivalent such that the priming fluid will evenly flow into each of the microfluidic flow paths 104 and the outlet 106, simultaneously priming the flow path 104 and outlet 106. In certain embodiments, each microfluidic flow path has a priming circuit (e.g., FIG. 3). In other embodiments, the entire microfluidic device is primed with a single priming circuit (e.g., FIGS. 11, 12 and 14).

In some embodiments, the microfluidic flow path 104, the outlet 106, and the priming circuit 108 are coupled at a single location 114.

The priming circuit 108 can include a check valve 118 adapted and configured to resist flow from the microfluidic flow path 104 into the priming circuit 108, especially when the priming circuit 108 is filled with priming fluid. Referring to FIG 2, the check valve 118 can be a single check valve in fluidic communication with a plurality of microfluidic flow paths 104. In some embodiments, the check valve 118 can be placed off the microfluidic device 100 and placed between a pumping means and the priming path inlet 108.

The microfluidic flow path 104, the outlet 106, and the priming circuit 108 can have a variety of cross-sectional profiles (as viewed axially in the direction of fluid flow). For example, the channels 104, 106, 108 can have a cross-sectional profile approximating one or more of circles, ellipses, triangles, quadrilaterals, rectangles, squares, trapezoids, parallelograms,
rhombuses, pentagons, hexagons, heptagons, octagons, nonagons, decagons, $n$-gons, and the like. Rectangular and square channels are exemplary embodiments. In one embodiment, microfluidic flow path 104 has a height between about 40 pm and about 100 pm (e.g., about 60 pm) and a width between about 80 pm and about 300 pm.

Referring now to FIG. 2, the microfluidic device 100 can include a plurality of microfluidic circuits 101 (e.g., 2, 4, 8, 12, and other quantities corresponding to multi-channel pipettes). In one example, the plurality of microfluidic flow paths 104 can converge to a spatially compact sensing region 116. For instance, referring to FIG. 2, the sensing region 116 can be arranged such that the microfluidic flow paths 104 are aligned in parallel a short distance from one another.

In embodiments having a plurality of microfluidic circuits 101, each microfluidic flow path 104 can have a substantially identical pressure drop between the inlet ports 102 and the spatially compact sensing region 116. In embodiments in which the cross-sectional area of the microfluidic flow paths 104 is substantially uniform, the length of the microfluidic flow path 104 will dictate the pressure drop such that microfluidic flow paths 104 having identical or substantially identical lengths will exhibit identical or substantially identical pressure drops. In one embodiment, the plurality of inlet ports 102 can be aligned with one another in a straight line, equidistant apart from their nearest neighbors. In such embodiments, the inlet ports 102 can be spaced along the single straight line such that a multi-channel pipette can be used to add material to the inlet ports 102. The microfluidic flow paths 104 can then converge to the spatially compact sensing region 116. In some embodiments, such as that shown in FIG. 2, microfluidic flow paths 104 optionally include appropriate lateral legs 120 to produce a uniform path length for microfluidic flow paths 104 having inlets 102 closer to the spatially compact sensing region 116.

