Endothelial cell culture in microfluidic devices for investigating microvascular processes

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Numerous conditions and disease states such as sickle cell disease, malaria, thrombotic microangiopathy, and stroke significantly impact the microvasculature function and its role in disease progression. Understanding the role of cellular interactions and microvascular hemodynamic forces in the context of disease is crucial to understanding disease pathophysiology. *In vivo* models of microvascular disease using animal models often coupled with intravital microscopy have long been utilized to investigate microvascular phenomena. However, these methods suffer from some major drawbacks, including the inability to tightly and quantitatively control experimental conditions, the difficulty of imaging multiple microvascular beds within a living organism, and the inability to isolate specific microvascular geometries such as bifurcations. Thus, there exists a need for *in vitro* microvascular models that can mitigate the drawbacks associated with *in vivo* systems. To that end, microfluidics has been widely used to develop such models, as it allows for tight control of system inputs, facile imaging, and the ability to develop robust and repeatable systems with well-defined geometries. Incorporating endothelial cells to branching microfluidic models allows for the development of “endothelialized” systems that accurately recapitulate physiological microvessels. In this review, we summarize the field of endothelialized microfluidics, specifically focusing on fabrication methods, limitations, and applications of these systems. We then speculate on future directions and applications of these cutting edge technologies. We believe that this review of the field is of importance to vascular biologists and bioengineers who aim to utilize microfluidic technologies to solve vascular problems. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5024901>

INTRODUCTION

As the site of numerous, continuous interactions between blood cells and the endothelium, the microvasculature plays a key role in the pathophysiology of a variety of disorders including sickle cell disease, malaria, microangiopathy, and stroke.^{1–4} Investigating these interactions and the hemodynamic forces which drive them is crucial to fully understanding the pathophysiology of these etiologies. The physical geometry of the microvasculature plays an important role in the hemodynamic forces and cellular interactions which occur within the microvasculature.⁵ As an example, studies have described the preferential aggregation and activation of platelets in vascular regions where wall shear stress is increased.^{6,7} Furthermore, many complications associated with these conditions originate in the microvasculature, such as pain crisis and stroke in sickle cell disease.⁸ Therefore, understanding the pathophysiology of these diseases requires a

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robust model of the microvasculature that is capable of recapitulating microvascular environments.

The inability to tightly control system geometry, blood flow rates, and experimental inputs, as well as imaging challenges, precludes the use of *in vivo* models for studying microvascular phenomena. Furthermore, *in silico* simulations of these interactions are difficult to achieve, as the number of interactions occurring at a given location and time frame in the microvasculature is vast.⁹ Thus, *in vitro* microvascular models are ideally suited platforms for investigating these microvascular events as they address all the shortcomings of *in vivo* systems and eliminate the difficulties associated with simulation models. To that end, microfluidics has been utilized to recapitulate complex environments found *in vivo*.^{4,10–12} In this review, we will summarize the growing field of “endothelialized” microfluidics, in which endothelial cell monolayers are incorporated into microchannels to generate blood vessels-on-a-chip (Fig. 1).¹³ We will specifically discuss the development and fabrication of branched microfluidic devices, as these devices best represent the complexities and branching nature of the *in vivo* vasculature. These systems are ideal for studying cellular interactions that occur within the vasculature, as branched structures mimic the forces found within the vascular systems. This enables cellular interactions to occur within these systems that correspond to the interactions that occur *in vivo*. We will particularly highlight the benefits, limitations, and applications of using these technologies to investigate the cellular interactions that occur within the microvasculature, as well as future directions that the field is headed in. By focusing on the fabrication of these technologies in particular, we provide a blueprint for researchers trying to enter or advance the field of endothelialized microfluidics.

