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Utility of microfluidic devices to study the platelet-endothelium interface

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Abstract

The integration of biomaterials and understanding of vascular biology has led to the development of perfusable endothelialized flow models, which have been used as valuable tools to study the platelet-endothelium interface under shear. In these models, the parameters of geometry, compliance, biorheology and cellular complexity are varied to recapitulate the physical biology of platelet recruitment and activation under physiologically relevant conditions of blood flow. In this review, we summarize the mechanistic insights learned from perfusable microvessel models and discuss the potential utility as well as challenges of endothelialized microfluidic devices to study platelet function in the bloodstream *in vitro*.

Keywords

vessel barrier; endothelial cell; shear; VWF

Introduction

The endothelial cell (EC) layer of the vessel wall serves an important function as the barrier between the blood and extravascular tissue space. At sites of vascular injury, blood platelets are rapidly recruited and activated to serve as a site of catalysis for thrombin generation and fibrin formation to form a hemostatic plug and staunch the loss of blood. Under physiologic laminar flow conditions, the ECs that comprise a healthy vessel prevent activation and aggregation of platelets in the bloodstream through membrane endonucleotidases (CD39/ATPase), which hydrolyzes adenosine diphosphate (ADP) molecules released by activated platelets, as well as by secreting prostacyclin (PGI₂) and, perhaps the most effective platelet inhibitor, nitric oxide (NO).[1–5] Moreover, the endothelium efficiently inhibits generation

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Declaration of interest

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of a potent platelet agonist, thrombin, through the catalysis of protein C to the anticoagulant serine protease, activated protein C (APC), in a thrombomodulin (TM)-dependent manner. [6] The roles of the pro/anticoagulants produced by the endothelium have been studied in reductionist approaches through, for example, the addition of a NO donor, soluble TM and other reagents in microfluidic platforms in order to study their individual roles in maintaining vessel patency and platelets in a quiescent state.[7–9]

Under physiologic conditions, the surface of the endothelium is covered by a glycosaminoglycan-rich layer, termed the glycocalyx, which facilitates EC mechanotransduction and expression of NO in a shear stress-dependent manner. Moreover, the glycocalyx serves as an antiadhesive barrier between the healthy vessel and cells in the bloodstream, including platelets and leukocytes (Figure 1 and Table 2).[10–12] Loss of the glycocalyx has been implicated in acute and chronic diseases, such as sepsis, atherosclerosis and diabetes, and is associated with thrombohemorrhagic complications.[13] The composition of the glycocalyx is dependent upon the physical biology of the vessel microenvironment including the cross-sectional area and shear rate.[10,14]

The subendothelial compartment of the vessel is lined with extracellular matrix (ECM) proteins, such as collagen and laminin, which when exposed to the bloodstream upon injury to the EC layer or loss of homotypic EC intercellular gap junctions facilitates platelet recruitment, activation, aggregation and thrombin generation in a shear-dependent manner (Figure 1).[15,16]. Moreover, platelets can bind directly to activated ECs at sites of inflammation.[17] Under these conditions, activated ECs facilitate recruitment of platelets through the secretion of the adhesive protein von Willebrand factor (VWF), expression of tissue factor (TF), the initiator of the extrinsic cascade of coagulation, and increased expression of E- and P-selectin, which facilitates recruitment of leukocytes in a PSGL-1-dependent manner.[18,19]

The biophysical properties of blood flow play a key role in regulating the distribution of blood cells within the bloodstream,[20] maintenance of EC layer barrier integrity[21,22] and EC gene and protein expression profiles.[23] Platelet interactions with ECs and other cells in the vasculature are profoundly altered by blood flow dynamics.[24–26] Thus, perfusable endothelialized microfluidic models (Table 1) hold potential for dynamic studies of the platelet-endothelium interface in health and disease states including evaluation of the efficacy and safety of pharmacological agents designed to treat or prevent cardiovascular diseases.

