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ARTICLE

Ultra-Thin and Ultra-Porous Nanofiber Networks as a Basement-Membrane Mimic

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Current basement-membrane mimics used for modeling endothelial and epithelial barriers in vitro do not faithfully recapitulate key in vivo physiological properties such as basement membrane (BM) thickness, porosity, stiffness, and fibrous composition. Here, we use networks of precisely arranged nanofibers to form ultra-thin (~3 µm thick) and ultra-porous (~90%) BM mimics for blood-brain barrier modeling. We show that these nanofiber networks enable close contact between endothelial monolayers and pericytes across the membrane, which are known to regulate barrier tightness. Cytoskeletal staining and transendothelial electrical resistance (TEER) measurements reveal barrier formation on nanofiber membranes integrated within microfluidic devices and transwell inserts. Further, significantly higher TEER values indicate a biological benefit for co-cultures formed on the ultra-thin nanofiber membranes. Our BM mimic overcomes critical technological challenges in forming co-cultures that are in proximity and facilitate cell-cell contact, while still being constrained to their respective sides. We anticipate that our nanofiber networks will find applications in drug discovery, cell migration, and barrier dysfunction studies.

Introduction

Physiological barriers are found throughout the body, and their integrity is paramount for maintaining healthy functionality[1]. Important physiological barriers include the alveolar–capillary barrier[2,3], intestinal-mucosal barrier[4], dermal barrier[5], renal-epithelial barrier[6], and the blood-brain barrier (BBB)[7-13]. Barrier properties are derived from junctional complexes that regulate transport between cells (paracellular transport) and from specialized transporter proteins that regulate transport across the cell membrane (transcellular transport)[11]. Barrier dysfunction has been implicated in many serious conditions, such as Alzheimer's disease[14], pulmonary edema[15], chronic kidney disease[16], and atopic dermatitis[17]. Physiological barriers also present significant blockades for therapeutic agents. Limited drug permeability across these barriers renders many promising therapies ineffective, with nearly 100% of large drugs and 98% of small drugs unable to penetrate the BBB[18]. Barrier properties are also key for immune cell trafficking, cancer metastasis, and nanoparticle transport[19].

Although barrier properties are maintained by endothelial and epithelial monolayers, a key component is the basement membrane (BM) [20]. The BM is a thin layer (about 50-300 nm) of extracellular matrix (ECM), composed of collagen, fibronectin, and laminin[20,21], that provides support for endothelial and epithelial cells, while also separating these cells from supporting cells. Endothelial and epithelial cells are regulated by cell-cell crosstalk from the supporting cells across the BM. The specialized brain vascular endothelial cells gain their unique transport-restricting properties when in close contact with adjacent cell types across the BM (Figure 1a). In vivo and in vitro studies have shown pericytes and astrocytes regulate endothelial barrier function. Brain endothelial cells make direct contact with pericytes through peg-and-socket connections (Figure 1a)[22]. Astrocyte endfeet surround capillaries in the brain and regulate endothelial behavior through the release of soluble factors such as VEGF, TGFβ, GDNF, and bFGF[23-25].

Many in vitro models have been developed for investigating barrier properties[26]. These models rely on endothelial or epithelial monolayers cultured on BM constructs, designed to mimic the in vivo BM. Although numerous BM mimicking materials have been developed, to date these membranes still have significant limitations[27]. Table 1 summarizes the advantages and disadvantages of several engineered BMs. The most widely used in vitro BM mimics are track-etched polycarbonate (PC), polyester or polyethylene terephthalate (PET), and polytetrafluoroethylene (PTFE)[28-39]. These semi-permeable membranes are commercially available with cylindrical pores of 0.4, 1, 3, 5, or 8 µm-diameter.

For barrier models, endothelial cells are cultured on one side of the membrane, and unless migration is desired, small pore sizes (typically 0.4 or 1 µm) are used[34,38]. These membranes, however, have limited physiological relevance in key aspects. Firstly, these membranes are generally 10-12 µm thick (or 30 µm for PTFE), over 100 times thicker than the BM in vivo. This inhibits direct cell-cell contact across the membrane and limits the transport of soluble factors between adjacent cell types. Secondly, these membranes

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Table 1. Membrane-mimics used for in vitro modeling.

Membrane Types	Desirable Properties	Undesirable Properties	Ref
Track-etched PC, PET, PTFE	Commercially available (0.4, 1, 3, 8 µm pores) Transparent (PET, PTFE) Biocompatible	Non-fibrous Thick (~ 10-12 µm) Low porosity (~ 0.5% - 15 %) Translucent (PC) Stiffer than *in vivo*	38 28-39
Silicon Nitride	Ultra-thin (50nm -1 um) to facilitate cell-cell contact High porosity (approaching 50%) Structured pore geometry and size (~400nm square pores) Transparent	Non-fibrous Poor cell adhesion to SiN Requires nanofabrication Stiffer than *in vivo*	40-42
Collagen or Vitrified Collagen	Fibrous Native ECM	Pore size determined by fabrication. Low porosity due to small pores Thick (>10µm)	43-48
Patterned PDMS/Mylar	Transparent Flexible (PDMS) Can be highly porous	Generally thick (5 µm) Non-fibrous Fragile Requires nanofabrication	2,49-53
Electrospun nanofibers	Fibrous Variable pore sizes Relatively high porosity Can be bioactive Can have good mechanical strength	Relatively thick (> 4 µm) Random arrangement of pores Wide pore size distribution Not transparent	54-57
STEP nanofibers	Ultra-thin (<3 µm) Ultra-porous (~90%) Transparent Fibrous No Nanofabrication or Cleanroom Excellent biocompatibility	Mildly distributed pore sizes Mildly Fragile	This Work

*PC, Polycarbonate; PET, polyethylene terephthalate; PTFE, Polytetrafluoroethylene

have inherently low porosity; track-etch membranes typically have maximum porosities of around 15% for 1 µm pores. Conventional track-etched membranes have porosities as low as 0.5% which can also obscure transendothelial electrical resistance (TEER) measurements[58]. Finally, the flat, sheet-like construction of these membranes cannot mimic the fibrous nature of the BM for physiological relevance[48]. Besides limited physiological relevance, track-etched membranes can obscure brightfield imaging (PC is translucent) and confocal imaging due to the thickness of the membrane.

Several BMs are under development as alternatives to track-etched membranes, including nanofabricated SiN membranes [40-42], electrospun nanofibers [54-56], patterned materials such as PDMS and mylar [49-52], and native ECM membranes such as collagen and collagen vitrigel membranes [43-48]. Nanofabricated SiN membranes can be extremely thin (50 nm to 1 µm), have evenly distributed pores, and are optically transparent[40-42]. However, these membranes require expensive microfabrication processes, are non-fibrous, and the SiN poses cell attachment problems[40]. Similarly, non-fibrous photolithographically-patterned Mylar[50] and PDMS[2,49,51] membranes have been fabricated with relatively high porosity (up to ~50%), controlled pore size/location, moderate thickness (generally 5-50 µm) and are optically transparent. Thinner PDMS membranes have been fabricated at 2 µm, at the consequence of low porosity (2%)[53]. To achieve fibrous membranes, electrospinning techniques have been used [54-56]. These membranes have disadvantages such as large thicknesses (> 10 µm) and randomly distributed fibers and pores. Native extracellular matrix (ECM) membranes have also been developed[43,48,59], such as vitrified collagen membranes[44-47], but these membranes have low porosity due to the vitrification process and are rather thick (≥ 10 µm).