MICROFLUIDIC TECHNOLOGIES ARE USED TO RECAPITULATE THE MICROVASCULATURE *IN VITRO*

Pioneering microfluidic technologies developed around the turn of the century proved that designing small fluidic channels that could potentially simulate the vasculature was possible.¹⁴ This technology typically requires the fabrication of small channels (channel dimensions on the scale order of microns) within a polymer substrate, which can be connected to syringe pumps for the purpose of fluid flow.¹⁵ Multiple advantages associated with microfluidic technologies make these systems ideally suited for recapitulating *in vivo* systems. The geometry of the flow channels can be tightly controlled using microfluidic fabrication techniques, allowing for the investigation of hemodynamic forces and cellular interactions which occur at specific geometric structures within the microvasculature. Investigation of vascular phenomena in these geometrically complex, bifurcating regions has led to important discoveries, such as the mechanisms of vessel wall remodeling reported by Meng *et al.*^{16–18} Moreover, utilizing microfluidic techniques to construct *in vitro* microvascular models facilitates robust imaging of these systems, as microfluidic devices are traditionally fabricated using optically transparent substrates that are highly compatible with various microscopy techniques.^{19,20} Furthermore, these optically transparent substrates are typically biologically inert, enabling investigation of a particular biological

FIG. 1. Endothelialized microfluidics simulates the *in vivo* microvasculature. (a) Macroscale view of a typical endothelialized microfluidic architecture. (b) Micrograph highlighting the formation of an endothelial monolayer lining microfluidic channels. Reproduced with permission from Myers *et al.*, J. Vis. Exp. (64), e3958 (2012). Copyright 2012 Jove.

interaction of interest without any unnecessary confounding variables introduced by the experimental setup. Finally, the ability to tightly control the system inputs in a microfluidic system allows researchers to isolate a particular biological interaction for investigation, representing a significant improvement over biologically complex *in vivo* models.

Microfluidic platforms for *in vitro* investigation of microvascular phenomena are typically developed using a technique known as soft lithography.^{21,22} This technology utilizes lithography techniques pioneered by the semiconductor industry, in which the patterned exposure of a photosensitive material is used to create master molds which are then used to define the structure of the microfluidic channels. Replica molding of the biologically inert, optically transparent, gas permeable polymer polydimethylsiloxane (PDMS) is the primary method used to fabricate microfluidic systems.²³ Soft lithography has been used to produce a variety of microfluidic systems capable of recapitulating a plethora of microvascular phenomena, geometries, and hemodynamic forces. Zilberman-Rudenko *et al.* reported a system in which microfluidic ladder networks with multiple bypass networks are used to simulate the complex fluid dynamics that occur in the branch points in the vasculature.²⁴ Lu *et al.* report a system in which they introduce vessel size and oxygen gradients into their *in vitro* system in order to simulate the various degrees of blood oxygenation throughout the *in vivo* vasculature.²⁵ Lamberti *et al.* reported a system in which a microfluidic channel is fabricated in close proximity to a large “tissue” compartment (i.e., a compartment in the microfluidic device that can be filled with different cell types to recapitulate multiple tissue environments) separated by a porous barrier.²⁶ This system enables the study of transport between the vasculature and the desired tissue of study. Stauber *et al.* have developed a microfluidic system consisting of a large chamber with regularly spaced posts which simulates the sheet flow found within the capillary vasculature of the lungs.²⁷ Overall, soft lithography has the capability to produce robust and repeatable geometries that mimic a wide variety of microvascular environments and conditions.

While this technique is very robust and repeatable, there are several limitations. Soft lithography requires specialized engineering training and expensive equipment. A researcher must have access to sophisticated cleanroom facilities and the training required to operate complex lithography tools and handle hazardous reagents. This labor and resource intensive technique restricts the ability to iteratively develop working systems and perform experiments within *in vitro* microvascular environments. This is a significant limitation in the field of microfluidics-based microvascular research. To that end, many innovative techniques have been developed which do not require photolithography to simulate microvascular environments. One such technique of particular importance due to its simplicity is the “do-it-yourself” method developed by Mannino *et al.*²⁸ This technique utilizes off-the-shelf materials commonly found or easily obtainable in bioengineering laboratories alone to generate robust and repeatable microfluidic channels. Poly(methyl methacrylate) (PMMA) optical fiber (500 μm diameter) is encased in, and subsequently removed from, PDMS in order to form microchannels that mimic the physical geometry of the *in vivo* environment. Simple adaptations and alterations can be made to the optical fiber generating different geometries found in the environment of the microvasculature. The resultant structures produced via this method display round lumens, a feature consistent with the *in vivo* microvasculature that is difficult to fabricate using traditional photolithography techniques.^{29,30} Similarly, Nguyen *et al.* have developed a technique utilizing needle extraction to fabricate channels in a biologically relevant hydrogel.³¹ Additionally, multiple sophisticated techniques such as 3D printing, dissolvable scaffold printing, inkjet printing, microfiber fabrication, and viscous fingering have all been used as alternatives to soft-lithography to generate *in vitro* microvasculature models.^{32–39} These techniques possess the distinct advantage of being able to simulate entire three-dimensional features rather than the two-dimensional planar features that traditional, photolithography-based fabrication methods are limited to. While these techniques mitigate some of the disadvantages of traditional photolithography based microfluidic systems, they suffer from their own disadvantages, including cost associated with various materials and equipment, as well as issues with repeatability of features.

