Ultrathin Dual-Scale Nano- and Microporous Membranes for Vascular Transmigration Models


Selective cellular transmigration across the microvascular endothelium regulates innate and adaptive immune responses, stem cell localization, and cancer cell metastasis. Integration of traditional microporous membranes into microfluidic vascular models permits the rapid assay of transmigration events but suffers from poor reproduction of the cell permeable basement membrane. Current microporous membranes in these systems have large nonporous regions between micropores that inhibit cell communication and nutrient exchange on the basolateral surface reducing their physiological relevance. Here, the use of 100 nm thick continuously nanoporous silicon nitride membranes as a base substrate for lithographic fabrication of 3 µm pores is presented, resulting in a highly porous (=30%), dual-scale nano- and microporous membrane for use in an improved vascular transmigration model. Ultrathin membranes are patterned using a precision laser writer for cost-effective, rapid micropore design iterations. The optically transparent dual-scale membranes enable complete observation of leukocyte egress across a variety of pore densities. A maximal density of ≈14 micropores per cell is discovered beyond which cell–substrate interactions are compromised giving rise to endothelial cell losses under flow. Addition of a subluminal extracellular matrix rescues cell adhesion, allowing for the creation of shear-primed endothelial barrier models on nearly 30% continuously porous substrates.

1. Introduction

Leukocytes, metastatic cancer cells, and recruited stem cells use the vasculature system to access peripheral tissues in vivo.[1–4] Transport into and out of the blood vessels (extravasation) is regulated by endothelial cells (ECs), pericytes, perivascular extracellular matrix (ECM), resident innate immune cells, a variety of cytokine signals, all working to maintain or restore vascular barrier homeostasis. The study of leukocyte egress through the vascular wall is the most widely studied transmigration event.[5] Pathogen-associated molecular patterns (PAMPs) trigger a response in the innate immune cells primarily residing in the peripheral tissues.[6] These immune cells, such as macrophages, release a variety of cytokine and proinflammatory molecules that stimulate the ECs lining the blood vessels within proximity to the pathogenic invasion. The resulting cytokine gradient, coupled with the expression of pro-transmigration molecules on the EC surface, attracts circulating leukocytes to the site of infection.[7]

As in vivo leukocyte transmigration cannot be viewed directly, current studies implement the use of specialized intra-vital microscopy systems to observe these events in real time in situ.[8,9] While these methods act as a powerful discovery tool in the field of leukocyte transmigration, unique imaging systems and intensive preimaging surgeries limit the scalability of these studies. The recent development of highly controlled, human cell based in vitro microvasculature models, however, have enabled leukocyte transmigration studies at the lab bench.[10]

Microfluidics-based vascular models have made use of planar microporous membranes as a selective support layer for EC growth.[11–14] These membranes are ideal for transmigration studies as they promote the growth of ECs into a continuous flat monolayer and can have optically favorable properties for real-time imaging. Conveniently, microporous membranes come in many forms (material, pore size, thickness) depending on the intended use.[11] Polyethylene terephthalate membranes with 3 µm pores have been used to facilitate neutrophil transmigration assays through an EC (human umbilical vein ECs (HUVECs)) monolayer.[15] Furthermore, polycarbonate filters with 8 µm and 12 µm pores were utilized to assess the migratory potential of melanoma cell lines.[16] Our lab has also previously developed an ultrathin, optically transparent silicon dioxide (SiO₂) membrane with tunable pore sizes for use in...
cell culture systems. Further use of planar membranes for organ-on-a-chip platforms has been reviewed in detail elsewhere.

While microporous membranes support monolayer integrity and transmigration events, they may not be ideal substrates when compared to the basement membrane (BM) in vivo. Traditionally, these polymeric and microfabricated membranes contain relatively large non-porous regions between micropores on which cells can adhere and proliferate. In some cases, these non-porous regions can span 40 \( \mu \text{m} \) between pores resulting in potentially non-uniform cell signaling and nutrient uptake on the basolateral surface of the endothelial monolayer. In studies aimed at observing subluminal stimulation of ECs or circulating leukocytes, pore non-uniformity could compromise the physiological fidelity of the transmigration model. One solution to this shortcoming is to increase substrate permeability through the addition of tightly-packed pores. In the microporous regime, a significant increase in pore density results in a decrease in membrane structural integrity, increasing the difficulty of integrating these membranes into cell culture devices, especially when physiological membrane thickness is desired.