We introduce ultra-thin, ultra-porous fibrous networks fabricated with the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) method[60,61]. Our BM mimicking STEP nanofiber networks offer several advantages compared to other membranes. Firstly, STEP membranes are ultra-thin and can be fabricated close to physiologically relevant thicknesses. Secondly, STEP membranes achieved porosities over 80%. Because our STEP membranes are thin and highly porous, significant crosstalk and physical contact can be achieved between cells cultured on adjacent sides of the membrane. Thirdly, STEP membranes are fibrous, like the native BM, and more relevant than commercial membranes. Lastly, STEP membranes are made of polystyrene, making them highly biocompatible and excellent for cell adhesion. They can be coated with ECM-like proteins to create a natural surface for cell growth. We demonstrate the physiological utility of our nanofiber membranes by modeling the BBB using primary human endothelial cells and pericytes in monoculture and contact co-culture models within both microfluidic and transwell devices. The two cell types can reach and make contact within the membrane, and TEER measurements robustly show the marked improvement over standard approaches. The STEP method may provide a more physiologically relevant BM than alternative membrane fabrication methods, leading to improved membranes for a vast range of applications.

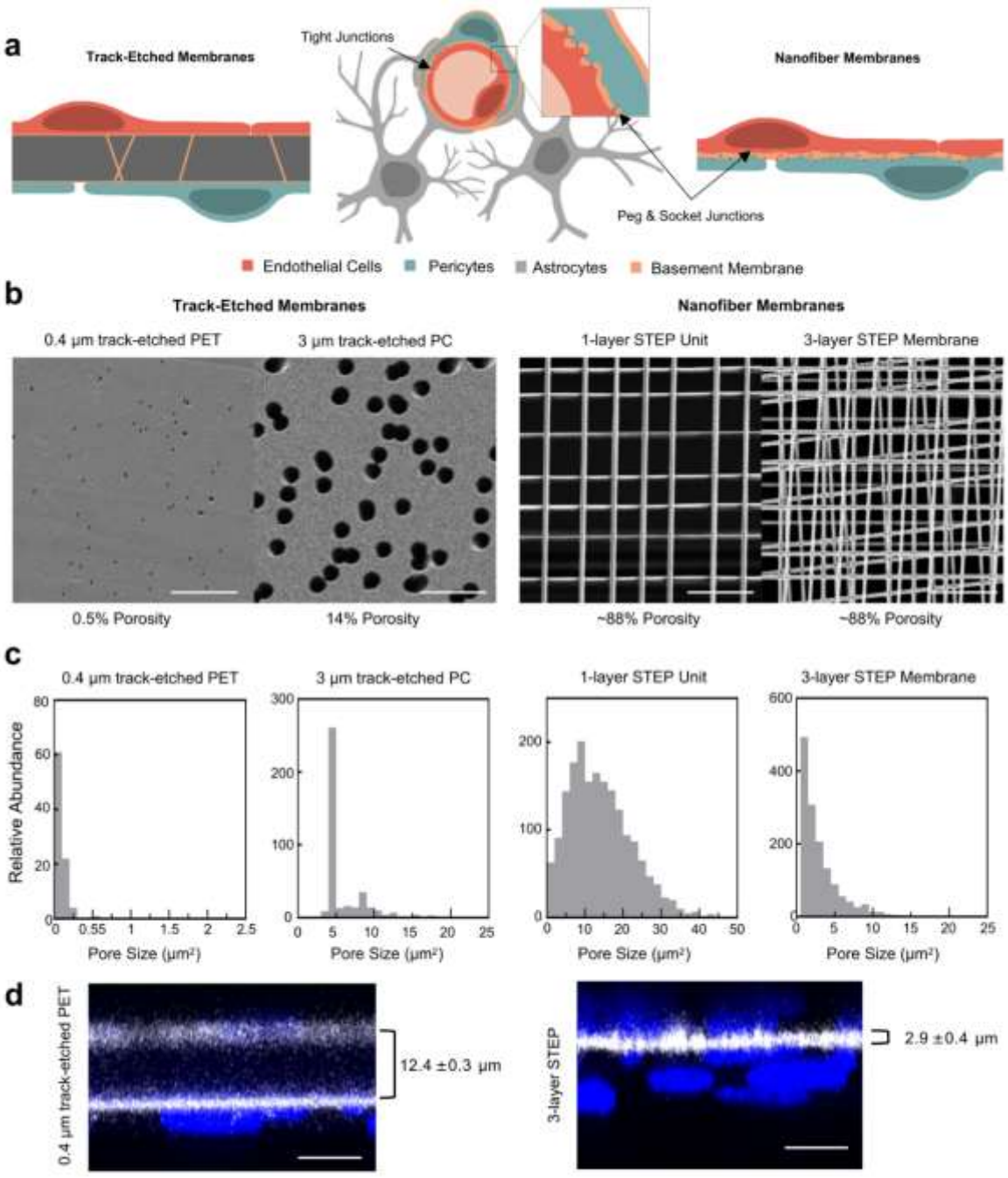

Figure 1. Ultra-thin, ultra-porous nanofiber networks offer significantly improved physical properties compared to track-etched membranes. a) Schematic representations of track-etched (left) vs STEP nanofiber membranes (right) as models of the blood-brain barrier in vivo (middle). b) SEM images reveal the pore structure of i) 0.4-µm diameter track-etched PET membranes, ii) 3-µm diameter track-etched PC membranes, iii) 1-layer crosshatch STEP unit, and iv) 3-layer STEP nanofiber membranes. The 1-layer crosshatch STEP unit was formed with 600-nm diameter fibers spaced ~4 µm apart within each layer, and the 3-layer STEP membrane is comprised of 3 crosshatch units spun on top each other and bonded. Track-etched membranes have randomly located pores of uniform size whereas the nanofiber membranes have precisely-organized pore structures with a greater distribution of pore sizes. c) Pore size distributions show nanofiber membranes have a wider pore size distribution than track-etched membranes. The 3-layer membranes have an average pore size significantly lower than the 3-µm diameter track-etched membrane. The pore size, thickness, and porosity of the nanofiber membranes can be controlled by fiber spacing, diameter, and the number of STEP units. d) The nanofiber membranes are significantly thinner than conventional track-etched membranes ($p < 0.001$). Conventional PET membranes are 10-12 µm thick (left) whereas 3-layer nanofiber membranes are approximately 3 µm thick (right). Rhodamine fibronectin (white) indicates membrane surfaces and blue indicates DAPI. Scale Bars 10 µm.

Materials and Methods

Nanofiber fabrication

Dense nanofiber membranes were fabricated according to the previously published STEP method[60,61]. Nanofibers were spun around scaffold supports that were laser-cut from plastic coverslips (Fisher Scientific, 0.22mm thick) and contained a hollow region 2 mm in diameter over which the nanofiber membrane was fabricated. Liquid prepolymer was dispensed from a microneedle and nanofibers were spun around a rotating and translating scaffold substrate. The scaffold was then re-oriented 90° and spun again to create the crosshatch unit. The 600 nm-diameter fibers were prepared using a 7 wt % solution of polystyrene (MW: 2000 000 g/mol; category no. 829; Scientific Polymer Products, Ontario, NY, USA) in p-xylene (X5-500; Thermo Fisher Scientific, Waltham, MA, USA). To decrease pore sizes, multiple nanofiber layers were spun across the scaffold. Nanofibers were then chemically fused together. To improve the throughput of the spinning process, and to eliminate the need to remove unwanted fibers from one side of the scaffold, we secured two scaffolds back-to-back by epoxying their stubs together. Prior to integrating them into the devices, the scaffold stub was carefully cutoff, and the two scaffolds were separated from each other.