In order to vascularize these micro fabricated channels, the channels are seeded with endothelial cells. In some cases, this step serves as its own fabrication step, a step we describe as

“self-assembly.” When devices are patterned in biologically inspired hydrogels, endothelial cells may be perfused into the device and allowed to self-assemble their own channels, a process known as angiogenesis, and the best accentuated studies were conducted by Chan *et al.* (Fig. 2) and Nguyen *et al.*^{31,40} In these cases, the inclusion of endothelial cells allows the devices to self-assemble their own channels, allowing for the development of quite complicated geometries and features found only *in vivo*. While these technologies offer considerable advantages, they suffer from the drawback of an inability to be perfused. Since these channels are randomly allowed to grow, it is not possible to perfuse reagents in a controlled manner. If the self-assembled channels do not have an inlet and an outlet, perfusion is not possible, whatsoever. Even if by chance the self-assembled channels fully form with an inlet and an outlet that can be connected to external tubing, allowing perfusion, it is difficult to measure the size of the resulting channels and calculate flow rates within them. This poses significant challenges for studying the delivery of reagents, dyes, and antibodies for characterization of cellular phenomena within these devices. A summary of the benefits and disadvantages of the methods described in this section can be found in Table I.

“ENDOTHELIALIZATION” ADDS A BIOLOGICAL COMPONENT TO AND IMPROVES THE PHYSIOLOGIC CONDITIONS OF *IN VITRO* MICROVASCULATURE MODELS

Thus far, we have discussed fabrication of the physical structure of these *in vitro* microvasculature models. However, in order to better recapitulate the microvasculature, microfluidic technologies must incorporate the primary biological component controlling the cellular interaction within the vasculature, the endothelium. The ability to “endothelialize” a microfluidic channel (i.e., lining the inner luminal surface with a three-dimensional endothelial cell monolayer) is the primary feature of *in vitro* microfluidic microvascular models. *In vivo*, endothelial cells surround the blood vessel lumen and provide the primary barrier function of the blood vessel. Furthermore, endothelial interactions with various blood cells represent a major group of

FIG. 2. Endothelialized microfluidics self-assembles through angiogenesis. (a) and (b) Pre- and post-vascularized macro-views of the self-assembled microfluidic channels. (c)–(e) Stained micrographs of these endothelialized microchannels highlighting the endothelialized lumen. Reproduced with permission from Chan *et al.*, PLoS One 7, e50582 (2012). Copyright 2012 Author(s), licensed under a Creative Commons Attribution 4.0 License.

TABLE I. Comparison chart of various endothelialized microfluidic fabrication methods.

Microchannel fabrication technique	Size scale (μm)	EC compatibility	Perfusion	Self-assembly	References
Photolithography—PDMS	~ 1	✓	✓	X	13
Needle/wire extraction—PDMS	~ 200	✓	✓	X	28
Needle/wire extraction—Hydrogel	~ 25	✓	X	✓	31 and 52
3D printing	~ 400	✓	✓	X	32
Dissolvable scaffold printing	~ 300	X	✓	X	35
Inkjet printing	~ 200	✓	✓	X	37
Microfiber fabrication	~ 1	✓	✓	X	36
Viscous fingering	~ 500	✓	X	✓	39

cellular interactions within the microvasculature.^{41–43} Endothelialized microfluidic systems are ideally suited to studying these interactions. In order to endothelialize a microfluidic device, an extracellular matrix (ECM) protein is typically allowed to adsorb to the inner surface of the channels within microfluidic systems, providing the endothelial cells with physiological adhesive ligands to adhere to Refs. 13 and 44 (Fig. 1). Multiple groups have reported success in using 3 of the primary ECM proteins collagen, fibronectin, and laminin in order to promote endothelial attachment and healthy endothelial phenotype in microfluidic devices.^{13,45,46} Furthermore, microfluidic devices have been fabricated from a variety of substrates (e.g., Polystyrene and PDMS).^{46,47} The adhesive ECM proteins used to anchor endothelial cells are perfused through the microfluidic channels. When cells are anchored uniformly along the entire three-dimensional inner surface of the microchannels, these cells are cultured with endothelial cell media under continuous flow conditions. Eventually, the cells spread such that they form a monolayer uniformly across the entire inner surface of the microchannels, at which point, the devices are ready for experimentation.¹³