Increased micropore density can also influence cell function, particularly the ability of the cell monolayer to remain adherent to the substrate under physiological shear forces. Previously, our lab presented the use of microporous silicon membranes as a model vascular substrate. In that study, HUVECs were seeded on microporous membranes with varying pore size and density. It was concluded that an increase in micropore density can result in decreased cell–substrate interactions (as measured by focal adhesion staining).

In a recent study of a blood–brain barrier (BBB) model, ECs were found to detach within minutes of the onset of physiological flow on microporous \( \text{SiO}_2 \) membranes, ultimately guiding the decision to utilize nanoporous membranes for the BBB model. Importantly, others have reported success in maintaining EC monolayers under shear on track-etched (TE) and PDMS microporous membranes. This discrepancy, however, may be due to the relatively low pore density and high contact area on TE and PDMS membranes that support stronger cell–substrate interactions. The tradeoff between membrane porosity, thickness, and firm cell adhesion continues to hinder cell barrier research. Considering physiological shear stress is known to modulate EC barrier formation in vitro, it is appropriate to conclude it is a necessary condition for the study of transmigration through barrier models. Therefore, to create a robust membrane solution with both high basolateral surface permeability and EC monolayer integrity under shear, it is necessary to change the paradigm of traditional microporous membrane fabrication.

Previously, our lab has developed a variety of ultrathin (50–100 nm), highly permeable nanoporous membranes for use in cell culture and biological separations. These membranes contain pores ranging in size from 15 to 100 nm with pore to pore spacing less than 100 nm. While these membranes have been shown to support cell culture despite high porosity, they do not provide pores large enough to permit leukocyte, stem cell, or cancer cell transmigration. However, as demonstrated by Morgan et al., the use of a highly porous material for fabrication of hierarchical support structures allows for drastically increased material porosity without suffering from complete loss of usability. To this end, it may be possible to implement our previously developed nanoporous material as a base substrate for micropore membrane fabrication.

To address the need for improved membrane permeability while simultaneously facilitating transmigration events, we set out to create an ultrathin, dual-scale (nano- and microporous) membrane. We developed methods that enable the lithographic patterning and etching of free-standing ultrathin nanoporous membranes, effectively transferring micropores directly into the highly permeable nanoporous surface. Despite porosities as high as 30\%, these membranes provided enough structural integrity to enable integration into microfluidic vascular model systems. We investigated leukocyte transmigration through a variety of pore densities as well as endothelial attachment and retention under shear.

2. Results and Discussion

2.1. Dual-Scale (Nano- and Microporous) Membrane Fabrication and Characterization

To closely mimic the uniform permeability and selective transport of the vascular BM in vitro, we explored the use of the highly porous and ultrathin nanomembrane as the base substrate for microporous membrane fabrication. These membranes are roughly equal in thickness to the BM (~100 nm) and contain a dense spread of nanopores much like the protein network that constitutes the BM itself. The membrane's uniform permeability can be attributed to the closely packed nanopores (spacing <100 nm) that allow for rapid passage of small molecules and proteins. The addition of micropores to the nanoporous membrane would facilitate motile cell transmigration through a membrane-supported endothelium.

Successful use of novel lithography techniques for vascular model design and membrane fabrication inspired us to explore its use in fabricating dual-scale (nano- and microporous) membranes. Standard lithography processes become more complex when coating an ultrathin, free-standing, porous membrane in place of a traditional silicon wafer monolith. The proposed lithography process begins with the spin coating of the suspended nanomembrane. A consequence of the high rotational velocities necessary to achieve even, thin resist coatings (7000 RPM) is the potential for thin film sag and membrane rupture. Remarkably, the spin coating process did not result in membrane fracture and formed a uniform 500 nm layer of positive photoresist over the entirety of the free-standing and support surfaces. This feat is not without appreciation, as it now opens to the door to an entire bases of lithographic membrane design, all on the surface of a 100 nm thick nanomembrane.

To this end, the photoresist was patterned using a MICROTECH LaserWriter system, effectively acting as a pore design template for etching. The use of a laser writer eliminates the need for high resolution mask production, allowing for rapid design iterations. Specifically, the laser writer system is unlimited in nature of design. While the focus of this study was to specifically pattern 3 \( \mu \text{m} \) pores for selective leukocyte passage, membranes could potentially be modified with pores of any
desired shape (triangle, square, hexagon, etc.) at several varying pore densities. While this may be achievable in other membrane fabrication techniques, our protocol is cost and time effective (≈2 h), and the immediate end result is an ultrathin membrane with the nanoporous background. Furthermore, the laser writer system, allows for in situ alignment, with minimum mechanical stage step sizes in the submicron range. This translates to the ability to precisely pattern micropores at any location on our nanoporous substrate. While it is partially conceptual at this point, a future study may explore the full potential of the patterning techniques described herein. Lastly, a dynamic focus feature of the writer permits consistent exposure over the resist surface, even in the case of resist nonuniformity.