Microfluidic fabrication

Microfluidic devices were fabricated from two polydimethylsiloxane (Dow Corning, PDMS) layers that enclosed the plastic nanofiber scaffold. PDMS was mixed 10:1 base to cross-linker and was cured in acrylic molds at 50°C for 4 hours. Acrylic molds were assembled from laser cut and solvent bonded layers of acrylic. The lower microfluidic channel and the indentation for the nanofiber scaffold were patterned using a top and bottom acrylic mold pressed together by binder clips. Access holes for the inlet and outlets of the lower channel were punched in the lower PDMS membrane using a 1.5 mm-diameter biopsy punch, and the PDMS layer was plasma bonded (Harrick Plasma) to a glass slide. The upper microfluidic channel was similarly formed in an acrylic mold, and once cured, inlet and outlet ports were punched in the upper layer using a 1 mm-diameter biopsy punch. Finally, a top well layer was cured in an acrylic mold and 6 mm diameter holes were punched with a biopsy punch. Upper and lower microfluidic channels were 0.22 mm high, 3 mm wide, and 18 mm long. Liquid PDMS glue (10:1 base to cross-linker) was used to bond the nanofiber scaffold between the upper and lower channel layers. Liquid PDMS was spun at 3000 RPM for 1 minute on a glass slide and de-gassed upper PDMS layers were placed channel-side down on the PDMS layer to absorb the PDMS. Liquid PDMS was loaded into a 1ml syringe connected to a dispensing needle. A small amount was spread on the lower PDMS layer where the indentation for the scaffold was located. The nanofiber scaffold stub was then carefully cut-off, and the scaffold was placed fiber-side down on the lower PDMS layer. The plastic scaffold containing the nanofiber network (or PET membrane for comparison) was then placed in the circular indentation and adhered in place with liquid PDMS glue.

Track-etched membranes

To enable a comparison between the nanofiber membranes, track-etched PET or PC membranes were also incorporated into the microfluidic device. PET membranes were removed from 6-transwell plates (Corning, #3450) and according to the manufacturer were 10 µm thick and had 0.4 µm diameter pores at $4x10^6$ pores/cm^2. PC membranes (Millipore Sigma, TSTP02500) were also tested and according to the manufacturer were 22 µm thick and had 3.0 µm pores with a porosity of 11.3%. Both PET and PC membranes were carefully cut into ~4x4 mm squares and bonded to the laser-cut nanofiber scaffolds using liquid PDMS glue. Liquid PDMS was spun at 3000 RPM on a glass slide. One side of the plastic scaffold was placed in contact with the glue, and then removed. The PET or PC membrane was carefully placed across the 2 mm hollow region, and then cured at 55°C for 2 hours.

Cell Culture

Human endothelial cells and pericytes were used to model the BBB. Cell culture was performed according to supplier protocols. Human cerebral microvascular endothelial cells (hCMEC/D3, EMD Millipore) were maintained in EngoGrow™-MV Complete media supplemented with 1 ng/mL FGF-2 (MilliporeSigma), and 1%

Table 2. Thickness, porosity, and average pore sizes and distribution for nanofiber and track-etched membranes.

Membrane Type	Structure	Thickness	Calculated 3D Porosity	Pore Size (µm²)	
				Median	Mean ± STD
1-Layer STEP	600-nm Nanofibers	≤ 1.2 µm	88 %	12.98	14.03 ± 8.18
3-Layer STEP	600-nm Nanofibers	≤ 3.0 µm	88 %	1.62	2.45 ± 2.55
Track-etched PC	3.0 µm cylindrical pores	22 µm*	11.3%*	4.54	6.07 ± 3.24
Track-etched PET	0.4 µm cylindrical pores	12 µm 10 µm*	0.5%*	0.09	0.11 ± 0.06

***According to manufacturer information**

penicillin/streptomycin (Life Sciences). Primary human brain microvascular endothelial cells (HBMECs, Cell Systems) were cultured in Cell Systems Medium supplemented with 5mL CultureBoost™. Primary human brain vascular pericytes (HBVP, ScienCell) were cultured in pericyte basal medium supplemented with 10 ml of fetal bovine serum, 5 ml of pericyte growth supplement, and 5 ml of penicillin/streptomycin solution. Cells were incubated at 37°C and 5% CO_2.

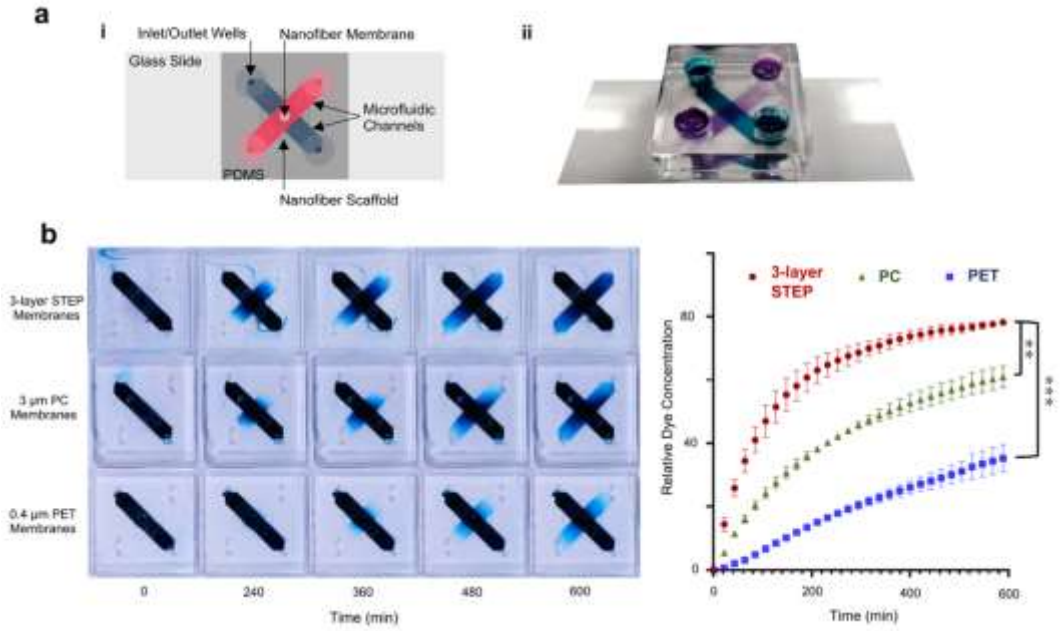

Figure 2. STEP nanofiber membranes enhance trans-membrane diffusion compared to track-etched membranes. a) A three-layer PDMS microfluidic BBB chip contains two orthogonal channels separated by a dense nanofiber network that replicates the basement membrane for co-culture BBB models. b) Diffusion of food dye from the lower channel to the upper channel shows that 3-layer nanofiber membranes enable enhanced diffusion compared with PC and PET membranes. Amount of dye concentration is quantified by color intensity and normalized relative to the bottom channel. STEP membranes have significantly higher rates of diffusion transmembrane compared to PET (p-value < 0.001) and PC (p-value < 0.01) track-etched membranes.

