**Proceedings Paper:**
Pensabene, V orcid.org/0000-0002-3352-8202, Crowder, SW, Balikov, DA et al. (2 more authors) (2016) Optimization of electrospun fibrous membranes for in vitro modeling of blood-brain barrier. In: Proceedings of the IEEE 38th Annual International Conference of the Engineering in Medicine and Biology Society (EMBC 2016). EMBC 2016, 16-20 Aug 2016, Orlando, Florida, USA. IEEE , pp. 125-128. ISBN 9781457702204

https://doi.org/10.1109/EMBC.2016.7590656

# Optimization of Electrospun Fibrous Membranes for *in vitro* modeling of Blood-Brain Barrier

V. Pensabene, S.W. Crowder, D.A. Balikov, J.B. Lee and H.J. Sung

**Abstract— The blood-brain barrier (BBB) plays a critical role in brain homeostasis at the cellular and global level. Mimicking the selective permeability and transport properties of the BBB to specific molecules and cells remains a significant challenge towards the development of a physiologically relevant in vitro BBB model. In this study, we developed electrospun poly (ε-caprolactone) (PCL) and polyethylene glycol (PEG) copolymer membranes that supported different cellular components of the neurovascular unit including human-derived endothelial cells, pericytes and astrocytes. Comparative analyses of thickness, morphology, biocompatibility and permeability of membranes were also conducted. We found that collagen coated 4%PEG-96%PCL membranes supported the growth of a confluent and tight endothelium confirmed by transendothelial electrical resistance measurements (TEER). Based on fabrication process and reported results, we finally discuss the adoption of these electrospun fiber membranes for in vitro and on-a-chip human BBB models.**

## I. Introduction

The blood-brain barrier (BBB) is a selectively permeable membrane, formed by various cell types that comprise the brain microvasculature. The BBB regulates brain function and metabolism by either permitting or blocking the transport of molecules [1]. Because the tight junctions of the vascular endothelium are responsible for blocking the passage of large drug molecules through the BBB, the development of new drugs for neurological diseases has been severely limited [2]. New models that mimic the properties of the BBB would be useful for testing drug interactions at this selectively permeable interface.

Different cell types constitute the human BBB: 1) endothelial cells form a tight physical barrier in the luminal side of the vessels and their abluminal side is surrounded by pericytes and astrocytes [3]. 2) Pericytes are contractile, phagocytic cells that are in direct physical contact with the brain endothelium. Pericytes also regulate capillary permeability by modulating endocytosis-mediated transport and tight junction stability of endothelial cells [4,5]. 3) Astrocytes serve as a physical contact between neurons and endothelial cells, and regulate tight junction expression and the localization of molecular transporters [6,7].

In order to reproduce the unique properties of the BBB in vitro, these three cell types must be grown in an organized fashion that mimics their in vivo physiology. This requires an appropriate scaffold that does not impair their viability or proliferation, and results in a functional, highly-selective cellular barrier.

The primary aim of this study was to synthetize permeable electrospun fiber membranes that support the growth of the three constituent BBB cell types, and to test the functional capacity of this tri-culture system as a synthetic BBB mimetic.

Copolymers of x% poly(ε-caprolactone) (PCL) and y% polyethylene glycol (PEG) (x, y: mole %) were used in this study. PCL is a semi-crystalline, biodegradable, FDA-approved, hydrophobic polymer [8]. PEG is a biocompatible, hydrophilic polymer that can repel protein and cells [9]. In our previous studies we showed that physicochemical, mechanical, and bioactive properties of electrospun fiber scaffolds can be tuned to promote cell growth and differentiation by combining these two components with different mole percentages [10, 11].

In order to produce an in vitro BBB model, the thickness and porosity of these membranes must be finely controlled to facilitate cell-cell contact, paracrine signaling and selective permeability. All of these properties can be manipulated by changing the processing parameters or chemical composition of the material.

With this goal, we employed copolymer substrates with promising properties for the desired application, and characterized the fiber architecture, pore structure, and overall morphology and permeability of electrospun membranes. We then evaluated the ability of copolymer membranes – both uncoated and coated with collagen type I - to support the growth and functionality of endothelial, pericytes and astrocytes as a synthetic BBB system.

## II. Materials and Methods

### 2.1 Polymer synthesis and BBB membrane fabrication and characterization

Copolymers of x% poly(ε-caprolactone) (PCL) and y% polyethylene glycol (PEG) (x, y: mole %) were synthesized as previously described in [11]. Briefly, PCL was synthesized via ring-opening polymerization of ε-caprolactone in bulk with benzyl alcohol and $Sn(Oct)_2$. The polymer structure was

*Research supported in part by NIH 1UH2-TR000491-01.