After resist development, the pore pattern was transferred into the free-standing nanoporous membrane using a reactive ion etcher (RIE), and the remaining photoresist was removed with an oxygen plasma ashing step (Figure 1B). The resulting membrane surface contained 3 μm pores etched directly into the highly permeable nanoporous material (dual-scale membranes; Figure 1C, Movie S1, Supporting Information). In using a thin layer of photoresist to protect the nanoporous membrane, there was concern that the resist might have entered the nanopores, making the membrane less permeable following development. We investigated this potential concern with the use of transmission electron microscopy (TEM; Figure 1C). We found that the oxygen plasma was sufficient to remove any residual photoresist. The use of the ash step to remove the photoresist was selected over an acetone wash as it allowed for batch production of the individual membranes, further pushing the rapid prototyping focus of this work.

One potential concern with the reactive ion etch process is the nonspecific etching of the nanoporous substrate, resulting in nanopore enlargement and substrate weakening. Transmission electron micrographs were obtained before (Figure 2A) and after (Figure 2B) micropore etching. The pore size distributions in both cases (Figure 2A,B) show a negligible change in pore size distribution (44.6 nm for native, 42.7 nm for postmodification). Furthermore, pore shape and porosity are also relatively unchanged.

Porosities of the dual-scale (nano- and microporous) membranes were determined using Equation (1).

\[ \phi_{\text{total}} = \phi_{\text{np}} (1 - \phi_{\text{mp}}) + \phi_{\text{mp}} \]  

(1)

Figure 1. Fabrication of dual-scale porous membranes. A) SEM image of NPN membrane prior to the addition of micropores (scale bar = 1 μm). B) The addition of micropores begins with the spin coating of Shipley 1805 onto the freestanding nanoporous membrane. The 500 nm coating is developed, forming the micropore pattern (scale bar = 10 μm). The pattern is transferred into the nanoporous membrane following an RIE process. The RIE ash process removes the Shipley 1805 mask (scale bar = 10 μm; I, II scale bars = 4 μm). C) SEM image of dual-scale (nano- and microporous) membrane (scale bar = 1 μm). Overlaid TEM images show further membrane detail including nanopore structure and micropore edge.

Figure 2. Micropore etching does not alter nanopores. Transmission electron micrographs show pore structure A) before and B) after micropore addition (scale bars = 50 nm). Histograms of pore size were obtained from lower magnification micrographs to obtain a higher pore distribution. Image analysis was performed in MATLAB to obtain average nanopore diameter, porosity, and roundness.
where ϕ_{np} represents the porosity of the nanoporous membrane, and ϕ_{µp} is the porosity of the equivalent microporous membranes (assuming only micropores). Porosities of all dual-scale membranes can be seen in Figure 3A. A unique feature of our dual-scale membranes is the baseline porosity provided by the nanoporous substrate. While porosity invariably declines as micropore density decreases with commercially available TE membrane inserts, the dual-scale membranes presented in Figure 3 are consistently porous (≈10–15%) until the micropore density exceeds 4500 mm⁻², at which point the micropores begin to drastically influence membrane porosity. The minimum porosity in this series is 10.3%, very close to the porosity of the underlying nanoporous silicon nitride (NPN; 9.4%) and roughly equivalent to the maximum porosity available for commercial 3 µm pore TE membranes (Corning; 3 µm pores, 2 × 10¹⁰ pores cm⁻²). The density of pores in TE membranes are limited by concerns about the merging of the randomly positioned pores,[39] while in our case micropores are precisely positioned on the nanoporous background. When micropore density is highest (27 777 pores mm⁻²), the total dual-scale membrane porosity is 27.2%, with micropores contributing to three-quarters of this total porosity (Figure 3C). By contrast, micropores contribute only one-tenth of the total membrane porosity at their lowest density (1371 pores mm⁻²; Figure 3D).