Fluorescence microscopy and cell staining.

Antibody staining was performed to investigate cell morphology. Cells were fixed and stained within the microfluidic devices. Cells were fixed in 4% paraformaldehyde for 15 minutes, washed with phosphate buffered saline (PBS), and permeabilized in 0.1% Triton-X-100 in PBS for 15 minutes. Cells were then washed with PBS and blocked with 5% normal goat serum in PBS for 15 minutes. Cells were incubated with primary antibodies mixed in antibody dilution buffer consisting of PBS supplemented with 1:100 w/v Bovine serum albumen (BSA) and 1:333 v/v Triton X-100. Endothelial cells were stained for ZO-1(ZO-1 Polyclonal Antibody, Invitrogen # 40-2200, 1:100) and VE-Cadherin (CD144 (VE-cadherin) Monoclonal Antibody (16B1), eBioscience, #14-1449-82, 1:100). Pericytes were stained for calponin (Abcam, Recombinant Anti-Calponin 1 antibody [EP798Y] (ab46794), 1:200). Cells were incubated with primary antibodies overnight at 4°C. Cells were washed with PBS, and secondary antibodies (Alexa Flour 488, 555, or 647 at 1:400 in antibody dilution buffer) were added to the device for 45 minutes in the dark at room temperature. Finally, cells were washed with PBS, incubated with 300 nM DAPI in PBS for 10 minutes, and then flushed with PBS for imaging.

Imaging was performed with a 63x water immersion objective (1.15 NA) in a Zeiss LSM 880 confocal microscope. Z-stacks were taken with step sizes 0.36 μm to 0.5 μm for STEP nanofiber and PET membranes, respectively. Optimal laser scanning settings were utilized for image acquisition.

Since >88% of our nanofiber membranes is free space, we observed that cell bodies and nuclei from both co-culture layers frequently settled into the free space within the nanofiber membrane. To calculate the effective distance between the two co-cultured layers, we imported the Z-stack images into ImageJ. There were clear borders for the cell membrane of both the pericytes and endothelial cells (see supplemental movies 1 – 4). We chose 20 random points across the region imaged (N = 4 devices, n = 80 for both PET and STEP nanofiber membranes) and measured the distance in the z-direction between the stained cell membranes across the nanofiber or PET membrane. The distance between nuclei was calculated as the z-distance between the two nearest nuclei (N = 4 devices, n = 20 for both PET and STEP nanofiber membranes).

Diffusion Experiments

Diffusion experiments were performed by adding McCormick Culinary Blue Food Color Dye (Mixture of Blue 1 (MW: 792.85 Da) and Red 40 (496.42 Da)). To prevent dye flow across the membrane, packing tape was used to seal the inlet and outlet ports of the upper channel while filling the device through the lower channel. To

decrease evaporation from the device, the top of the device was then sealed with packing tape. Images were collected every minute for 10 hours at room temperature. Diffusion data were then analyzed in ImageJ to determine the time constants of diffusion.

Experimental methods

To prepare the microfluidic devices for experiments, vacuum-degassed devices were sterilized with ethanol and then washed with PBS. The devices were incubated overnight at 37 °C with 4 µg/ml of human fibronectin in PBS. For imaging experiments, rhodamine-conjugated fibronectin (Cytoskeleton Inc) was used at the same concentration. The following day, devices were flushed with endothelial media and incubated for another hour at 37 °C. Endothelial cells were passaged according to supplier instruction and suspended at $1x10^7$ cells/ml (2,200 cells/mm^2) for the hCMEC/D3 cells or at $5x10^6$ cells/ml (1,100 cells/mm^2) for the HBMECs cells in cell culture media. Before pipetting the cells into the lower channel of the device, the inlet and outlet of the upper channel were blocked with PDMS-clogged pipet tips to prevent fluid flow across the membrane. Cells were then added to the lower channel and the devices were immediately inverted and incubated at 37 °C for three hours. The devices were then place right-side up, the pipet tips removed, and additional media was added to the device wells. Devices were incubated overnight to allow endothelial monolayer formation. The following day, pericytes were passaged according to supplier instructions and resuspended in pericyte media at $5x10^6$ cells/ml (1,100 cells/mm^2). PDMS-clogged pipet tips were used to block the lower channel inlet and outlets, and the cell suspension was added to the upper channel. After incubation for three hours, the pipet tips were removed, and additional media was added. Pericytes were allowed to spread and interact with the endothelial cells for 24 hours, after which time the device was fixed with 4% paraformaldehyde for imaging.

