Endothelialized Microfluidics for Studying Microvascular Interactions in Hematologic Diseases

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Abstract

Advances in microfabrication techniques have enabled the production of inexpensive and reproducible microfluidic systems for conducting biological and biochemical experiments at the micro- and nanoscales [1,2]. In addition, microfluidics have also been specifically used to quantitatively analyze hematologic and microvascular processes, because of their ability to easily control the dynamic fluidic environment and biological conditions[3-6]. As such, researchers have more recently used microfluidic systems to study blood cell deformability, blood cell aggregation, microvascular blood flow, and blood cell-endothelial cell interactions[6-13].However, these microfluidic systems either did not include cultured endothelial cells or were larger than the sizescale relevant to microvascular pathologic processes. A microfluidic platform with cultured endothelial cells that accurately recapitulates the cellular, physical, and hemodynamic environment of the microcirculation is needed to further our understanding of the underlying biophysical pathophysiology of hematologic diseases that involve the microvasculature.

Here, we report a method to create an "endothelialized" *in vitro* model of the microvasculature, using a simple, single mask microfabrication process in conjunction with standard endothelial cell culture techniques, to study pathologic biophysical microvascular interactions that occur in hematologic disease. This "microvasculature-on-a-chip" provides the researcher with a robust assay that tightly controls biological as well as biophysical conditions and is operated using a standard syringe pump and brightfield/fluorescence microscopy. Parameters such as microcirculatory hemodynamic conditions, endothelial cell type, blood cell type(s) and concentration(s), drug/inhibitory concentration etc., can all be easily controlled. As such, our microsystem provides a method to quantitatively investigate disease processes in which microvascular flow is impaired due to alterations in cell adhesion, aggregation, and deformability, a capability unavailable with existing assays.

Video Link

The video component of this article can be found at http://www.jove.com/video/3958/

Protocol

1. Fabrication of the Endothelial Microdevice

1. Create a photomask by submitting a computer assisted design (CAD) drawing of the microfluidic device to an outside mask vendor. The mask used was composed of a chrome layer on soda lime glass. In this case the microfluidic channel width was 30 μm.
2. Clean a bare silicon wafer with piranha (10:1 ratio of sulfuric acid and hydrogen peroxide) for 15 minutes and dip in hydrofluoric acid for 30 seconds. Rinse with deionized (DI) water for approximately 10 seconds.
3. Using a spin coater, spin Microchem SU-8 2025 photoresist onto the wafer to a height of 30 μm. For SU-8 2025, a spin speed of 3000 rpm is recommended. Other viscosities of SU-8 are available which will achieve this height. Full instructions for SU-8 use are available at www.microchem.com
4. Place the wafer with SU-8 on a hotplate at 95 °C for 5 minutes to drive off excess solvent.

   Note: An oven will dry the wafer differently than a hotplate and is not recommended.
5. Place a mask with the desired feature shape over the wafer, and expose to UV light (160 mJ/cm^2 measured at 365 nm) in a mask aligner (Karl Suss, MA-6). This cross links the photoresist.
6. Place the wafer back on a hotplate at 95 °C for an additional 5 minutes to further accelerate the polymerization of SU-8.
7. Immerse the wafer in SU-8 Developer, composed primarily of PGMEA (propylene glycol methyl ether acetate) for 4 minutes to remove the non-cross-linked SU-8.
8. Rinse the newly developed wafer with 100% isopropyl alcohol (IPA) for 10 s. The wafer may then be dried using pressurized nitrogen or by allowing the solvent to evaporate from the wafer in a clean fume hood for several minutes.

9. Tape the edges of the dry wafer with patterned SU-8 into a petri dish to prevent movement.
10. Apply 1 mL of Sigmacote to the wafer using a pipette, cover with the top of the petri dish, and swirl wafer to ensure complete coating of the wafer, remove cover, and allow wafer to dry for several minutes until all solvent has evaporated.