Annular perfusion models

The ‘explant’ single channel chamber devices were initially introduced into the platelet research field in the 1970s. The original chambers consisted of inverted isolated vessel segments mounted on a central rod (Table 1) within a perfusion tube and were utilized to study platelet adhesion under flow as compared to *in vivo* studies using balloon catheters in murine models.[27–29] In annular perfusion models, regions of a blood vessel are denuded to expose ECM proteins. These studies were among the first to validate the concept that perfusable *ex vivo* systems could be used to study platelet dynamics and subsequently

spiked interest in studying the platelet-EC interface under flow. Specifically, these annular perfusion model studies were among the first to identify the contribution of the von Willebrand factor (VWF)–coagulation factor VIII axis to platelet interactions with subendothelial ECM under flow conditions.[28,29]

Annular perfusion models are still used to study platelet function under shear. For example, an annular perfusion model was recently employed to demonstrate the role of platelet internalization of recombinant coagulation factor VII in hemostasis.[30] This technique allows for the study of the contribution of native components of the vessel wall; however, the evaluation of platelet adhesion requires sectioning of the perfused vessel and extensive histological processing, which limits the real-time evaluation of platelet-endothelial cell interactions.

Parallel-plate flow chambers and microcapillaries

Introduction of EC culture into optically-clear chambers to be used under flow enabled real-time studies and evaluation of interactions between blood cells and the endothelium.[26] In early work, flat parallel-plate flow chambers consisted of a microscope cover slip coated with ECs and integrated into a plastic flow chamber, as shown in Table 1.[31,32] Later, Nash *et al.* introduced a simplified protocol using endothelializeable borosilicate glass capillaries that allowed for expansion and increased throughput of studies of platelet-leukocyte-endothelial cell interactions, in particular.[33]

The ability to conduct experiments under shear flow conditions allowed researchers to discriminate the roles of platelet receptors and proteins in the (patho)physiology of platelet dysfunction-related diseases, such as Bernard-Soulier syndrome (BSS). BSS is a bleeding diathesis disorder associated with a qualitative or quantitative deficiency in the platelet receptor, glycoprotein (GP)Ib, which binds VWF in a shear-dependent manner and thus is required for the initial step of platelet recruitment from the bloodstream during hemostasis in arteries and arterioles.[34] As platelets are able to bind to ECM proteins, such as collagen and laminin, via integrins $\alpha_2\beta_1$ and $\alpha_6\beta_1$, respectively, to activate via the GPVI receptor under static conditions, the role of GPIb only became evident when experiments were performed under shear.[25,35,36] In particular, platelet recruitment to the endothelium was found to be mediated by the shear-dependent binding of the platelet receptor complex GPIb-IX-V with VWF which has undergone conformational activation to expose the A1-domain.[37,38]

VWF monomers are stored in Weibel-Palade bodies of ECs and α -granules of platelets. VWF multimers are released upon cell activation by the serine protease thrombin, hormones or inflammatory cytokines.[39,40] Parallel-plate flow chamber studies showed that ultra-large multimer forms of VWF are efficiently processed and removed by the VWF-cleaving protease, ADAMTS13 (a disintegrin and metalloprotease thrombospondin type 1 motif, member 13), on cultured ECs under shear.[41,42] These studies provided a mechanism behind the thrombotic complications seen in patients with Thrombotic Thrombocytopenic Purpura (TTP), which is associated with conformational changes in ADAMTS13 and thus results in the persistence of ultra-large VWF multimers.[43] Imaging of activated VWF in

endothelialized parallel-plate flow chamber studies, validated by studies in harvested vessels, are examples of the utility of microfluidic device technologies towards the development of non-invasive diagnostic approaches to assess disease states such as TTP.[44] Similar studies have revealed potential mechanisms of thrombotic complications in atherosclerosis, associated with platelet and EC activation and increased VWF expression. [45,46]

The utility of parallel-plate flow chambers in combination with micropatterning technologies have been used to study the role of the physical biology of ECs in regulating barrier function and vessel patency. These studies have shown that increased levels of shear stress upregulate gene and protein expression of anticoagulant and platelet inhibitory factors, including TM, tissue factor pathway inhibitor (TFPI) and nitric oxide synthase 3 (eNOS).[23] The biophysical properties of blood flow were further shown to play roles in maintaining EC layer integrity. For example, as compared to stasis or perturbed flow, laminar flow has been shown to promote enhanced EC migration towards denuded regions of EC monolayers. [47,48]