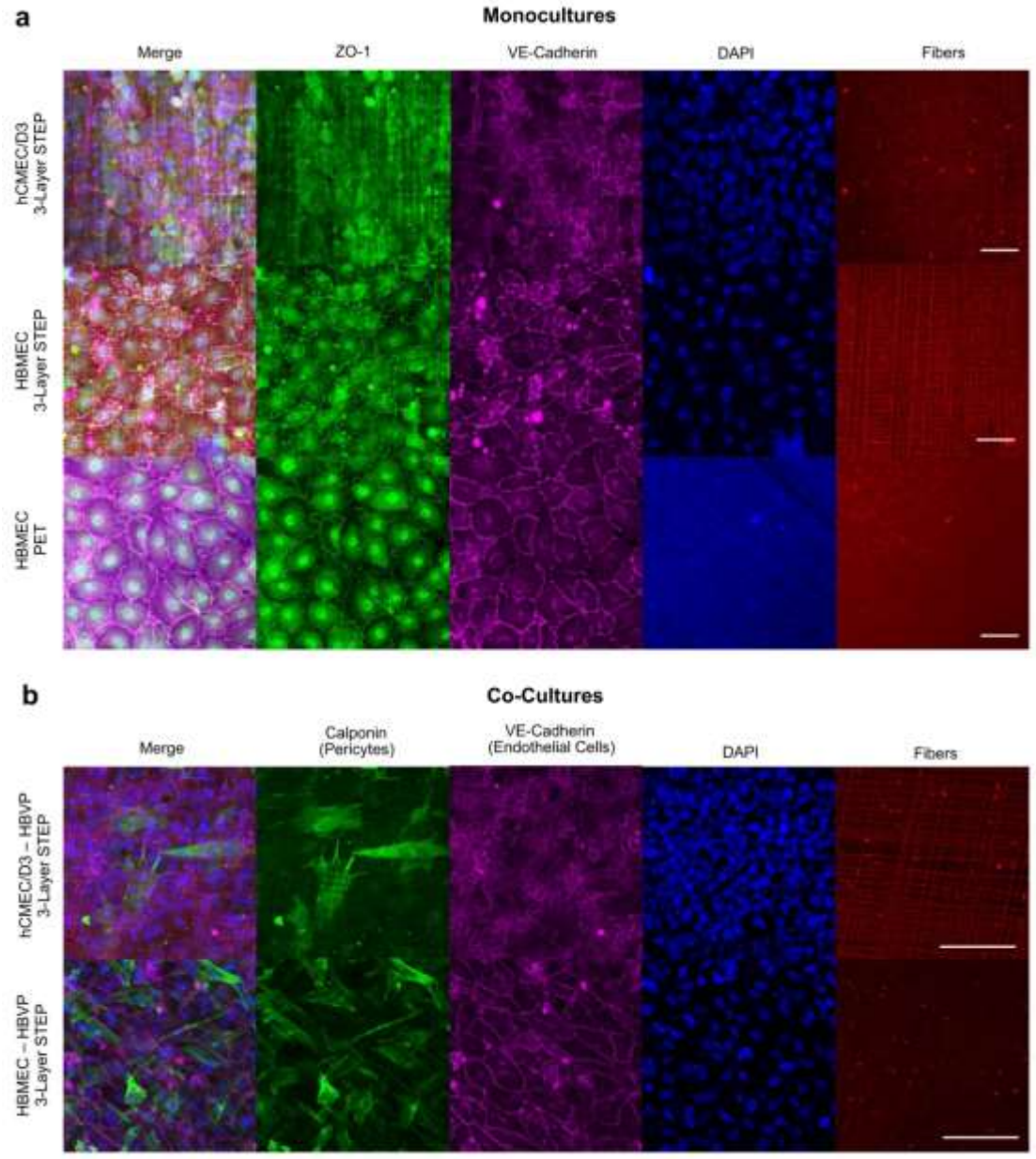

Figure 3. Endothelial cells form complete monolayers on nanofiber scaffolds and can be co-cultured with pericytes. a) Immortalized endothelial cells (hCMEC/D3s, top row) and primary human brain microvascular endothelial cells (HBMECs) cultured in monolayer on nanofibers (middle row) and PET membranes (bottom row). Expression of ZO-1 (green), VE-Cadherin (magenta), DAPI (blue), and rhodamine-conjugated fibronectin (red) are shown. b) hCMEC/D3s (top row) stained with VE-Cadherin and primary pericytes (HBVPs) stained for calponin demonstrate successful co-culture across the nanofiber membrane. HBMECs (bottom row) likewise show characteristics of monolayer formation along with pericyte localization. Scale bars are 20 µm.

Transwell Experiments

To explore tight junction integrity for both mono- and co-culture models, we incorporated our custom scaffolds into a 24-well transwell system. The same scaffolds and membranes for the microfluidic devices were used, as the scaffolds are the same diameter as the bottom of a 24-well plate transwell insert (CellTreat, #230635). The manufactured membranes on the bottom of the transwell inserts were removed and liquid PDMS was used to bond our custom scaffolds in place, with the membrane facing the bottom of the insert. The transwells were sterilized with ethanol and UV light. 4 µg/ml of human fibronectin was added to the transwell and incubated over night at 37 °C. Warm media was added, and initial TEER values were taken to measure the membrane resistances. TEER values were obtained using the EVOM3 epithelial TEER meter with stick electrodes (World Precision Instruments). The values were obtained at 12.5 Hz with a 10 µA applied current. The transwells were then removed and inverted. Endothelial cells and pericytes were

passaged and seeded at the same concentration as with the microfluidic devices. The endothelial cells were added and allowed to attach for two hours at 37 °C. The transwells were then placed into the 24-well plate with the media. HBMEC attachment was verified with a light microscope. The transwell devices were allowed to incubate overnight. HBVPs were added to the inside of the transwell the following day. TEER measurements for day 1 were taken prior to adding HBVPs. TEER measurements were then taken every day for 5 days, directly after removing from the incubator to maintain the temperature at 37 °C. Barrier formation was verified on day 2 with 4 µM Calcein-AM live-dead stain added directly to the transwell.

Porosity, pore size and nominal thickness calculations.

Pore size distributions for the nanofiber membranes and track-etched membranes were determined by thresholding Scanning Electron Microscope (SEM) images of the membranes using ImageJ's particle analysis menu. Porosity values were estimated based on fiber diameters and fiber spacing. Porosity can be calculated as:

$$Porosity = \frac{V_S}{V_M} \cong \frac{V_M - V_F}{V_M}$$

where V_M is the total volume of the membrane, V_F is the total volume of the fibers, and V_S is the volume of free, accessible space within the membrane. V_F depend linearly on the number of fibers within a layer. The number of layers does not affect porosity for thin membranes with a low number of layers since it can be assumed that every point within the membrane is accessible from any other point[62]. Therefore, porosity is determined by the fiber diameter and fiber spacing. The porosity of our 1-layer and 3-layer membranes were estimated using nominal 600-nm diameter fibers and 4 µm spacing.

Nominal thicknesses of the nanofiber and PET membranes were calculated using SEM images as the thickness from the topmost point of the membrane to the bottom most point of the membrane.

Statistical Analysis

All data are presented as mean ± STD. Statistical analysis was performed in Graphpad Prism 9.4.0 (GraphPad Software, San Diego, California USA). Student t-tests were performed to determine the significance of membrane thickness. One-way ANOVA with post hoc tests were performed to find the significance between data sets for porosity, diffusion, and TEER measurements. The significance level was α=0.05.

Results

Ultra-thin, ultra-porous nanofiber membranes are fabricated by the STEP method.

To replicate the ultra-thin and ultra-porous properties of the in vivo BM, we used the STEP method[60,61] to deposit dense networks of biocompatible polystyrene nanofibers with controlled fiber diameters (100 nm – 10 µm) and controlled fiber spacing (as low as 3 µm)[63]. Suspended crosshatch nanofiber patterns were fabricated by stacking orthogonal fiber layers on top of previously deposited fibrous layers to form one crosshatch unit (Figure 1b). One crosshatch unit has too large of pores to constrain cell monolayers to their respective sides, so multiple crosshatch units can be spun on top of each other at an angle (0-90°) to form thicker but more confining membranes. The nanofibers were then chemically fused together to improve the integrity of the membrane. We demonstrate the depositional control for the 3-layer step membrane and quantify the fiber orientation for the two orthogonal arrays (Supplemental Figure 2). Through the STEP process, fiber orientations are tightly controlled in both arrays with a standard deviation <0.25° between the intended and actual deposition angle.

STEP nanofiber membranes were compared against the conventional barrier-modeling PET membranes with 0.4 µm pore sizes with an extremely low porosity of less than 0.5% and 3 µm diameter pore PC membranes with a porosity of ~15% (Figure 1b). The STEP nanofiber membranes have extremely high porosities for two nanofiber membrane configurations investigated: 1- and 3-layers of crosshatch unit membranes with 600-nm fiber diameters (586 ± 58.9 nm; range 503 – 703 nm). For both the 1-layer and 3-layer membranes, we found the theoretical porosity to be ~88%. This represents a 220x increase over the 0.4 µm pore PET membranes and a 6.5x increase over the 3-µm PC membranes. Further, analysis of the Z-projected (2D) from SEM images reveals the average pore size is 14.0 ± 8.2 µm^2 for the 1-layer membrane and 2.5 ± 2.5 µm^2 for the 3-layer membrane (Figure 1c). One single crosshatch layer has roughly equivalent pore sizes as a 5-µm diameter track-etched membrane (pore size ~19.6 µm^2), and our 3-layer nanofiber membranes have pore sizes roughly between 1-µm and 3-µm diameter track-etched pores (pore sizes 0.8 µm^2 and 7.1 µm^2, respectively). Our 3-layer STEP membrane has a significantly smaller pore size than 3-µm diameter PC membrane ($t = 22.4$, $p < 0.0001$).