In certain embodiments, at least one of the inlet 102 and the microfluidic flow path 104 can contain one or more chemical agents. The chemical agents can be present in a dried form. The chemical agents can be a reagent or chemical reactant that interacts with a blood sample. In embodiments having a plurality of microfluidic circuits 101, at least a portion of the microfluidic circuits 101 can contain a different chemical agent, reagent or reactant. Examples of chemical agents can include matrix proteins, enzymes, polymers, small molecule compounds, fluorescent labeling probes especially for labeling platelets, neutrophils, fibrin, thrombin,
chelating agents (EGTA, EDTA), deoxygenating agents (e.g., a dithionite salt, an ascorbate salt, or a sulfite salt), modulators of platelet function (aspirin, thromboxane receptor antagonists, integrin antagonists that block alpha-2b/beta-3, P2Y12 inhibitors, P2Y1 inhibitors, PAR1 and PAR4 inhibitors) and modulators of coagulation function (inhibitors of Factor Xla, Xla, Xa, IXa, Villa, Vila, Va, thrombin, or tissue factor). Exemplary reagents for coating a surface of path 104 in the sensing region 116, include fibrillar collagen, von Willebrand factor (VWF), lipitated tissue factor (TF), kaolin, silica, vitronectin, fibronectin, laminin, and activators of Factor Xla. In certain embodiments, the chemical agents can be compounds that allow for the monitoring of platelets, thrombin, fibrin, RBCs, white blood cells, enzyme activity, clot stability, clotting rate, fibrinolysis, fibrinolytic resistance, NETosis, or a specified biological process indicative of drug response or disease progression. The chemical agent can be patterned on to at least a section of the microfluidic flow path 104, especially across the location of the sensing region 116. In some instances, the section of the microfluidic flow path 104 is about 100 pm to about 1,000 pm long.

The microfluidic device 100 can be made of substantially any material adapted for and compatible with storing and transport of blood. Exemplary materials include polymeric (e.g., PDMS, PMMA, PTFE, PEEK, PE, epoxy resins, thermosetting polymers), amorphous (e.g., glass), crystalline (e.g., silicon, silicon dioxide) or metallic (e.g., Al, Cu, Au, Ag, alloys) materials. Suitable polymers for fabricating the device component include glass, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), polycarbonate, acrylic, polyethylene, polystyrene, and the like.

The microfluidic device 100 can be fabricated by substantially any means common in the art, including but not limited to photolithography (e.g., UV photolithography), micromachining, additive manufacturing, laser cutting, laser ablation, drilling, molding, casting, chemical vapor deposition, electron beam evaporation, and reactive ion etching. The microfluidic device can be made of two or more pieces: a chip containing the elements of the microfluidic circuit(s) 101 and a flat substrate to which the chip is bonded to. The one or more microfluidic flow paths 104 may be separated on the chip by a distance of up to about 10 pm, about 10 pm to about 5000 pm, and/or greater than about 5000 pm. The chip and the flat substrate can be bound through a method selected from ultrasonic welding, adhesives (e.g. one or more adhesive coatings as understood in the art), vacuum bonding, thermal treatment, and plasma treatment. The bonding
process should be a method which does not disrupt, disturb or destroy the one or more chemical agents present in the inlet 102 and/or the microfluidic flow path 104, especially in the location of the sensing region 116.

The substrate may be flexible or rigid, transparent or nontransparent. The substrate may be constructed from one or more suitable materials that may include polymeric (e.g., PDMS, PMMA, PTFE, PEEK, PE, epoxy resins, thermosetting polymers), amorphous (e.g., glass), crystalline (e.g., silicon, silicon dioxide) or metallic (e.g., Al, Cu, Au, Ag, alloys) materials. Suitable polymers for fabricating the device component include glass, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), polycarbonate, acrylic, polyethylene, polystyrene, and the like.

Biochemical-Printed Substrates

Referring now to FIG. 10, the one or more adhesives and/or adhesive coatings 1004 may be coated with one or more biochemical constituents 1006, including for example, one or more polymers, biopolymers, proteins, small molecules, antibodies, antibody fragments, nucleic acids, liposomes, or other subcellular constituents, including mixtures containing one or more of collagen, fibrillar collagen, fibronectin, vitronectin, laminin, von Willebrand factor (VWF), lipitated tissue factor (TF), kaolin, silica, activators of Factor X11a, and the like. Coatings can be soluble in order to interact with the priming fluid and/or the sample.

The biochemical coating 1006 may be deposited directly onto the adhesive coatings 1004, such that the biochemical coating 1006 is sandwiched between the microfluidic device 1008 and the substrate 1002, which are fluidly sealed by the adhesive coating 1002. The biochemical coating 1006 may have a thickness of about 0.1 pm to about 25 pm. The biochemical coating 1006 can be dry, semi-wet (e.g. in hygroscopic media such as glycerol), or wet (e.g. in one or more buffer solutions) once the microfluidic device is assembled before using the device for assaying a sample.