2.2. Leukocyte Transmigration on Nanoporous and Dual-Scale Membranes

The presented porosities of our dual-scale membranes demonstrate the benefits of the combined pore sizes from a material properties perspective. Importantly, the continuity of the nanopores might also have added benefits when making comparisons to the vascular ECM in vivo. The vascular ECM is a network of proteins that supports EC structure and growth. The porous nature of the ECM facilitates cytokine and inflammatory signal delivery directly from the peripheral tissue to the basolateral surface of the EC monolayer.[40] In the case of a strictly microporous membrane, the presence of discrete micropores create artificial “chimneys” that restrict diffusion to highly localized regions of the membrane. In our dual-scale membranes, nanopores permit uniform diffusion of molecules across the endothelial monolayer, much like the in vivo ECM counterpart.

To validate the use of our dual-scale membranes in a leukocyte vascular transmigration model, freshly isolated human neutrophils were introduced to the luminal (top) channel of our cell culture microfluidic device (Figure S1B,C, Supporting Information). Neutrophils settled on the HUVEC monolayer and were attracted to the abluminal (bottom) channel with the addition of N-Formylmethionyl-leucyl-phenylalanine (fMLP), a potent chemotactant. As expected, 3 µm pores facilitated complete neutrophil migration as confirmed by presence of cells on the coverslip 430 µm below the dual-scale porous membrane (abluminal side of device) 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4A). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B). When unmodified nanoporous membranes were used, the presence of neutrophils was not observed on the coverslip 30 min post fMLP addition (Figure 4B).
devices (Figure 4A) eliminates the concern that neutrophils are not exposed to sufficient fMLP to complete transmigration. Furthermore, the real-time imaging of neutrophil migration on dual-scale membranes shows the mechanism of complete transmigration (Figure 4B), in which human neutrophils probe and migrate under the EC monolayer, locate a micropore, and egress through the micropore to reach the abluminal side of the device. The videos of both processes can be seen in Movies S2 and S3 in the Supporting Information. Once under the membrane, neutrophils may crawl, remaining adhered to the silicon nitride membrane, or detach and descend to the abluminal well, as observed by the presence of ripples formed by the motile cells falling out of focus (Movie S4, Supporting Information). Overall, the observable complete migration on dual-scale membranes

Figure 4. Complete leukocyte transmigration facilitated by dual-scale porous membrane. A) Time-lapse phase contrast images were recorded of neutrophils migrating through a nanoporous membrane supported HUVEC monolayer (scale bars = 20 μm). Representative frames are presented. Phase images were taken 2 μm above the membrane (EC focus) and 430 μm below the membrane (coverslip focus; scale bar = 200 μm) postexperiment to objectively observe neutrophil complete migration. B) Experiments were repeated for dual-scale membranes. Time-lapse videos can be found in the supplemental material.
demonstrates the power of this platform as a tool for studying vascular transmigration kinetics. The ability for neutrophils to complete migration to the coverslip also adds a level of convenience when conducting experiments that involve quantifying leukocyte (or cancer cell and stem cell) migration. While previous studies explore the use of neutrophil collection and hemocytometer assisted counting of extravasated cells,[44] our mimetic eliminates possible collection error by allowing for in situ counting. Furthermore, the complete transparency of our system from above the EC layer down to the coverslip allows for direct observation of all steps of leukocyte egress. This is especially beneficial when exploring leukocyte regulation, as an observed reduction of neutrophils through the vascular barrier does not inform the study on what step of the transmigration process is halted.[42]

2.3. Cell Adhesion on Varying Micropore Densities Under Shear

Another important aspect of a microphysiological system is the recapitulation of in vivo mechanical stimuli.[26] In the vascular system, this mechanical stimulus can come in many forms, including shear stress, matrix stiffness, and cyclic strain, some of which are more influential depending on the accompanying organ system of interest.[43] Shear stress has presented itself as an important contributor to the formation of a complete EC barrier,[22,26] and has also been hypothesized to influence lymphocyte migration.[44] Shear stress ranges from 4 to 30 dyn cm^{-2} in the arteries, 10 to 20 dyn cm^{-2} in the capillaries, and 1 to 4 dyn cm^{-2} in veins.[45] For our study, we selected a shear stress level of 4.5 dyn cm^{-2}.

Previously, we have observed ECs that had reduced cell–substrate interactions (as measured by focal adhesion staining) when micropore density increased.[22] At the same time, these ECs increased cell–cell interactions (as measured by VE-cadherin staining). This suggests a mechanism by which ECs downregulate their interactions with a highly microporous surface. In the context of a microfluidic shear system, this has indirectly been shown to translate to EC loss under physiological levels of flow.[23] This result is undesired, as it greatly disrupts the establishment of a physiological EC barrier. However, the threshold of micropore densities at which cell–substrate interactions are insufficient to maintain an adherent EC monolayer under shear has yet to be elucidated.