In summary, custom-built parallel-plate flow systems have been widely used for mechanistic studies of the role of flow dynamics in maintaining EC layer morphology and patency of mid-diameter vessels (100 – 200 micron separation gap).[49] Recently a number of commercially-available flow chamber systems have become available, increasing the adoption, standardization and miniaturization of flow studies.[50,51] Limitations remain for parallel-plate flow devices largely based on the fact that many of this platforms are made of glass or silica and are thus gas-impermeable, stiff and unamenable for fabrication for high-throughput studies,[52–54] not to mention cost-prohibitive in the case of commercially-available single-use products.

Compliant straight channel devices

The advent of the use of an optically-clear, inert, non-toxic, gas permeable and cost-effective polydimethylsiloxane (PDMS) polymer for the production of flow chambers in the 1990s expanded device design capabilities to nanometer dimensions.[55–58] Building on a broad knowledge of biorheology, channels were designed to achieve specified shear and specific flow dynamics, and printed onto a master mold using photolithographic techniques. Microfluidic devices are typically prepared by plasma or vacuum bonding the PDMS chambers, made by curing PDMS over master molds, onto a microscope cover slip.[59] The use of a compliant material such as PDMS helped to mimic vessel deformation and changes in blood viscosity due to shear thinning and pressure drops found in the microvasculature *in vivo* under high-flow conditions.[60–62]

Endothelialized PDMS microfluidic devices have facilitated simultaneous studies of multiple shear conditions from high physiologic to pathologic rates of 500 – 30,000 s⁻¹, regimes which have been shown to promote VWF multimer formation.[63,64] A channel design mimicking vessel narrowing, such as occurs due to atherosclerotic plaque formation, tested in parallel to an *in vivo* murine model of atherosclerosis has shown that vessel constriction leads to increased VWF release and VWF-dependent platelet aggregation to the intact

endothelium immediately downstream of the constriction.[65] PDMS-based platforms have shown promise for use as clinical diagnostic devices in assessing the thrombotic potential of patients on antiplatelet therapy[66] or to perform mechanistic studies to define the ADAMTS13 domains required for VWF cleavage.[67] These studies may assist in development of targeted therapies for the treatment of patients with debilitating diseases such as chronic TTP by allowing high throughput screening of known ADAMTS13 mutations.[68]

Another advantage of PDMS microfluidic technology is the ability to have user-defined spatial control of the microenvironment properties. For instance, these platforms allow for the control of the spatial organization of surface coatings of adhesive or ECM proteins, cells and the controlled release of agonists and inhibitors.[7,69–72] As an example, in one study human umbilical ECs were focally injured by an electrode in order to study the interaction of platelets with activated and adjacent healthy ECs.[73] The results showed that while activated ECs lost their tight junctions and released their Weibel-Palade bodies to promote platelet recruitment and adhesion, ECs upstream and downstream of the site of focal injury maintained their quiescence state and barrier function under physiological flow conditions.

ECs play an important role in the progression of many diseases including sepsis and cancer, both of which are multi-faceted dynamic processes. In sepsis, the dysfunction of vascular ECs has been correlated with multiple organ failure and poorer outcomes.[74] Recently, 3D perfusable, functional microvessels have been produced with cylindrical microvessels embedded in an ECM material.[75,76] These models were used for the study of endothelial barrier leak, which presents in both cancer and infection, in a quantitative and kinetic manner with direct observations of a role for platelets in immunity-related processes within the vasculature.[75,77–79] Incorporation of subendothelial matrix proteins in these 3D microvessel devices expanded the capacity of the microfluidic studies to investigate metastatic cancer cell and lymphocyte intravasation and extravasation, respectively, [76,80,81] and enabled the control of physical parameters such as transmural pressure and interstitial flow through the ECM[82] to study platelet-endothelium dynamics. Lastly, these microvessels chambers exhibit a physiologically relevant cylindrical geometry, which may prove useful for the modeling of smaller diameter microvessels where curvature has been shown to affect endothelial morphology and response to shear stress.[83]