In addition to the ultra-high porosities of our nanofiber membranes, our membranes are also significantly thinner than conventional membranes. Conventional track-etched PET and PC membranes range from 10 to 12 µm in thickness, over 100x the thickness of the BM in vivo. Our nanofiber networks offer at least a 3-fold decrease in thickness (Figure 1d). Our 1-layer membranes are nominally 1.0 µm thick, while our 3-layer membranes are nominally 2.9 µm thick. At ≤ 3 µm nominal thickness and highly porosity, our membranes can enable significant cell-cell interactions across the membrane, by allowing easier transmembrane diffusion and co-cultures to reach into the membrane without crossing over. Overall, we demonstrate a significant advancement in fabrication of BM mimics through precise patterning of suspended fiber networks in multiple layers. These properties are summarized in Table 2.

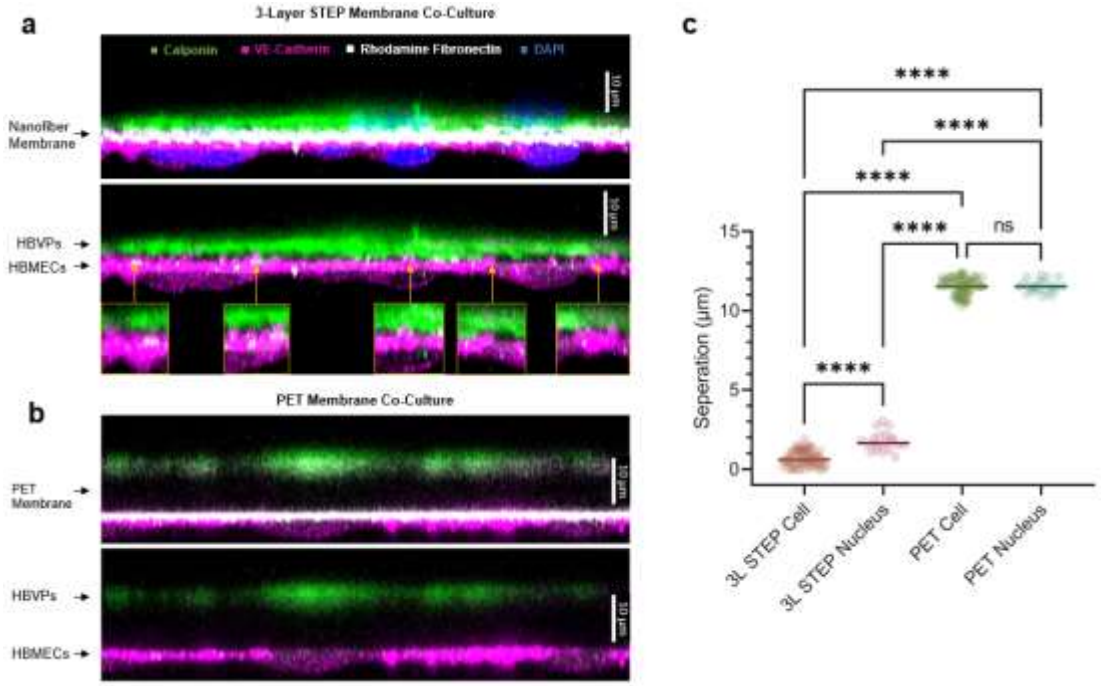

Figure 4. Nanofiber co-cultures of endothelial cells with pericytes enable cell-cell interactions across the membranes. a) Z-stack images of co-cultures taken with a 63x water lens demonstrate the close-contact of pericytes (green, calponin) and endothelial cells (cyan, VE-cadherin) across the nanofiber membranes. In multiple instances, the endothelial cells and pericytes are reaching into the free space within the membrane (yellow-dashed boxes). b) PET track-etched membranes show significant separation between pericytes (top) and endothelial cells (bottom) which prevents cell-cell interaction. Notice that the imaging quality on the upper side of the PET membrane is significantly reduced due to the mismatch in refractive index of the plastic. In contrast, there is minimal loss of resolution across the nanofiber membranes. c) Measured separation between the stained cellular membranes and nuclei for the nanofiber membrane and the PET membrane. The effective separation between cells (0.68 µm) is significantly smaller than the nominal thickness of the membrane (3 µm) as the cell can reach into the free space within the membrane. Rhodamine-fibronectin on the membranes is white and the DAPI is blue. (**** $p < 0.0001$)

Nanofiber membranes enhance trans-membrane diffusion.

Next, we inquired if the combination of high porosity and low thickness of our nanofiber membranes would enable enhanced molecular diffusion across the membrane and support the marked increase in theoretical porosity. To visually demonstrate increases in transmembrane diffusion, we performed time-lapse diffusion experiments by incorporating the nanofibers in a microfluidic device (Figure 2). To create our microfluidic model, we fabricated two patterned PDMS channel layers that enclosed the scaffold with a nanofiber membrane at the intersection so diffusion from one channel to another could only occur by passing through the membrane. For comparison studies, conventional track-etched PET or PC membranes were bonded to identical laser-cut scaffolds (see methods). Food dye was injected into the bottom channel and imaged every minute for 10 hours (Figure 2b). The 3-layer nanofiber membrane had a significantly increased rate of dye diffusion from the lower channel to the upper channel. The time constants of diffusion were calculated to be 72.3 ± 10.3 min, 199 ± 11.5 min, and 574 ± 45 min for the 3-layer nanofiber, 3-µm PC, and 0.4 µm PET membranes, respectively. The 3-layer nanofiber membrane has almost a 3x increase in the diffusion rate over the PC membrane and an 8x increase over the PET membrane. Despite the 3-layer nanofiber membrane having a much smaller calculated pore size compared to the PC membrane, it has a 3x higher rate of diffusion, supporting the significantly higher theoretical porosity.

Nanofiber membranes support endothelial monolayer formation.

Given the improved physical properties of our nanofiber membranes compared to track-etched alternatives, we investigated whether they could be used to recapitulate critical BBB physiology in vitro. We found that two human brain microvascular cell lines were able to form intact monolayers on the 3-layer nanofiber membrane: immortalized human cerebral microvascular endothelial cells (hCMEC/D3) and primary human brain microvascular endothelial