The biochemical coating 1006 can be applied in a variety of patterns and can include a variety of constituents. For example, one or more lines 1010 can be applied that each include a different composition. Such lines 1010 can be collinear with the microfluidic channels 1012 or can be angled (e.g. orthogonally) relative to the microfluidic channels 1012. Angled
biochemical coating lines 1010 advantageously relax tolerances for mating the substrate 1002 and the channel-defining chip 1008.

Although the biochemical coating 1006 in FIG. 10 is depicted with a uniform thickness after mating with microfluidic chip 1008, the biochemical coating 1006 and/or adhesive 1004 may compress or flow laterally in regions contacting the microfluidic chip 1008.

Applicant has invented a process such that even with angled biochemical coatings extending laterally beyond the sidewalls of the microfluidic channels 1012 (e.g., between channels 1012), the microfluidic channels 1012 remain liquid-tight at least over the course of a typical microfluidic experiment (e.g., for at least 1 minute, for at least 5 minutes, for at least 10 minutes, for at least 15 minutes, for at least 20 minutes, for at least 30 minutes, and the like).

Biochemical coating(s) 1006 can be applied using a variety of fluid handling techniques including spraying, printing (e.g., using ink-jet techniques), pipetting, silk screening, masking, and the like.

Coupling With Other Devices

The microfluidic device 100 can be coupled to an imaging device capable of collecting imaging data along the one or more microfluidic paths 104. The imaging device can include at least one device selected from a visible light camera, a photodiode, a diode array detector, a UV-Vis spectrometer, an infrared camera, an infrared spectrometer.

The devices of the invention can be operated automatically and can be made to be compatible with robotic liquid dispensers and automated sensing equipment. The microfluidic device 100 can further include a computer for storing information, controlling robotic components, regulating applied pressures, operating imaging devices, and recording collected data.

The devices of the invention provide a number of notable benefits over devices common in the art. Notably, the microfluidic device can be incorporated on to a chip that remains stable under dry storage for over three months. Additionally, the devices can be loaded and manipulated without extensive training as will be discussed below. As such, the devices are particularly useful for translation to clinical laboratories.
Methods

The invention further provides methods of measuring a fluid (e.g., blood, urine, saliva, water sample) using the microfluidic devices of the invention.

The methods of the invention include injecting a volume of a priming fluid (e.g., water, saline, and the like, a saline solution containing added protein such as albumin, or other suitable fluid including one or more blocking buffers as understood in the art) through the priming circuit 108 of the microfluidic device 100, thereby priming the one or more microfluidic circuits 101. The microfluidic device may be primed with a volume of priming fluid including up to 100 μL, 100 to 200 μL, 200 μL to 500 μL, 500 μL to 1000 μL, and the like. One or more samples (e.g., blood) is then loaded into one or more of the inlet ports 102. A pressure source is then applied to the one or more inlet ports 102 in order to force the blood into the one or more microfluidic flow paths 104 while imaging the flow along the one or more microfluidic paths 104. The samples moving through the paths 104 cannot enter the priming path 108 due to the check valve 118 and due to the incompressibility of the priming fluid resident in path 108.

A defined volume of priming fluid can be injected to prime the device. Alternatively, priming fluid can be injected until priming fluid is visible in inlets 102.

A defined volume of sample (e.g., 50 μL to 100 μL, 100 μL to 200 μL, 200 μL to 500 μL, or up to 1 mL) can be loaded into inlets 102. Alternatively, the sample can be loaded by sight (e.g., until the inlets 102 are filled to a visualized level).

The imaging can take place at the spatially compact sensing region 116, if present, allowing for imaging of multiple microfluidic paths 104 simultaneously. The priming step can take place up to about 1 hour before the loading of the blood sample into the one or more inlet ports 102. For example, a technician can prime a number of devices before loading those devices with samples.