Compliant branching network and multi-bypass devices

Microvessel network geometries dictate shear gradients and blood cell distribution profiles as well as biotransport of proteins and other molecules within the vasculature, all of which influence the dynamics of the platelet-endothelium interface.[84–86] *In vivo*, ECs at vascular branch points and bifurcations have been shown to endure disturbed blood flow profiles,[87] which have been implicated in vessel susceptibility to thrombosis and atherosclerotic plaque formation or rupture.[52,88,89] PDMS microfluidic devices have been used to show that perturbations of flow due to engineered stenosis and vessel branch points directly affect EC expression parameters of vascular cell adhesion molecules, such as VCAM-1, NO and the integrity of gap junctions between ECs.[90,91] Furthermore,

engineered microvessel network geometries have been used to show the role of differential VWF multimer assembly and shear on platelet recruitment and activation.[92–94]

Perfusable endothelialized PDMS microfluidic devices have been developed to study the role of flow in complex microvessel network niches. Recent developments include studies of blood cell interactions in complex microvascular geometries in hematologic diseases,[95] lung assist devices,[96] the development of platelet bioreactors mimicking bone marrow venules[8] and biodegradable implantable devices for studying the cardiac and hepatic microvasculature.[97–99] A concern about the PDMS material absorbance of small hydrophobic molecules and subsequent toxicity in long-term culture still remains.[100,101] A further limitation of current PDMS microfluidic technologies is the difficulty to utilize these platforms to study flow regimes characterized by high Reynolds numbers without requiring the use of supraphysiological shear rates, thus making it difficult to emulate inertial driven secondary flows that could potentially occur at stenoses, valves, or bifurcations in larger veins and arteries.[102]

Conclusions and future perspectives

There is strong evidence for the potential of microfluidic device technologies to be used to study dynamic interactions of platelets and ECs under flow conditions.[49,76,103] Current flow chamber design models (Table 1) allow for mechanistic studies of platelet adhesion to the vessel wall at sites of injury or inflammation. Endothelialized microfluidic devices have been utilized to demonstrate that shear stress is an important factor in regulating endothelial barrier integrity and vessel patency. Advances in microfluidic device design allow for high-throughput and dynamic real-time studies of cellular and molecular mechanisms that regulate platelet-endothelium interactions in disease states ranging from atherosclerotic plaque formation to sepsis and cancer angiogenesis and metastasis (Table 2). These studies may provide insight into the (patho)physiology of different disease states and serve as an expedient platform for therapy design and testing.

The platelets-endothelium interface research is complex, as platelets and ECs exhibit a bidirectional relationship which is heavily dependent on physiological factors that can be recreated in microfluidic devices in a reductionist manner. Inflammation and exposure of the vasculature to oxidative stress, via reactive oxygen species (ROS), has been shown to decrease TM production[104–106] and CD39 activity,[107] promote assembly of hyperadhesive VWF[104,108] and thus increase the recruitment of platelets.[109] Tissue necrosis factor α (TNF α), a prevalent cytokine associated with sepsis, has been shown to directly promote EC activation, decrease barrier function and increase procoagulant TF expression.[66,81,110] Platelets have been shown to assist metastasis and the intravasation of tumor cells across the endothelial cell layer through the expression and secretion of lysyl oxidase (LOX) and matrix metalloproteinases (MMPs).[80] Moreover, platelet granules contain an abundance of growth factors, such as VEGF and PDGF, which has been shown to promote tumor cell growth and angiogenesis *in vitro* and *in vivo*. [111–113] Furthermore, endothelial cell-bound platelets have been shown to directly recruit circulating tumor cells, which may play a role of seeding of cancer cells in specific vascular beds.[113–116]