To fill this gap in knowledge, dual-scale membranes with a gradient of micropore densities were designed and fabricated. These membranes contain a ramp of eight different 3 µm pore spacings (6 to 27 µm center to center; Figure 5A). The ability to pattern all eight pore spacings on a single free-standing nanoporous membrane permitted highly controlled experiments with all the results in a single field of view. As anticipated, high micropore densities reduced cell–substrate interactions, resulting in EC loss after 24 h exposure to shear (Figure 5A). Postshear cell densities on the highest and lowest micropore densities were equal to 787 and 1172 cells mm^{-2} respectively (Figure 5C). Interestingly, the variation in EC density between micropore densities of 6944 and 1371 pores mm^{-2} was minimal (mean EC density of 1195 cells mm^{-2}, standard deviation of 101 cells mm^{-2}). We hypothesize this is due to the little variation in porosity between these two micropore densities (15.4% vs 10.3%) compared to that of the two extremes (27.2% vs 10.3%).

To address concerns that flow patterns near the entrance or exit of the microfluidic channel might be responsible for patterns of EC loss, side-by-side membranes with different micropore densities were created. Specifically, the highest (27 777 pores mm^{-2}) and lowest (1371 pores mm^{-2}) pore densities tested on the gradient membranes were patterned over the long axis of the nanoporous membrane, resulting in two microporous regions that extended the full length of the membrane (Figure 5B). Once again, EC loss was only observed on the high micropore density region, while cells were retained on the low density micropores. Quantification of postshear cell density on gradient dual-scale membranes in Figure 5A revealed a maximal number of micropores per cell of =14, above which cell delamination was observed (Figure 5C). This number will provide insight when selecting micropore densities in future shear flow experiments.

2.4. Subluminal Extracellular Matrix Gel for Improved Cell Adhesion

For many transmigration experiments a low density of micropores may be sufficient, especially considering the membranes will still be highly porous because of the nanoporous substrate. For experiments that may need a higher density of micropores and shear priming, these membranes in their bare form may not be suitable. However, the remarkable structural robustness of the dual-scale membrane may permit the use of an underlying physiological matrix to “rescue” the loss of cell adhesion on regions with a high density of micropores. To test this hypothesis, a collagen I gel was added to the abluminal side of the dual-scale membrane, providing additional cell–matrix attachment within the exposed micropores (Figure 6A). The collagen gel did not obstruct phase imaging (Figure 6B), and completely rescued cell adhesion on all micropore densities tested following 24 h shear (Figure 6C). The use of an ECM-like gel to back-fill the dual-scale membranes, as opposed to a continuously nanoporous membrane, is advantageous because it permits selective immune cell (or cancer cell and stem cell) transmigration through the matrix via matrix metalloproteinases (MMPs).[46]

While leukocyte migration on and through the vascular endothelial barrier is largely regulated by interactions between endothelial expressed ICAM1 and leukocyte expressed LFA1 and MAC1 integrins, the movement of these immune cells through the underlying ECM involves the combined interactions of other beta 1 integrins, MMPs, and ECM proteins.[7] In vitro studies observing the migratory patterns of leukocytes through collagen gels have allowed for the investigation of mechanisms involved in this behavior.[47–50] In order to observe the effects of a supplemented ECM on the ability to observe neutrophil migration within our mimetic, HUVECs were seeded on collagen gel-backed dual-scale membranes (1736 pores mm^{-2}) and neutrophil migration experiments were performed. The presence of the gel did not affect the ability to observe neutrophil transmigration or restrict the formation of a sufficient fMLP gradient to induce transmigration.
As expected, the collagen gel restricts the “fall-off” of neutrophils as they pass through the micropores. Instead, the neutrophils take on an additional step in the innate immune response cascade, in which they must crawl along and into the proteinaceous ECM. This result can be observed in Movie S5 in the Supporting Information. MMPs have also been hypothesized to influence tumor cell migration through the ECM.\textsuperscript{[51,52]} Benbow and colleagues previously discovered the role of MMP-1 in the invasion of type I collagen gels by an A2058 melanoma cell line.\textsuperscript{[53]} Future studies may explore the use of our gel-supplemented vascular mimetic in investigating tumor cell migration through both ECs and the supporting ECM. Overall, the addition of an underlying collagen gel both rescues cell adhesion and allows for the study of the next step in the innate immune response and tumor metastasis.