This general endothelialization technique had been adapted to multiple unique approaches. In order to endothelialize larger devices, rotational seeding was utilized by Mannino *et al.*²⁸ In traditional microfluidic systems, capillary and viscous forces (i.e., cells will spread in all directions within channels regardless of the orientation with respect to gravity) are the primary force interacting with cells.⁴⁸ However, in larger microfluidic devices (on the order of 500 μm diameter channels), gravity plays a larger role, pulling endothelial cells to the bottom of the channels and preventing proper monolayer formation. In these cases, rotation about the central axis of the microchannel is critically necessary to ensure successful seeding of endothelial cells and formation of the endothelial monolayer. Furthermore, some nontraditional techniques have been reported for endothelializing microfluidic devices. Hewes *et al.* modified an inkjet printer in order to bioprint free-standing microvessels via endothelial cell-laden alginate drops into a cross-linker bath. These drops were placed in a circular pattern which eventually formed a microchannel.³⁷ This novel microfluidic fabrication method and the endothelialization technique are unique in that the vector material used to add endothelial cells also creates the structure for the microchannels.

ENDOTHELIALIZED MICROFLUIDICS HAVE A WIDE VARIETY OF APPLICATIONS

Endothelialized microfluidic technology enables detailed investigation of cellular interactions which occur within the microvasculature, namely, interactions between blood cells and endothelial cells.^{28,49} Moreover, these techniques have been utilized to study complex microvascular pathophysiologies of a variety of etiologies. Jain *et al.* utilized a chemically preserved endothelium to evaluate platelet aggregation and hemostasis under different physiological and pharmacological conditions after prolonged storage.⁵⁰ Prolonged storage of biological microfluidics opens up the possibility that these technologies may be used in point-of-care settings. Degradable hydrogel materials, such as collagen and fibrin gels, can also be integrated into

microfluidics. The enzymatic degradability of these hydrogel matrices by cells enables the study of endothelium remodeling and cell transendothelial migration. These techniques have been adapted to oncological disorders. Jeon *et al.* reported a microfluidic system in which an endothelialized channel is placed in contact with a hydrogel simulating the extracellular space and is perfused with tumor cells.⁵¹ This system enables the study of tumor cell extravasation, which is a key component to understanding cancer metastasis.

As different organ systems contain physiologically distinct endothelial cell and vessel wall phenotypes, the composition of the endothelial monolayer within microfluidic devices can be customized to recapitulate the vasculature of a specific body region of interest. This concept is known as organ-on-a-chip. Multiple groups have employed hydrogel-integrated microfluidics to co-cultured different endothelial cells with organ specific cells to simulate the organ-specific microvasculature.^{52,53} For example, Chonan *et al.* developed a microfluidic system in which glioma initiating cells are co-cultured with endothelial cells in order to investigate the invasive properties of glioblastomas.⁵³ Wang *et al.* and Prabhakarpandian *et al.* described a neurological co-cultured microfluidic system that recapitulates the selective barrier function of the blood brain barrier (BBB).^{49,54} Jain *et al.* have recently demonstrated the co-culture of alveolar epithelial cells along with vascular luminal endothelial cells and shown organ-level responses to pulmonary injury, vascular inflammation, and thrombus formation.⁵⁵ These systems also have the capability to determine their own geometry. When imprinted into a collagen hydrogel, new microchannels, i.e., endothelial channels, will begin to branch off the original, designed, endothelialized channels, a process known as angiogenesis⁵⁶ [Fig. 3(a)]. Finally, Tsai *et al.* have modeled microvascular occlusion in microangiography by stimulation with tumor necrosis factor-alpha (TNF- α) and quantification of the resulting occlusions [Fig. 3(b)].⁴