A major question to still be answered is how to sustain platelet activity required for endothelial barrier repair whilst avoiding (patho)physiologic platelet function at the vessel wall under different disease states. Endothelialized microfluidic flow platforms represent advantageous models for the identification of safe and effective targets that could attain this balance; however, as with any *in vitro* system, a healthy skepticism remains concerning the physiological significance and translational potential of the findings attained in endothelialized microfluidic systems. Investigators employing microfluidic device technologies must keep in mind the physiological factors, such as presence of pulsatile flow, [117] microvascular network geometries [118] and thrombin generation that significantly affect the platelet-endothelium interface *in vivo*. Initial studies utilizing microfluidic technologies to study the platelet-endothelium interface have been performed either in purified systems or utilizing anticoagulated whole blood. [73,79,119,120] A limitation of these initial studies surround that fact that the right-angles of the microfluidic devices do not recapitulate the physical biology of the vasculature. Relatively few attempts have been made to include pulsatile flow in microfluidic studies, [121] despite the physiological relevance of pulsatile flow in the study of the platelet-endothelium interface.

Special attention needs to be given to the role of coagulation in any studies assessing the platelet-endothelium interface, as thrombin generation is essential for the maintenance of endothelial barrier function, while increased thrombin generation is a hallmark of a number of pathological conditions associated with cancer, inflammation and sepsis. Thrombin directly activates platelets, cleaves fibrinogen to form fibrin, and promotes ADAM10-dependent VE-cadherin proteolysis leading to EC dissociation and gap formation, all of which are hallmarks of acute inflammatory responses associated with sepsis and may lead to multiple organ failure and poorer outcomes. [74,122–125] Current studies utilizing microvascular networks are focused on understanding the role of intravascular thrombin generation and flux under shear [126] on EC layer barrier function and platelet activation and consumption downstream of sites of EC injury and inflammation. [127]. Multiple approaches have been explored for the identification of safe and effective inhibition of thrombin generation to preserve EC barrier function in inflammatory and infectious diseases, however clinically the primary focus has been on inhibiting the extrinsic and the common pathways of coagulation [128–130] or platelet aggregation via inhibition of GPIIb/IIIa. [131] These however are at the risk of limiting the physiologically relevant functions of platelets and ECs in hemostatic, immunologic and other processes which require the generation of thrombin. New studies are emerging employing endothelialized microfluidic platforms to evaluate the anticoagulant potential of using recombinant thrombin molecules that retains non-thrombotic thrombin functions [132,133] and the development of targeted therapies to inhibit thrombin generation via the contact activation pathway of the coagulation pathway. [134–138]

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Figure 1. The platelet-endothelium interface

Under laminar flow (depicted with arrows on left), healthy endothelial cells prevent platelet adhesion and aggregation by secreting soluble nitric oxide (NO) and prostacyclin (PGI₂). The surface of the endothelium is covered by an anti-adhesive layer of glycocalyx and expresses endonucleases (CD39), which hydrolyzes adenosine diphosphate (ADP), and thrombomodulin (TM), which promotes reduction of thrombin and an increase in antithrombotic activated protein C (APC), all of which serve to maintain platelets in their quiescent state. Upon vessel injury, platelets are rapidly recruited to subendothelial extracellular matrix (ECM) proteins via platelet glycoprotein (GP) Ib binding to the adhesive protein von Willebrand factor (VWF). Platelets are activated downstream of the collagen receptor GPVI leading to glycoprotein IIb/IIIa (GPIIb/IIIa)-dependent aggregate formation to prevent blood loss. Activated platelets release adenosine diphosphate (ADP) and thromboxane (TXA₂) and provide a surface for thrombin generation, all of which feedback to promote secondary recruitment and activation of platelets from the bloodstream to form a hemostatic plug.