The microfluidic device may be placed in a frame 500, shown in FIG. 15, which includes one or more troughs 502 for containing sample (e.g. blood), one or more wells 504 containing buffer, including priming solution, and one or more waste traps 508 for collection of outflow fluid. The device may be loaded while mounted in chip slot 506 of frame 500. The device may be imaged while mounted in the frame.

In certain embodiments, the sample is pre-mixed with one or more reagents. In other embodiments, the sample is contacted with one or more reagents present within at least one of
the inlet port 102 and the microfluidic flow path 104. In still other embodiments, the sample is both pre-mixed with one or more reagents and contacted with a reagent within the microfluidic circuit 101.

The sample can be added to the inlet port 102 through substantially any means common in the art, including, but not limited to, via syringe or pipette. In embodiments that have inlet ports 102 aligned in a single line as described elsewhere herein, the samples can be added to the inlet ports via multi-channel pipette.

Pressure can be applied using a variety of devices. For example, a manifold can be pressed or clamped over the inlets 102 and pressurized. In certain embodiments, the applied pressure is maintained at a sufficient pressure to establish a center line wall shear rate of about 10 to about 10,000 s⁻¹, or about 10 to about 2,000 s⁻¹. The applied inlet pressure can be maintained at a constant pressure in a range of about 1 mm-Hg to about 500 mm-Hg. In another embodiment, a constant pressure less than atmospheric pressure (a negative pressure) can be applied to the exit location in order to pull blood from the inlet across the device to the outlet. In other embodiments, a syringe pump can infuse a hydraulic fluid such as a salt water buffer to push blood at a constant flow rate across the device from the inlet to the outlet. In another embodiment, a syringe pump can be attached to the outlet of the device for withdrawal of blood at constant flow rate.

In certain embodiments, the method can be an automated method carried out by one or more computer controlled robots.

The sample can be derived from any animal. In some instances the animal is a mammal; in other instances, the animal is a human.

EXAMPLES

Materials and Methods

The device can be configured with all inlets, outlets, priming path and collagen-patterned surface in the sensing region using layers that are bonded together. In FIG. 5, a device is shown assembled from components. The microchannels are molded into a polymer such as PDMS that is polymerized on a wafer patterned by etching with small features to create channels within the polymer. The wafer is obtained by standard lithography techniques whereby a mask allows the exposure of a precise pattern on a photosensitive coating such as SU8 that can then be etched.
The polymer device is removed from the wafer and configured with an inlet pressure chamber, outlet reservoir, and off-chip priming tubing and check valve. The device is bonded to a flat substrate presenting a patterned surface in the sensing zone of the fluidic device.

Use of the device is shown in FIG. 5 where priming fluid is delivered through the check valve, blood samples are delivered to 8 inlet wells, and the inlet pressure chamber is sealed by an upper lid that is attached to a pressure source. In FIG. 6A, the device is first primed with a red-dye priming liquid, then the inlet wells are filled with a blue-dye test liquid (FIG. 6B), after which a pressure source is attached (FIG. 6C) to apply 10 mm-Hg of pressure to drive the blue-dye test liquids through the 8 individual paths, thereby displacing the red-dye priming liquid as the blue-dye test liquid flows from the inlet to the outlet of each channel. In FIG. 13A, an exemplary device is first primed with a green-dye priming liquid. Then, the inlet wells are filled with a red/orange-dye test liquid (FIG. 13B), and the test liquid displaces the green-dye priming liquid as the red/orange-dye test liquid flows from the inlet to the outlet channel while not mixing with the priming circuit (FIG. 13C).