Virginia Pensabene is now with the School of Electronic and Electrical Engineering at the University of Leeds, LS2 9JT, Leeds, UK, and was with Department of Biomedical Engineering at Vanderbilt University, 37235, Nashville, TN, USA (phone: 615-343-8978; e-mail: virginia.pensabene@vanderbilt.edu).

S. W. Crowder was with the Department of Biomedical Engineering, Vanderbilt University, 37235, Nashville, TN, USA, and is now with the Department of Materials, Department of Bioengineering, Imperial College London, London, UK (e-mail: s.crowder@imperial.ac.uk).

D. A. Balikov is with the Department of Biomedical Engineering at Vanderbilt University, 37235, Nashville, TN, USA (e-mail: daniel.a.balikov@vanderbilt.edu).

J.B. Lee is with the Department of Biomedical Engineering and with the Department of Mechanical Engineering, Vanderbilt University, 37235, Nashville, TN, USA (e-mail: jung.bok.lee@vanderbilt.edu).

H.J. Sung is with the Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University Medical Center, with the Department of Biomedical Engineering at Vanderbilt University, 37235, Nashville, TN, USA, and with Severance Biomedical Science Institute, College of Medicine, Yonsei University, Seoul 120-752, Republic of Korea (e-mail: hak-joon.sung@vanderbilt.edu).

verified by $^1$H NMR spectra: ($CDCl_3$)=$\delta$ 4.06 (t, 3H,-$OCH_2$), 2.31(t, 2H, -$CH_2$), 1.66 (m, 2H, -$CH_2$), 1.37 (m, 4H,-$CH_2$) ppm. 4%PEG-96%PCL was prepared with the same method except for a stoichiometric amount of in the presence of 2kDa monomethoxy-PEG and showed a spectra as 1H NMR ($CDCl_3$)=δ 4.06 (t, 3H,-$OCH_2$), 3.65 (s, 4H,-$OCH_2$), 2.31(t, 2H,-$CH_2$), 1.66 (m, 2H,-$CH_2$), 1.37 (m, 4H,-$CH_2$) ppm.

ε-caprolactone was dried and distilled over $CaH_2$ immediately before polymerization. Tin (II) ethyl hexanoate was distilled under high vacuum. Benzyl alcohol was dried and distilled over $CaH_2$. ε-caprolactone and benzyl alcohol were purchased from Alfa Aesar (Ward Hill, MA, USA). Tin (II) ethyl hexanoate ($Sn(Oct)_2$), benzyl alcohol, monomethoxypoly(ethylene glycol) (PEG) (MW =2000), anhydrous tetrahydrofuran (THF), lithium diisopropylamide (LDA) (2M in THF/n-heptane), anhydrous toluene, dichloromethane, and diethyl ether were purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA) and were used as purchased unless otherwise noted.

To generate fibrous membranes by electrospinning, the polymers were dissolved in trifluoroethanol at 10% w/v and placed into a syringe with a 2 mL stainless steel needle. The solutions were ejected at a flow rate of 1 mL/hour with an applied voltage of 10 kV and collected on a grounded drum (diameter = 10 cm), spinning at a rate of 500 rpm placed at 10 cm of distance. After 30 minutes, the electrospun membranes were cut and carefully peeled off the drum. The thickness was measured with a stylus profilometer (Veeco Dektak 150), as average of 5 measurements. Morphology and pore size were measured by Scanning Electron Microscopy (Hitachi S4200).

In order to measure water absorption, dry membranes were cut into 1 x 1$cm^2$ pieces and weighted ("dry mass"). Samples were then incubated in deionized $H_2O$ overnight at 37°C and thus weighted again ("wet mass"). The reported values of mass fold increase (wet/dry) are averages of n≥4 measurements.

### 2.2 Membrane conductivity measurements

Impedance was measured with an AD5933 1 MSPS, 12-Bit Impedance Converter Network Analyzer (Analog Devices, Norwood, MA) with 2 electrodes fixed on a printed circuit board in order to fit on a 24 well plate (or 6-well snapwell plate). Measurements were performed in PBS. Impedance values were reported after subtraction of background PBS impedance. 3 measurements were recorder for each mat with frequency ranging from 20 kHz to 21 kHz.