In addition to recapitulating geometries, microfluidic technologies have been integrated with mechanical systems to recapitulate more sophisticated vascular phenomena. For example, microfluidic ratchets have been used to generate unidirectional liquid propulsion systems to tightly control the fluid flow in microchannels.⁵⁷ Furthermore, valves have been incorporated into microfluidic devices in order to tightly control fluid flow, fluidic resistance, mixing, and trapping particles such as cells.⁵⁸ One group, Sakurai *et al.*, has incorporated a valve into an endothelialized microfluidic in order to induce the opening of a channel perpendicular to the main endothelial channel.⁵⁹ This causes flow through the endothelial channel to be diverted through the new channel upon actuation of the valve, causing the endothelial monolayer to rupture, effectively producing an *in vitro* bleeding model. This bleeding model enables the study of hemostasis in real time. Overall, as the microvasculature is implicated in the pathophysiology of a myriad of disease states, these endothelialized microfluidic systems serve as key *in vitro* test beds for understanding the role of the microvasculature in disease.

CONCLUSIONS AND FUTURE DIRECTIONS

In vitro endothelialized microfluidic technologies provide researchers with a powerful tool for investigating the cellular interactions and the biological impacts of hemodynamic forces within the microvasculature. These techniques offer the ability to isolate specific, desired aspects of the microvasculature and shield experiments from the inherent variability of *in vivo* systems. As these *in vitro* systems offer tight control over the system geometry and inputs, they have been adapted to study a wide variety of diseases and microvascular phenomena including cancer, angiogenesis, sickle cell disease, and hemostasis. Typically, these devices are fabricated using photolithography and replica molding of PDMS. However, as these techniques suffer from time, training, cost, and equipment requirements necessary to develop them, non-traditional fabrication techniques (e.g., 3D-printing, electrospinning, inkjet printing, and “do-it-yourself” replica molding) have been used as alternatives to develop *in vitro* microvascular models. In spite of any drawbacks, these systems represent an important tool in the tool belt of microvascular biologists for studying the microvasculature and its role in disease.

In the future, endothelialized technologies will continue to be adapted to study a wide range of pathological and physiological phenomena which occur within the microvasculature.

FIG. 3. Endothelialized microfluidics may be applied to study many microvascular etiologies. (a) Endothelialized microfluidic model of angiogenesis. (i) Endothelialized microvessels begin to branch off main channels and sprout new channels in a hydrogel substrate with proangiogenic stimuli. (ii) The endothelial monolayer is highly permeable at locations of new sprouting points. Scale bars represent $100\ \mu\text{m}$. (a) Reproduced with permission from Zheng *et al.*, Proc. Natl. Acad. Sci. U. S. A. **109**, 9342 (2012). Copyright 2012 National Academy of Sciences. (b) Blood flow decreases and microvascular occlusions occur when endothelial cells are activated with TNF-A (Bottom) vs health control vasculature (Top). (b) Reproduced with permission from Tsai *et al.*, J. Clin. Invest. **122**, 408 (2012). Copyright 2012 American Society for Clinical Investigation.

Technological advances in microfluidic fabrication methods used to develop these technologies will lead to the development of more complicated and variable vascular geometries (e.g., blood vessels exhibiting complex tortuosity).⁶⁰ Fabrication of these physiologically accurate geometries facilitates more accurate recapitulation of the *in vivo* environment than is currently possible, enabling more physiologically relevant research. Furthermore, the advancements in microfluidic fabrication techniques may lead to rapid evolution in the field of personalized medicine. As an example, the endothelialized microfluidic techniques we have highlighted may be combined with clinical imaging (e.g., computed tomography, magnetic resonance imaging, and angiography) to develop patient specific models. In this case, clinical imaging may be used to provide accurate scans of patient-specific physiology, which created *in vitro* using microfluidic technologies and then used as a test-bed for potential therapies.^{61,62} Furthermore, patient cells may be combined with these technologies for patient specific organs-on-a-chip, facilitating the study of the impact of patient-specific treatment approaches.⁶³ Finally, utilizing the multi-cell co-culture of microfluidic devices will enable the development of more physiological environments that encompass multiple cell types and organ systems.^{64,65} Overall, endothelialized microfluidic technology has enabled researchers to study microvascular phenomena that were previously inaccessible via conventional models. As the field of endothelialized microfluidics continues to mature and advance using sophisticated technologies, researchers will have new avenues at their disposal to study the complex cellular interactions that occur within the microvasculature.

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