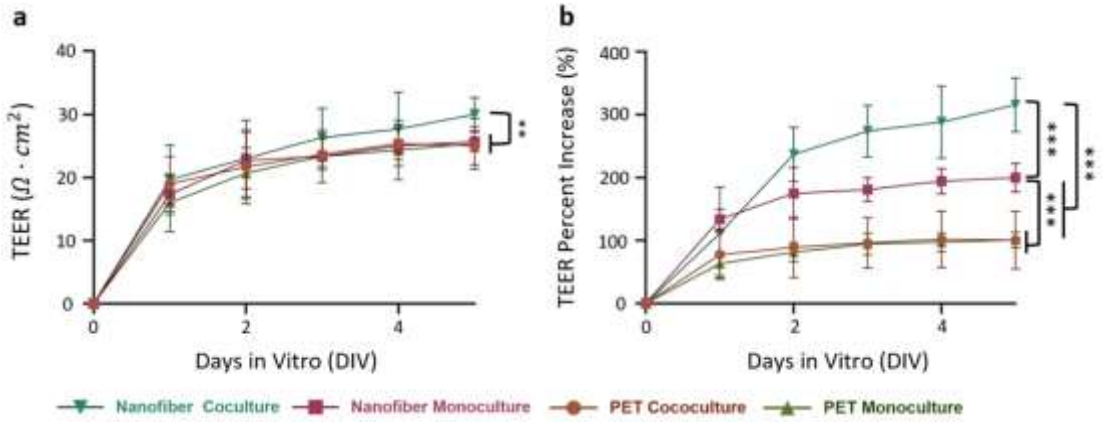

Figure 5. TEER measurements of HBMEC and HBMEC-HBVP co-cultures indicate a benefit of using STEP nanofiber membranes. Custom transwell inserts were fabricated to generate either a monolayer of endothelial cells (HBMECs) or a co-culture of endothelial cells (HBMECs) and pericytes (HBVPs). a) TEER measurements with stick electrodes on HBMEC monolayers indicate equal TEER values between the 3-layer STEP nanofiber and PET membranes. Co-cultures on the PET membranes had a negligible rise in TEER compared to the monoculture, while the nanofiber membrane co-cultures had a significant increase in TEER values from the monoculture condition (p-value < 0.01). b) The 3-layer nanofiber membranes had a more significant percent increase from base measurements than PET membranes for both monoculture (p-value < 0.001) and coculture conditions (p-value < 0.001). Each data point is given as mean ± std. with n >= 3. (** p < 0.01, *** p < 0.001)

cells (HBMECs). Endothelial cells were seeded at high densities on the fibers (see methods), and

monolayers began forming within 24 hours. Figure 3 shows complete monolayers of HBMECS and hCMEC/D3s formed after 72 hours of culture on the fibers. Furthermore, confocal microscopy reveals that endothelial cells were confined to one side of the membrane (See Movie 1). Nanofiber networks were stained with rhodamine-conjugated fibronectin.

To explore the integrity of the monolayers, we interrogated the expression of tight junction markers ZO-1 and VE-cadherin for both

monolayer cultures of HBMECs and hCMECS/D3s. Both endothelial cell lines had a significant expression of ZO-1 and localization of VE-Cadherin (Figure 3a), showing both tight junction expression and monolayer formation. The 3-layer nanofiber membranes and 0.4 µm PET membranes both showed ubiquitous ZO-1 and VE-Cadherin expression for HBMECs with well-defined boundaries between cells.

Nanofiber co-cultures of endothelial cells with pericytes enable cell-cell interactions across the barrier.

Due to the low thickness and high porosity of our membranes, we next sought to explore the utility of the STEP nanofiber membrane to form a physiologically relevant co-culture model. We cultured human brain vascular pericytes (HBVPs) and endothelial cells on opposite sides of the nanofiber membrane to mimic the spatial configuration of these cells in vivo. As shown in Figure 3b, pericytes were successfully cultured opposite endothelial monolayers for both hCMEC/D3 and HBMEC cells.

We used rhodamine fibronectin (white) to promote cell adhesion and visualize the membranes, and cell nuclei are stained with DAPI (blue). For the track-etched membranes, there are two distinct lines of rhodamine fibronectin expression on the top and bottom of the membrane, with the bottom being noticeably more visible. The track-etched membranes are not optically transparent, which can lead to loss of image resolution. Further, they are auto-fluorescent and emit blue light when excited (Figure 3a). This may impede imaging with a similar excitation wavelength. The nanofiber membranes, however, do not share either disadvantage. We did not observe a visible separation of rhodamine expression across the 3-layer STEP membrane and found minimal loss of image resolution through the STEP membranes, indicated by the increase in resolution for cells on top of the membrane.

Unlike conventional tracked-etched membranes which are flat, the fibrous nature of our nanofiber membrane allows for a 3D environment for cell attachment[63,64]. The interface between the monolayers and the track-etched membrane is clearly visible in the confocal Z-stack image.

While the nominal thickness of the nanofiber membranes was calculated to be 2.9 ± 0.4 µm, the effective thickness is smaller due to cells settling and occupying space within the nanofiber membrane. We observed that the nuclei (DAPI) for both pericytes and endothelial cells are within the free space of the nanofiber membrane (Figure 4a). The average separation between nuclei across the membrane was 1.75 ± 0.60 µm, with separations ranging from 0.75 – 3 µm (Figure 4c). Importantly, we observed no instances of nuclei from one cell layer crossing over to the other side.

Further, we observed that the cytoplasmic membranes of these cells penetrated deeper within the STEP membranes, with many instances where there is no observable separation between the endothelial and pericyte plasma membranes. We calculated the effective distance between the two co-culture layers and found an average separation of 0.69 ± 0.47 µm with a range of 0 – 1.8 µm. The

separation between the two co-cultured layers is significantly less (p-value < 0.0001) than the separation between the nuclei. This supports the observations that the nuclei are confined to their respective sides, but the cells are allowed to reach into the free space to come into contact. From confocal images, we calculated the average nucleus diameter to be 12.4 ± 1.5 (n = 40; range 10.1 – 16.7 µm). Migration studies frequently employ membranes with pore diameters >3 µm[65], so it isn't surprising that cells can partially enter the porous STEP membrane, without crossing over. The calculated separation between nuclei and co-culture layers for the PET membrane was 11.5 ± 0.40 µm and 11.4 ± 0.57 µm, respectively. There was not a significant difference between the nuclei and membrane separation for the PET membrane.

Further, multiple experiments were observed demonstrating the close proximity of the co-cultures on the STEP nanofiber membrane and the ability of cells to come into contact within the membrane (Supplemental Figure 1c and Movies 2-4), with no visible separation between the two cell monolayers. Movies 2 and 3 give Z-stacks that show HBMEC monolayers confined to the bottom of the membrane, with pericyte processes reaching through the nanofiber membrane. This is not seen for the track-etched membrane in Movie 4, where there is large separation between the cell monolayers and a loss of resolution for the pericytes on the top of the membrane.

TEER measurements indicate a biological benefit of the ultra-thin and ultra-porous nanofiber co-culture.

BBB integrity is commonly assessed by TEER measurements[66,67]. Endothelial monolayers resist electrical current by restricting free ion transport with tight-junction formation. Thus, by applying a small potential across the monolayer and sensing the corresponding electrical current, the resistance of the monolayer can be deduced. Since PET membranes are thick with minimal porosity, pericytes have not been shown to increase TEER measurements as meaningfully as either astrocytes or neurons[68]. This is in part because astrocytes and neurons upregulate tight junctions through soluble factors, while pericytes upregulate tight junctions through peg-and-socket junctions. Soluble factors can pass through the small pores in the thick track-etched membranes, while cells cannot fully reach across for physical contact.

Since our membranes are ultra-thin and ultra-porous, we sought to explore the impact that pericytes may have on tight junction formation. The membranes on conventional 24-well plate transwell inserts were removed and replaced with the scaffolds holding our custom nanofiber membranes (see methods). HBMECs were cultured on the basolateral side of the membrane, and if co-culture was desired, HBVPs were grown on the apical side. We used stick electrodes to measure the resistance across the membrane. TEER measurements were taken prior to seeding the cells (control) and every day after for 5 consecutive days. The TEER measurements were normalized by the control values to find the contribution of the endothelial cells. The nanofiber membranes had initial resistance values ~3x lower than the PET membranes; however, their nominal TEER increase was equal to or higher than with the PET membrane (Figure 5a). We observed TEER values for the HBMEC monolayers are within the range previously characterized ($20 - 100\ \Omega \cdot \mathrm{cm}^2$)[69]. The TEER values were equal between monocultures of HBMECs on nanofibers and PET membranes. Since nanofibers had a much lower initial resistance, the percent increase in TEER is significantly larger (Figure 5b). Next, we looked at the contributions that HBVPs may have on HBMECs tight junction formation. We saw that the co-culture of HBMECs and HBVPs on the PET membrane followed the same trend of no increase in TEER values. The nanofiber co-culture, however, had a significant increase in TEER values over both monoculture conditions and the PET co-culture conditions. This indicates that the nanofiber BM more accurately replicates the physiology of the BBB than conventional track-etched membranes.