2. PDMS (Polydimethylsiloxane) Preparation

1. Mix PDMS polymer and curing agent at a 10:1 ratio (w/w) and remove air bubbles using a vacuum desiccator. The length of time needed to degas the PDMS varies on the strength of the available vacuum system, but typically ranges from several minutes to one hour. For a six inch petri dish with no previously poured PDMS, a total volume of 60 mL of polymer is recommended.
2. Pour the mixture onto the wafer, approximately 5 mm thick. Also pour onto a flat bottom dish to create a thin sheet of PDMS, approximately 1 mm thick. Cure at 60 °C in an oven overnight.
3. Using a knife or scalpel, cut out around the cured PDMS device and remove it from the wafer. Additionally, cut a thin sheet of PDMS slightly larger than the device.
4. Create inlet and outlet holes in the PDMS device using a 1.0 mm hole punch. This may be accomplished using either a pin vice, Harris Uni-Core, or similar device.
5. Clean the surfaces of the device and the sheet using scotch tape.
6. Using a plasma cleaner, expose the surfaces of the PDMS device and PDMS sheet to oxygen plasma for 30 s. The oxygen plasma creates reactive species on the exposed surface of the PDMS which bond when brought into physical contact.
7. Connect smaller tubing to a small length (several centimeters) of large tubing, which in turn is connected to a syringe with a blunt-point needle, filled with 50 μg/ml fibronectin from human plasma in PBS. The tubing and needle are sized such that a friction fit is created between the tubing. Insert smaller tubing in the inlet of PDMS microdevice and apply pressure to the syringe to fill the channels completely with fibronectin solution and to create a small 100 μL drop at the outlet port, to ensure that the channels stay wet. Incubate the device at 37 °C for 40-60 min.
8. Connect a fresh syringe filled with PBS to the blunt point needle. Apply pressure to the syringe to rinse the device with PBS.

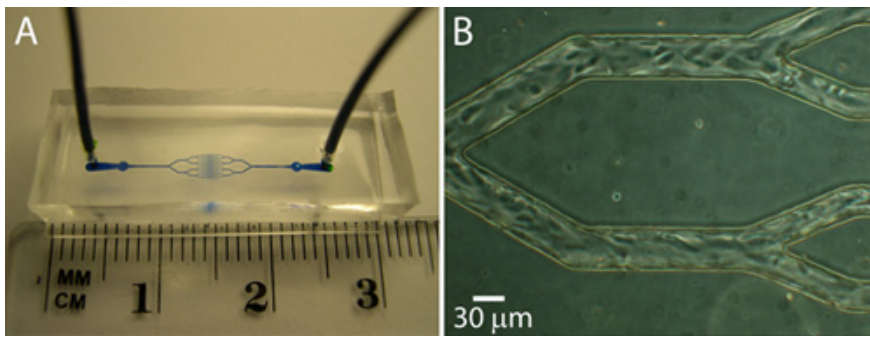## 3. Seeding the Microfluidic Device with Endothelial Cells

1. Prepare 1,000,000 cells/mL of human umbilical vein endothelial cells (HUVECs) in endothelial growth media with 8% dextran. A range of 500,000 to 2,000,000 cells/mL has been successfully used. Addition of dextran to the cell loading medium increases the viscosity of the fluid, which decreases the velocity of endothelial cells as they enter the fibronectin-coated microfluidic system. This, in turn, increases the likelihood that the cells will adhere and culture successfully within the microdevice.
2. Connect the new syringe and tubing as in Step 2.7. but with a longer tubing (approximately 1 meter in length).
3. To optimize the performance of this system, it is critical at this point to prevent any leaks or bubbles in the entire perfusion system; any leakage of solution or presence of bubbles in the media will change the flow and prevent successful seeding of endothelial cells. Therefore, tight fitting of the tubing is essential to eliminate leakage between any connections. Bubbles in the syringe and/or tubing must be eliminated before cells are introduced in the microdevice and the entire perfusion system should be primed with the cell solution before attaching the tubing to the microfluidic to avoid the introduction of air bubbles.
4. Using a syringe pump, infuse cell suspension into PDMS device at volumetric flow rate of 1.23 μl/min for 2 hours at 37 °C and 5% CO_2. Optimal results were achieved when the syringe pump was at the same height as the PDMS device. For the inlet, the longer tubing, which can be coiled within the incubator, ensures that the cells and media are adequately warmed before coming in contact with the device. In our system, the volumetric flow rate of 1.23 μl/min approximates a midstream velocity of ~1 mm/s in the smallest microchannels, corresponding to a wall shear stress of ~1 dyne/cm^2 at those channels using whole blood.
5. Using the same syringe pump and long tubing, perfuse fresh growth media for 2-8 days at ratio of 1.23 μl/min. A successful device will have a monolayer of endothelial cells growing on the inside of the device within 24-48 hours. Previous experiments have shown that confluent monolayers appropriately express VE-cadherin at cell-cell junctions throughout the device[14].
6. Inject blood or cell suspension into system for experimentation.

4. Representative Results

Using this protocol, standard lithographic microfabrication techniques are used to create the mold needed to produce the microfluidic channels that physiologically mimic the sizescale of the microvasculature (**Figure 1A**). Using an optimized perfusion technique, endothelial cells then seed and confluently culture the entire inner surface of the microfluidic system within 24-48 hours of cell seeding (**Figure 1B**). As the microfluidic system is transparent, the entire microdevice can be placed on a brightfield/fluorescence microscope stage for imaging and data collection.