To operate the chip, the priming step is shown in FIG. 7 for manual delivery of a priming fluid to the device where a syringe is connected to the priming inlet (FIG. 7A), and the syringe is actuated manually to deliver the priming fluid through a check valve and into the device (FIG. 7B) resulting in the completing priming of the device including priming to the inlet reservoirs where the test samples will be delivered (FIG. 7C). These manual steps can easily be replaced with a robotic fluid dispenser system or a preattached prefilled syringe that is actuated by automated movement of the syringe plunger. The use of a priming fluid at the time of use allows the device to be stored under a dry condition (FIG. 7D) and then to be fully primed with a priming fluid (red-dye priming fluid in FIG. 7E) in preparation for use of the device with individual test samples such as blood.

Example 1

In tests of platelet function, a fluorescent antibody to label platelets can be added to the blood sample and the blood sample can be delivered to the primed device. Application of pressure to the inlet will drive the individual samples across the collagen-coated strip in sensing region of the device. All individual channels can be imaged simultaneously (FIG. 8) as platelets accumulate on the collagen. Images can be obtained a video rates (30 frames per sec) or at
slower sampling rates of 1 frame every 1 to 30 sec. Image processing allows regions of interest
to be defined in the image that correspond to the individual clotting zones in the sensing region
of the device. In these defined regions of interest, the fluorescence intensity can be determined
with time using standard image analysis techniques. In FIG. 9, all the individual sensing regions
are averaged together to give a mean fluorescence and standard deviation of fluorescence for 8
unique replicate clotting events for the course of clotting from 0 to 600 sec of chip operation.

Example 2

In some experiments, blood is treated with d-phenylalanyl-prolyl-arginyl chloromethyl
ketone (PPACK) to inhibit thrombin and/or apixaban to inhibit Factor Xa. Other anticoagulants
could include heparin, citrate, hirudin, and the like. The blood is further treated with a platelet
label and perfused over collagen to evaluate platelet response to collagen. In variations of this
experiment, increasing doses of antiplatelet agents are added to some of the blood samples and a
dose-response and patient drug sensitivity can be established for a given antiplatelet agent using
a single device. Common anti-platelet agents include aspirin, indomethacin, P2Y12 inhibitors,
prostacyclin, prostacyclin analogs, integrin antagonists, and the like.

In some experiments, blood is treated with trypsin inhibitor to inhibit Factor Xlla.
The blood is further treated with a platelet label and a fibrin label (anti-fibrin or fluorescent
fibrinogen) and a thrombin label (low doses of 1 to 600 nM fluorescent PPACK or platelet
targeting thrombin sensors) and perfused over collagen/TF surfaces to evaluate platelet response
to collagen in the presence of robust thrombin generation and fibrin polymerization. In
variations of this experiment, increasing doses of antiplatelet agents are added to some of the
blood samples and a dose-response and patient drug sensitivity can be established for a given
antiplatelet agent using a single device. Common anti-platelet agents include aspirin,
indomethacin, P2Y12 inhibitors, prostacyclin, prostacyclin analogs, integrin antagonists, and the
like. In variations of this experiment, increasing doses of anticoagulant agents are added to some
of the blood samples and a dose-response and patient drug sensitivity can be established for a
given anticoagulant agent using a single device. Common anticoagulant agents including, but
not limited by, those targeting Factors Xlla, XIa, Xa, IXa, Villa, Villa, Va, Ila (thrombin) or
tissue factor, TFPI, Protein S, Protein Z, activated protein C, or PAR 1 or PAR4 inhibitors.
In some experiments, blood from patients with known or suspected hemophilias or bleeding disorders can be applied to the device along with agents that promote blood clotting such as those enhancing the production of thrombin and fibrin during a clotting event or enhancing platelet adhesion such as von Willebrand Factor.

In some experiments, blood from patients suffering acute trauma can be applied to the device to evaluate platelet function, thrombin generation rate, fibrin polymerization rate as a measure of bleeding risk and as a tool to guide choices of transfusion products or pharmacological agents.

In some experiments, after a fixed time of perfusion and clotting, the pressure of the inlet can be acutely reduced to 0 PSIG such that flow across the microchannels stops due to the absence of a pressure drop. In this situation, platelet released products such as ADP, ATP, and thromboxane are not washed away by the flow field and instead accumulate to high levels to drive platelet retraction and overall clot retraction, which is easily observed by image analysis whereby the clot reduces its area over time.