**Figure 5.** High micropore density leads to EC loss under fluid shear. HUVECs were seeded on A) gradient and B) side-by-side dual-scale (nano- and microporous) membranes and grown to confluency. 24 h static images represent microfluidic devices that were replenished with fresh media and returned to the incubator for an additional 24 h. 24 h shear images represent cells that were integrated into the flow circuit and sheared at 4.5 dyn cm\(^{-2}\) for an additional 24 h. Phase and fluorescent (DAPI, blue) images were recorded postflow (scale bar = 100 µm) for the same experiment. C) FIJI-assisted quantification of cell nuclei post shear. *Micropore/cell represents the number of micropores located directly underneath a given EC based on an estimated spread area of 2000 µm\(^2\).
While it might be concluded that the use of a collagen gel may eliminate the need for a membrane all together, it must be noted that the membrane is essential for scaffolding gel polymerization and maintaining the position of the viscoelastic gel once flow begins. Furthermore, the planar membrane provides a consistent focal plane for clear phase contrast imaging even in the presence of flow pressures.

2.5. Leukocyte Transmigration Through Shear-Primed Endothelial Cell Monolayers

Shear stress is known to enhance EC barriers as shown by the direct relationship between increased transendothelial electrical resistance and prolonged shear stress.[54] This increased barrier function has been shown to also modulate neutrophil

Figure 6. Submembrane collagen maintains endothelial monolayer for all micropore densities and permits leukocyte transmigration experimentation. A 300 µm collagen gel was added to the backside of a dual-scale membrane. A) SEM images were obtained of glutaraldehyde fixed samples without cells (top scale bar = 2 µm; bottom scale bar = 1 µm). B) Phase images were obtained of HUVECs seeded on collagen gel back dual-scale membranes and left to grow for 24 h after reaching confluency (scale bar = 20 µm). C) Additionally, HUVECs seeded on gel back dual-scale membranes were grown to confluency and sheared at 4.5 dyn cm$^{-2}$ for 24 h and phase images were recorded (scale bar = 20 µm). D) Submembrane collagen gel permits neutrophil migration under an fMLP gradient, with an additional ECM crawling step. Roman numerals in panels B and C correspond to the following 3 µm pore densities (pores mm$^{-2}$): I = 27 777, II = 12 345, III = 6944, IV = 4444, V = 3086, VI = 2267, VII = 1736, VIII = 1371.
HUVECs grown on dual-scale membranes (1736 pores mm$^{-2}$) and sheared at 4.5 dyn cm$^{-2}$ for 24 h (scale bar = 100 µm). B) Neutrophil migration experiments were performed on the shear-primed EC monolayers. Time-lapse phase contrast imaging revealed some instances of reverse leukocyte migration and probing without complete transendothelial migration.

Figure 7. Dual-Scale membranes support shear priming for enhanced barrier function in leukocyte migration experimentation. A) HUVECs were seeded on dual-scale membranes (1736 pores mm$^{-2}$) and sheared at 4.5 dyn cm$^{-2}$ for 24 h. B) Neutrophil migration experiments were performed on the shear-primed EC monolayers. Time-lapse phase contrast imaging revealed some instances of reverse leukocyte migration and probing without complete transendothelial migration.

Transmigration in response to TNFα, as demonstrated by a marked decrease in transendothelial migration events through a shear stress influenced EC monolayer.$^{[55]}$ With the goal of using our platform to expand on such findings, dual-scale membranes were investigated here for their ability to support long-term shear and modulate endothelial phenotype. HUVECs grown on dual-scale membranes (1736 pores mm$^{-2}$) were sheared at 4.5 dyn cm$^{-2}$ for 24 h. Phase images show the EC transition from cobblestone morphology to increased elongation over the 24 h shear incubation (Figure 7A). Neutrophil transmigration in response to fMLP showed an observable decrease in neutrophil transmigration events, with prominent instances of reverse migration (transendothelial migration from the abluminal to luminal side) and probing without migration (Figure 7B). This result validates the ability of the dual-scale pore membranes to support shear-induced changes to vascular EC phenotype prior to the study of leukocyte migration.