Our system can then be applied to study hematologic diseases that involve altered biophysical properties, such as sickle cell disease, in which the increased rigidity of sickled red cells and aberrant leukocyte and endothelial adhesion contribute to microvascular obstruction. A clinically approved medication, hydroxyurea, ameliorates symptoms but its direct effect on microvascular flow is unknown. Our assay takes into account both cell rigidity and adhesion, and demonstrates that hydroxyurea significantly ameliorates flow in sickle cell disease (**Figure 2**).

Sickle cell disease is only one example of an application for the microvasculature-on-a-chip, as this system is ideally suited to study any hematologic process in which blood cells interact with each other and endothelial cells in the microvasculature. Other clinically relevant applications include inflammatory disorders, sepsis/lung injury, thrombotic microangiopathies, malaria, and cancer metastasis while more basic applications include leukocyte biology and hematopoietic stem cell biology, among many others.

Figure 1. A) the initial PDMS microfluidic device before endothelialization. Here, the microdevice is injected with food coloring to illustrate sizescale and overall design of the system. B) Brightfield microscopy shows the microfluidic system is completely endothelialized within 48 hours of cell seeding using the protocol described here.
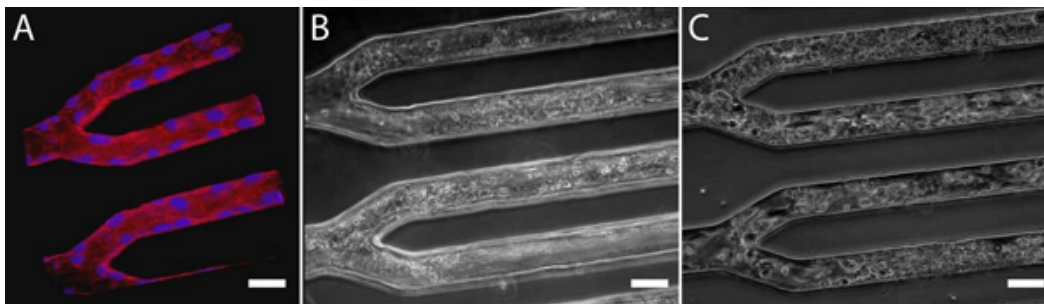
Figure 2. A) The microvasculature-on-a-chip with microchannels approximates the size of post-capillary venules (30 μm), the site of most sickle cell microvascular obstructive events. Whole blood from sickle cell patients receiving hydroxyurea and from patients not receiving hydroxyurea is flowed through two different microvasculature-on-a-chip devices. B) Whole blood from sickle cell patients receiving hydroxyurea flows with relative ease within the endothelialized microchannels. C) Under the same hemodynamic conditions, whole blood from sickle cell patients not receiving hydroxyurea, however, exhibits much more sluggish flow with microchannel obstruction. The bottom channel is completely obstructed with no flow and the flow velocities in the other endothelialized microchannels are significantly lower than in the hydroxyurea condition. The scale bar in all three images is 30 μm.

Discussion

Our endothelialized microdevice system is best suited when used in conjunction with *in vivo* experiments, and its reductionist approach may help elucidate the biophysical mechanisms of hematologic processes that are observed in humans and animal models. Furthermore, our system is not without limitations. For instance, our microfluidic channels are square in cross-section. Although technically circular microchannels can be fabricated[10,11], we opted to use a more simplified and standard fabrication procedure to allow other researchers to readily apply this system to their own work. In addition, the presence of the cultured endothelial cells naturally "rounds out" the effective lumen, enabling the system to be more physiologic. Furthermore, our fluid dynamic modeling reveal that the flow conditions in our system are comparable to that in the *in vivo* microvasculature. Finally, recent work characterizing blood flow in square and rectangular microchannels has shown that those geometries are suitable for blood rheology experiments[15].

Finally, our assay is not intended to measure, in isolation, distinct cellular biophysical properties that lead to microvascular occlusion. Techniques such as atomic force microscopy, micropipette aspiration, and optical trapping have been well characterized for those types of experiments. Instead, the value of our microsystem is its capability to recapitulate, simultaneously and within a single *in vitro* system, an ensemble of physiological processes and biophysical properties, including adhesion molecule expression, aberrant blood cell-endothelial cell interactions, blood cell aggregation (e.g. thrombosis), cell deformability, cell size/shape, microvascular geometry, and hemodynamics, all of which contribute to pathologic microvascular cellular interactions in different disease states.

Disclosures

No conflicts of interest declared.

Acknowledgements

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