In some experiments, after a fixed time of perfusion and clotting, the pressure of the inlet can be acutely increased by 1.5 to 10-fold such that flow across the microchannels increases and the shearing forces on the clots in the sensing region increases. In this situation, clot embolization can occur as a metric of weak clot structures. Embolization is easily observed by image analysis whereby the clot reduces its mass over time as indicated by a decline in clot associated fluorescence. In variations of this experiment, modulators of platelet function including contraction, thrombin generation, or fibrin polymerization can be added to some of the wells to understand the effect of those pathways on overall clot strength.
CLAIMS

1. A microfluidic device comprising:
   an inlet port adapted and configured to receive a fluid sample;
   a microfluidic flow path in fluidic communication with the inlet port;
   an outlet in fluidic communication with the microfluidic flow path, the outlet:
      having a smaller cross-sectional area than the microfluidic flow path; and
      adapted for communication with a pressure sink; and
   a priming circuit in fluidic communication with the microfluidic flow path such that
when a priming fluid is applied under pressure to the priming circuit, the priming fluid will flow
through the microfluidic flow path to the inlet port due to low resistance to laminar flow in the
microfluidic flow path relative to the outlet.

2. The microfluidic device of claim 1, wherein the outlet comprises:
   an outlet channel in fluidic communication with the microfluidic flow path; and
   an outlet port in fluidic communication with the outlet channel.

3. The microfluidic device of claim 1, wherein the outlet channel has a cross-sectional area
   that is less than about 60% of a cross-sectional area of the microfluidic circuit.

4. The microfluidic device of claim 1, wherein the microfluidic flow path, the outlet, and
   the priming circuit are coupled at a single location.

5. The microfluidic device of claim 1, wherein the priming circuit comprises a check valve
   adapted and configured to resist flow from the microfluidic flow path into the priming circuit.

6. The microfluidic device of claim 1, wherein the check valve is a single check valve in
   fluidic communication with a plurality of microfluidic flow paths.

7. The microfluidic device of claim 1, further comprising:
   one or more reagents dried within at least one of the inlet and the microfluidic flow path.
8. A microfluidic device comprising:
   a plurality of microfluidic circuits, each microfluidic circuits comprising:
   an inlet port adapted and configured to receive a fluid sample;
   a microfluidic flow path in fluidic communication with the inlet port; and
   an outlet in fluidic communication with the microfluidic flow path, the outlet:
   having a smaller cross-sectional area than the microfluidic flow path; and
   adapted for communication with a pressure sink;
   a priming circuit in fluidic communication with each of the microfluidic flow paths such
   that when a priming fluid is applied under pressure to the priming circuit, the priming fluid will
   flow through the microfluidic flow paths to the inlet ports due to low resistance to laminar flow
   in the microfluidic flow path relative to the outlet.

9. The microfluidic device of claim 8, wherein the microfluidic flow paths converge to a
   spatially compact sensing region.

10. The microfluidic device of claim 9, wherein the microfluidic flow paths each have a
   substantially identical pressure drop between the inlet port and the spatially compact sensing
   region.

11. The microfluidic device of claim 9, wherein the microfluidic flow paths each have a
    substantially identical distance between the inlet port and the spatially compact sensing region.

12. The microfluidic device of claim 8, wherein the plurality of inlet ports are arranged in a
    single line.

13. The microfluidic device of claim 8, wherein the plurality of inlet ports are spaced along
    the single line at an inter-port distance compatible with a multi-channel pipette.

14. The microfluidic device of claim 8, wherein each of the microfluidic circuits further
    comprises:
    one or more reagents dried within at least one of the inlet and the microfluidic flow path.
15. The microfluidic device of claim 14, wherein the one or more reagents differs amongst the plurality of microfluidic circuits.