3. Conclusion

We have developed a dual-scale (nano- and microporous) ultrathin membrane technology that facilitates cell transmigration studies, in a vascular model, while also providing a continuously porous substrate. This unique membrane design contains hundreds of 3 µm pores on a background of millions of closely spaced ($\approx$100 nm) nanopores. The porosities of the membranes range from 9% to nearly 30%. Whereas existing microporous membranes may limit uniform small molecule passage due to large nonporous interpore regions, our membranes remedy this issue through the lithographic addition of micropores on a highly permeable nanoporous substrate. In the case of our vascular transmigration model, this creates a physiologically relevant presentation of chemotactant to both the endothelium and circulating immune cells. Our rapid, iterative membrane fabrication protocol ($\approx$2 h) allowed us to study the effects of micropore density on EC adhesion. It was determined that micropore densities larger than $\approx$14 per cell limit cell–substrate interaction, resulting in EC loss under shear, but that the addition of a physiologically relevant collagen gel under the micropores eliminates this loss. While the benefits of our dual-scale membranes in a vascular model setting are explored in detail here, future work will explore the use of these membranes in a variety of settings, not limited to organ-on-a-chip platforms, separations and capture kinetics, and sensor systems.

4. Experimental Section

Transfer of Micropores: NPN,$^{[34]}$ membranes (Figure S1A, Supporting Information) were purchased from SIMPore Inc. (Rochester, NY) with average pore size of 44.6 nm and membrane thickness equal to 100 nm. Membranes were adhered to a standard glass slide using carbon double-sided adhesive tape. Glass slides were then attached to a 20 mm spinner vacuum chuck for the resist spinning process. The use of an intermediate glass slide allowed for coating of free-standing membranes without the influence of the vacuum pulling on the nanomembrane (Figure S2, Supporting Information). To enhance resist adhesion, a P20 hexamethyldisilazane solution was spin-coated onto the nanomembrane at 7000 RPM for 50 s. Using the manufacturer protocol, Shipley 1805 positive photoresist was spun down onto the membranes to create a uniform 500 nm coating. The spin coat process was followed by a 60 s soft bake at 115 °C. Photoresist-coated membranes were patterned using a laser writer system (MICROTECH, Palermo, Italy). Membranes were loaded directly into the laser writer sample tray without modification. Using a pore pattern designed in the accompanying CleWin5 software, resist is selectively exposed to a 233 mJ cm$^{-2}$oluminated beam and subsequently developed in Microposit MF-319. Various pore patterns and densities (1371–27 777 pores mm$^{-2}$) were written onto the nanoporous membranes. Patterned membranes were then etched in an RIE system (South Bay Technology Inc., San Clemente, CA). A three-gas silicon etch recipe (15 SCCM O$_2$, 10 SCCM CHF$_3$, 30 SCCM SF$_6$, 200 s, 30–50 mTorr, 100 W) was used to remove exposed NPN, resulting in a successful transfer of the micropore pattern into the free-standing nanoporous membrane. A subsequent oxygen plasma ash step in the RIE (55 SCCM O$_2$, 15 min, 200 mTorr, 100 W) was used to selectively remove the remaining photoresist, leaving a clean, dual-scale (nano- and microporous) membrane.

Microfluidic Device Fabrication: For cell culture and transmigration experiments, dual-scale membranes were integrated into a silicone based microfluidic device. Pressure sensitive adhesive (3 µm, Maplewood, MN; 130 µm thick) and silicone gasket (Trelleborg, Trelleborg, Sweden; 300 µm thick) were cut to specification (Figure S1B, Supporting Information) using a Silhouette Cameo craft cutter system.$^{[56]}$ Layers were irreversibly bonded together following a 15 min UV-ozone exposure and 2 h thermal curing at 70°C. The dual-scale membrane was sandwiched in between layers, creating defined luminal and abluminal channels. Polydimethylsiloxane made from a Sylgard 184 kit
was used as a pipette/tubing access layer. The assembled device (Figure S1C, Supporting Information) was bonded to a #1.5 coverslip, allowing for unobstructed optical imaging. In flow experiments, devices were connected in circuit to a media reservoir (placed postdevice), fluidic capacitor (placed predevice), and peristaltic pump (placed in-between reservoir and capacitor; Figure S1D, Supporting Information). The inclusion of the air-tight fluidic capacitor (Figure S1E, Supporting Information) dampens pressure fluctuations introduced by the peristaltic pump, while also acting as a bubble trap upstream of the cell monolayer. The capacitor was designed around a 1.5 mL cryovial with modifications that include a fluid inlet, fluid outlet, and glass slide base. Media flow rate was set to achieve a cell layer shear stress equal to 4.5 dyn cm⁻² as determined by Equation (2)

\[
\tau = \frac{6Q \mu}{w^2} \quad (2)
\]

where \(Q\) represents the input flow rate, \(\mu\) is the media viscosity, \(h\) is the height of the channel directly over the cell monolayer, and \(w\) is the width of the channel.