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Table 1

Summary of perfusable endothelialized models

Diagrams and photographs of the basic set up for various microfluidic techniques are summarized. Advantages, limitations, and major findings are listed for each platform. For annular perfusion model, an electron microscopy image of a processed vessel section post perfusion is shown.[44]

Basic set up		Notes
<div><div>Annular</div><div> </div><div> </div></div>	<p><i>Advantages:</i> Use of native vessel layers to study the platelet-endothelium interface.</p> <p><i>Limitations:</i> Visualization requires histologic processing; use requires large volumes.</p> <p><i>Major findings:</i> von Willebrand Factor (VWF)-VIII complex mediates platelet adhesion to denuded vessels under shear.[27–29]</p> <p>Platelet internalization of recombinant coagulation factor VII.[30]</p>	
<div><div>Parallel-plate</div><div> </div><div> </div></div>		
<div><div>Microcapillaries</div><div> </div><div> </div></div>	<p><i>Advantages:</i> Real-time studies of platelet-endothelium interaction under shear; simple set-up.[26,31–33]</p> <p><i>Limitations:</i> Non-compliant, non-gas permeable, macroscopic scale, expensive fabrication of microvessel network geometry.[52–54]</p> <p><i>Major findings:</i> Identification of the roles for platelet receptors under shear, for instance a shear-dependent role for the platelet receptor GPIIb in the pathophysiology of Bernard-Soulier syndrome (BSS);[25,35–38,139]</p> <p>VWF multimer processing by ADAMTS13 under shear.[41,42,44–46]</p> <p>Effect of shear on endothelial barrier function and receptor expression.[23,47–49]</p>	

Notes

Advantages: Compliant, gas permeable and cost-effective material; ability to form microscale vessels; study of 3D cylindrical channels surrounded by an ECM layer; study complex blood vessel network morphoelogy, shear gradients and platelet distribution.[7,55–62,69–72,76]
Limitations: Potential for the absorption of hydrophobic or toxic substances by PDMS after prolonged use.[100,101] limitation in high Reynold number flow designs.[102]
Major findings: Role of shear on VWF multimer expression and processing and VWF-mediated platelet aggregation on an intact endothelium immediately downstream of a region of high shear stress.[63–65]
Identification of specific ADAMTS13 domains required for VWF processing.[67]
Study of endothelial barrier leak under (patho)physiological conditions.[75,77–79]
Effect of vessel geometry and flow disturbance on EC morphology.[83] EC receptor expression profile.[90,91] and thrombus formation.[92–94]
Study of complex microvasculature networks and bioreactors.[8,95,97–99]

Basic set up

Table 2

The effect of the physical biology of blood flow on the endothelium and platelets.

Setting	Endothelium	Platelets
Laminar flow, intact endothelial barrier	Anti-adhesive layer, glycocalyx[10–14] CD39/ATPDase expression[1,4] Soluble antiplatelet molecules: nitric oxide (NO) and prostacyclin (PGI ₂)[2–4] Anticoagulants: thrombomodulin (TM) and activated protein C (APC)[6]	Circulate as quiescent surveyors of the vessel wall[20]
Endothelial damage	Exposure of extracellular membrane (ECM) proteins: collagen, laminin, etc.[15,16] Release of von Willebrand factor (VWF)[40]	Adhesion to ECM proteins via GPIb and GPIIb/IIIa homodimers[36] Platelet activation and release of ADP and TxA ₂ [5] Platelet aggregation via GPIIb/IIIa homodimers[36]
Perturbed flow	Development of gap-junction leak[21,22,91] Decrease in NO and PGI ₂ secretion[23] Release of VWF[40,65]	Shear-induced platelet activation[65,86,94]
Inflammation Sepsis	TNFi-induced activation, VWF release and TF surface expression[66,81,110] ROS-mediated hyperadhesive VWF assembly[104,108] and TM inactivation[104–106] CD39/ATPDase inactivation[107]	ROS-induced platelet activation[109] Thrombin-induced platelet activation[6] Heterotypic platelet-leukocyte aggregate formation and adhesion to vessel wall[81]
Tumor Metastasis	Similar phenomena associated with inflammation Tumor cell-induced lysyl oxidase (LOX) and matrix metalloproteinases (MMPs) upregulation which facilitates intravasation[80]	Binding and protection of circulating tumor cells (CTCs)[115,116] Release of PDGF and VEGF[113]