Discussion

Here we have shown that precisely aligned nanofiber networks can be used to create physiologically relevant basement-membrane mimics. Nanofiber network properties, such as fiber spacing, fiber diameter, fiber orientation, and number of fiber layers can be precisely controlled to create membranes with variable thicknesses and porosities. To our knowledge, our nanofiber membranes significantly exceed the highest reported porosities for membranes used in barrier modeling. At almost 90% porosity, these membranes are near the limit of what might be experimentally possible, and higher porosity membranes are not likely to significantly increase transport across the membranes. While the described 1-layer and 3-layer nanofiber membranes form BM mimics with enhanced properties, it may be possible to further optimize these membrane properties by exploring additional fiber diameters, especially smaller diameter fibers. Nanofibers as small as 100-nm in diameter can easily be fabricated with the STEP method. Incorporating smaller diameter fibers could theoretically produce membranes less than 200 nm, reaching the upper end of the in vivo brain BM thicknesses. Further, thinner fibers would decrease the surface area for focal adhesion formation, allowing cell-cell contact to dominate compared to cell-surface contact. However, fabricating membranes with thinner fibers may compromise membrane strength. Our previous study with a single crosshatch STEP membrane demonstrated a bending stiffness of 0.45 N/m and an elastic modulus of 150.6 kPa[63]. Our observed elastic modulus is lower than those observed for other fibrous membranes[70] and relative to the lower end of measurements for the in vivo basement membrane[71]. Future studies should be conducted to tune the STEP membranes elastic modulus through manipulating fiber layer numbers and thicknesses to achieve a desired value.

Recent work has been performed to create thin fibrous membranes using electrospun poly(e-caprolactone)[57] membranes coated with collagen. However, due to the electrospinning process, the fibers are not precisely aligned, leading to variability in pore size and local thicknesses. STEP nanofiber membranes overcome this challenge due to the precise control over fiber diameters, spacings, and orientations across multilayered structures. This is demonstrated with a smaller skew in the measured pore sizes than other fibrous membranes[54-57,66], with < 1 µm² difference between the median and mean pore size. Consistent generation of intended

pore sizes is paramount for applications in transmembrane migration and transport.

Non-fibrous membranes are also popular for BM modelling. Poly(lactide-co-caprolactone) membranes have a nominal thickness of 0.95 µm, due to the spin coating method[72]. However, their calculated porosity was between 35 and 40%, significantly lower than our membrane.

We found for the STEP membrane that most nuclei were separated by less distance (1.75 ± 0.60 µm, range 0.75 - 3 µm) than the nominal membrane thickness, suggesting the cells partially sit within the membrane. This has been observed previously for STEP membranes, where the nucleus shape will remodel around the nanofiber membranes[64]. Jana *et. al.* observed that the size and curvature of the nanofibers affects the evagination by the cell and modeling of the nucleus. Briefly, cells sense the interfiber spacing and wrap around and align along the fiber axis, form varying shapes[63,73]. These findings have been validated to match various in vivo bahaviors[74-76].

We integrated our nanofiber membrane into a PDMS microfluidic model; however, absorption and adsorption of molecules by PDMS makes it unfavorable for drug transport studies[77]. Since our nanofibers are polystyrene, an entirely plastic device could be designed and assembled for commercial use. Furthermore, our nanofibers can be integrated into conventional transwell plates, enabling use with current well plate-based equipment to enable rapid adoption. As a proof-of-concept, we modified a conventional 24-transwell plastic insert to integrate the nanofiber scaffold and used them for collecting TEER measurements with stick electrodes.

We cultured endothelial monolayers on the nanofiber scaffolds and demonstrated that the monolayers express key junctional proteins such as ZO-1 and VE-Cadherin. Confocal imaging shows that endothelial cells were constrained to only one side of the nanofiber membrane. Furthermore, we were able to co-culture pericytes on the opposite side of the membrane and show that our highly porous and thin membrane enables close contact between endothelial cells and pericytes. From confocal images, it appears that the two monolayers can reach into the membrane to come into direct contact. The benefit of having pericytes in close proximity is supported by the significantly higher TEER measurements of HBMEC and HBVP co-cultures on the nanofibers which suggests that this membrane has advantages over other membranes when replicating in vivo physiology. Due to the high porosity of the STEP membrane, the initial resistance values measured were 3-5x lower than for the PET membrane. This resulted in a higher percent increase for TEER measured using the STEP nanofiber membranes. The significantly higher TEER values indicate tighter barrier formation, while the percent increase in TEER measurements suggests that barriers formed on STEP membranes may be more sensitive to changes in TEER values. This would have a benefit in pharmaceutical testing as it allows for more resolution in measuring effects.

Previous studies have shown that migratory astrocytes that cross track-etched membranes for pores as small as 3-µm in diameter[38]. Migratory pericytes or astrocytes across the membrane clearly are not consistent with the in vivo configuration but are more realistic for our thin membranes. Our nanofiber networks have significant advantages for studying cell and nanoparticle migration across the BBB. The controllable pore size and high porosity may allow for small cell and nanoparticle migration while keeping large cells to their respective sides of the membrane. Conventional track-etched membranes are unsuitable for nanoparticle transport studies due to the adhesion of nanoparticles to the membranes and within track-etched pores[43]. Our highly porous membrane should minimize interference of the membrane to nanoparticle transport. Likewise, modeling cancer metastasis and immune cell migration across the BBB is of significant importance[78], and our models should enable high-levels of migration. The larger pore sizes (generally ≥ 3µm pores) used for cellular transmigration studies have the disadvantage of allowing endothelial cells to cross the membrane and form a second monolayer on the opposite side of the membrane[34,38].

Conclusions

Using our suspended nanofiber networks, we achieved high porosity (88%) in ultrathin fibrous (< 3µm nominal) BM mimics compared with reported literature or commercially available. Our approach achieved low separation between the cell membrane and nuclei (0.69 µm and 1.75 µm, respectively), thus providing unrestrained opportunity for the cells on either side to contact each other without permitting migration, as would be expected in vivo. We expect future studies to further optimize the physical membrane properties and investigate the utility of these networks. We anticipate that our nanofiber networks will improve barrier modeling with implications for drug discovery, cell migration, and disease studies.

Data Availability and Supplemental

The data that supports the findings of this study are available within the article and its supplementary material. See supplementary material for additional device images of endothelial cells forming monolayers and co-cultures with pericytes**.** Movies of Z-stack images are also provided to demonstrate the isolation of monolayers while being in close proximity for monoculture and co-culture conditions on nanofiber and PET membranes.

Author Contributions

Conceptualization: PMG, EJJ, AJ, RVD, ASN. Methodology: PMG, EJJ, AJ, AA. Materials: AJ, AA Formal Analysis: PMG, EJJ. Data Curation: PMG, EJJ. Resources: ASN, RVD. Supervision: ASN, RVD.

Conflicts of interest

The authors have pending patents and current patents on the work presented within the manuscript.

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