16. A method for measuring blood, the method comprising:
   injecting a priming fluid through the priming circuit of the microfluidic devices of any one or claims 1-7;
   loading blood into one or more of the inlet ports;
   applying a pressure source to the one or more inlet ports to force the blood into the microfluidic flow paths; and
   imaging flow along the microfluidic paths.

17. The method of claim 15, the blood is pre-mixed with one or more reagents.

18. A microfluidic device comprising:
   a plurality of microfluidic circuits, each microfluidic circuit comprising:
   an inlet port adapted and configured to receive a fluid sample; and
   a microfluidic flow path in fluidic communication with the inlet port, wherein the microfluidic flow paths iteratively converge pairwise to form a converged single microfluidic flow path;
   an outlet channel in fluidic communication with the converged single microfluidic flow path;
   an outlet port in fluidic communication with the outlet channel, the outlet port adapted and configured to collect a fluid sample; and
   a priming circuit in fluidic communication with the converged microfluidic flow path and the outlet channel at a single location, such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid flows simultaneously through the microfluidic flow paths to the inlet ports and through the outlet channel to the outlet port.

19. The microfluidic device of claim 18, wherein the converged single microfluidic flow path and the outlet channel have cross-sectional dimensions within 10% of each other.
20. A microfluidic device comprising:
   a substrate having a surface;
   an adhesive coating deposited onto the surface of the substrate;
   a biochemical coating deposited in a pattern directly onto a portion of the adhesive coating; and
   a chip comprising:
       a plurality of microfluidic channels having an open boundary, each microfluidic channel comprising:
           an inlet port adapted and configured to receive a fluid sample;
           a microfluidic flow path in fluidic communication with the inlet port;
           an outlet channel having an open surface, the outlet channel in fluidic communication with the plurality of microfluidic channel,
           an outlet port in fluidic communication with the outlet channel, the outlet port adapted and configured to collect a fluid sample;
           a priming circuit having an open surface, the priming circuit in fluidic communication with the microfluidic channels and the outlet channel, such that when a priming fluid is applied under pressure to the priming circuit, the priming fluid flows simultaneously through the microfluidic flow paths to the inlet ports and through the outlet channel to the outlet port;
   wherein the chip is mounted onto the adhesive coating such that at least a portion of the microfluidic flow paths overlaps with the biochemical coating, and
   wherein the adhesive coating bonds the chip to the substrate, thereby fluidly sealing the open surface of the microfluidics channels, the outlet channel, and the priming circuit.

21. The microfluidics device assembly of claim 20, wherein the biochemical coating is deposited onto the adhesive coating by printing.
FIG. 6A

FIG. 6B

FIG. 6C

Primed

0 mmHg

10 mmHg

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120
FIG. 10

Flow in channels (top view)

0.1 to 25 micron thickness
bonding of sandwich device
(side view)

printing
FIG. 13A

FIG. 13B

FIG. 13C

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8 sample wells aligned for multi-channel pipetting

Single outlet

Prime inlet

FIG. 14
FIG. 15

- Fluidic connect waste trap
- Buffer, dry, wet, mix wells (sealed)
- 8-tip
- Blood trough

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INTERNATIONAL SEARCH REPORT

International application No. PCT/US2019/022965

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(8) - B01L 3/00; B01B 1/00; B01F 13/00 (2019.01)
   CPC - B01L 3/5027; B01L 3/5027/53; B01L 3/5027/69; B01L 3/5027/15; B01F 13/0059; B01L 2200/027 (2019.02)

   According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

   Minimum documentation searched (classification system followed by classification symbols)
   See Search History document

   Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
   USPC - 422/50; 422/502; 422/504 (keyword delimited)

   Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
   See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>A</td>
<td>US 2015/0024965 A1 (ILLUMINA INC) 22 January 2015 (22.01.2015) entire document</td>
<td>1-21</td>
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□ Further documents are listed in the continuation of Box C. □ See patent family annex.

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