**Cell Culture:** HUVECs were purchased from VEC Technologies (Rensselaer, NY; Product# HUVECP). Pooled HUVECs were selected over single source to prevent result bias due to cell population homogeneity. HUVECs were maintained in T25 flasks at 37 °C, 5% CO₂ in MCDB-131 Complete media (VEC Technologies) and used between passage numbers 3 and 7 as recommended by the supplier. Following microfluidic device assembly, silicon membranes were coated with 0.17 mg mL⁻¹ human fibronectin (BD Biosciences, Franklin Lakes, NJ) for 1 h at room temperature to facilitate cell adhesion. HUVECs were resuspended and perfused into the luminal channel at a density necessary to achieve 40 000 cells cm⁻². The perfusion was performed with a P20 pipette placed directly in the PDMS access layer port. Gentle introduction of the cell bolus ensured uniform cell seeding. Following perfusion, cells were allowed to adhere and grow to confluency for 24 h under standard culture conditions listed above. In experiments involving shear stress, microfluidic devices were added to the flow circuit 24 h post cell perfusion. Cell counting, 4% paraformaldehyde fixed cell nuclei were stained using a 1 μg mL⁻¹ solution of 4-6-diamidino-2-phenylindole (DAPI) in 1X phosphate-buffered saline (PBS). Epifluorescence images were imported into FIJI and converted to 16-bit. The image threshold was adjusted to isolate DAPI staining. “Touching” nuclei were divided using the watershed plugin (Process > Binary > Watershed). Cell nuclei were automatically counted in regions of interest using the particle analysis feature in FIJI (Analyze > Analyze Particles). Cell counts were normalized to area of the membrane region specified in our results. Cell culture experiments were performed in triplicate.

**Neutrophil Transmigration:** Human neutrophils were isolated from consenting healthy donors using a previously described, IRB approved protocol.[15] Briefly, whole blood collected in sodium heparin vacuum tubes was density separated using 1-Step Polymorph (Accurate Chemical & Scientific Co., Westbury, NY). The collected neutrophil rich solution was washed and rid of red blood cells using a lysis step. The resulting neutrophil pure pellet was stored in Hank’s balanced salt solution with added 10 × 10⁻³ M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 5 mg mL⁻¹ bovine serum albumin to prevent nonspecific activation prior to experimentation. Neutrophils were used within 3 h postisolation (5 h postblood draw). Immediately prior to device perfusion, neutrophils were removed from their suspension buffer and transferred to MCDB-131 complete media (VEC Technologies; HUVEC media) at a concentration equal to 6 million cells mL⁻¹. Neutrophils were then introduced to the luminal channel and allowed to settle on the HUVEC monolayer. To promote chemotaxis, 10 nM fMLP (Sigma, Darmstadt, Germany) in MCDB-131 complete media was added to the abluminal channel. The prepared device was then transferred to a modified incubation chamber (PECON, Erbach, Germany) surrounding a Nikon TS100 microscope to allow for time lapse imaging in a controlled environment. Time from introduction of fMLP to initiation of time lapse imaging was less than a minute. All transmigration videos were recorded at 40x, 4 fps over 30 min.

**Collagen Gel:** A 2 mg mL⁻¹ solution of type I rat tail collagen (Enzo Life Sciences, Farmingdale, NY) was prepared on ice. A combination of NaOH, diH₂O, and 10× PBS was used to facilitate the gelling process. While cold, the collagen solution was pipetted into the 300 μm “trench” between the bottom of the silicon supports surrounding the free-standing membrane, and the membrane itself. Membranes were placed in an incubator (37 °C, 5% CO₂) for 1 h to permit gelling. Gel-backed membranes were then used in the same fashion as the clean membranes for cell culture. A 2.5% glutaraldehyde solution was used to fix gel-backed membranes for scanning electron microscopy (SEM).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare the following competing financial interest(s): J.L.M. and T.R.G. are co-founders of SiMPore, an early-stage company commercializing ultrathin silicon-based technologies.

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dual-scale, nanoporous silicon nitride, shear stress, transmigration, vascular mimic

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