### 2.3 Live/Dead staining of cells on membranes

Telomerase immortalized microvascular endothelial cells (TIME) cells (ATCC CRL-4025, Manassas, MA) were maintained with Vascular Cell Basal Medium (ATCC PCS-100-030) supplemented with Microvascular Endothelial Cell Growth Kit-VEGF (ATCC PCS-110-041) which contains VEGF (5 ng/ml), EGF (5 ng/ml), FGF basic (5 ng/ml), IGF-1 (15 ng/ml), 10 mM L-glutamine, heparin sulfate (0.75 Units/ml), hydrocortisone (1 µg/ml), ascorbic acid (50 µg/ml), 5% fetal bovine serum and penicillin-streptomycin (0.5 mL, 10 units/mL) and amphotericin B (25 ng/mL).

Human brain astrocytes (ATCC CRL 8621-SVG p12) were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC 30-2003) modified to contain Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine,1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate and Penicillin-Streptomycin-Amphotericin B.

Human vascular brain pericytes (ScienCell, Carlsbad CA) were cultured in pericytes medium (ScienCell) containing basal medium, 2% of fetal bovine serum, 1% of pericyte growth supplement and 1% penicillin/streptomycin solution.

Cell culture experiments were performed in a 6-well plate containing modified transwell membrane-less inserts ("snapwells", Corning, Tewksbury, MA). One fiber membrane was secured on each insert by a polystyrene ring. Astrocytes, pericytes and TIME cells were seeded at $10^5$ cells/transwell on 100%PCL and 4%PEG-96%PCL membranes and on glass (as control). Seeding densities are live cell densities as trypan blue was used during cell counting. After 7 days of culture, viability of the cells was investigated using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Life Technologies, Carlsbad, CA). The cultures were rinsed with PBS and then treated for 10 min at 37°C with 2 µM calcein AM and 4 µM EthD-1 in PBS. Cells were imaged with an inverted fluorescent microscope (Nikon Eclipse Ti, Nikon Instruments, Melville, NY) equipped with a cooled CCD camera (DS-5MC USB2, Nikon Instruments). Images were process in NIS Elements imaging software.

### 2.4 Membrane diffusion experiments

Diffusion of compounds with different molecular weight through the fiber membranes was measured by using a UV-Vis Spectrophotometer (Varian Cary 50, Agilent Technologies, Santa Clara, CA). FITC-Dextran (70kDa and 150kDA) and 2kDa FITC-Inulin (Sigma Aldrich, St. Louis, MO) were diluted in deionized water and added to nude fiber mats and on fiber membranes coated with purified type I collagen (50 µg/ml, ScienCell).

The same method was used to measure diffusion of the different compounds through the same membranes in the presence of a confluent layer of endothelial cells. Prior to cell seeding, membranes were assembled in the snapwell inserts, sterilized for 15 minutes under direct UV light, and divided in two groups (with and without collagen type I coating). Membranes were first hydrated with full medium for 15 minutes. 100 µL of the media was then removed from the apical side and replaced with 100 µL of TIME cell suspension ($10^6$ cells/mL). Cells were allowed to grow for 72 hours. Once the monolayers were formed, the media were collected from the apical side and replaced with FITC dextran (70 kDa) solution, as previously described. The permeability was evaluated by analysis of fluorescence intensity of media collected from the basal side after 20 minutes of incubation. In the same way, in the second experiment, the cell layers were permeabilized by adding 4% DMSO to the apical side. After 23 hours the media were replaced by fresh media containing dextran (2.5 µg/mL) and permeability was measured.

2.5 Transendothelial electrical resistance (TEER)

A Millicell ERS-2 Voltohmmeter (Millipore, Billerica, MA) with a silver/silver chloride (Ag/AgCl) electrode was used to collect TEER measurements. Membranes only and membranes coated with purified type I collagen were secured into snapwell inserts. $10^5$ TIME cells were seeded onto each membrane and grown for 6 days. TEER was measured every 2 days after 48 hours from seeding.

## III. Results

100%PCL and 4%PEG-96%PCL polymers were electrospun to form fiber membranes with the same electrospinning conditions in order to keep diameter (~0.5 μm), morphology and alignment of the fibers in the same range (Fig. 1). Table 1 summarizes thickness and pores size measured by analysis of SEM images. Water uptake data show higher hydrophilicity of the PEG-PCL membrane, as expected, due to the physical properties of. The impedance, however, was higher for the 100%PCL, suggesting a more compact microarchitecture that was not as disrupted by PEG.

TABLE I. Fiber mats characterization

| | PCL | 4%PEG-96%PCL |
|---|---|---|
| **Thickness (μm)** | 5.888±0.045 | 5.855±0.101 |
| **Pore size (μm)** | 0.627±0.053 | 0.612±0.038 |
| **Water uptake*** | 16.65±5.68 | 40.07±5.78 |
| **Impedance (Ω)** | 365.73±36 | 296.97±1.9 |

* in fold changes.
** average values: 15 measurements; 5 samples for each type of polymer.

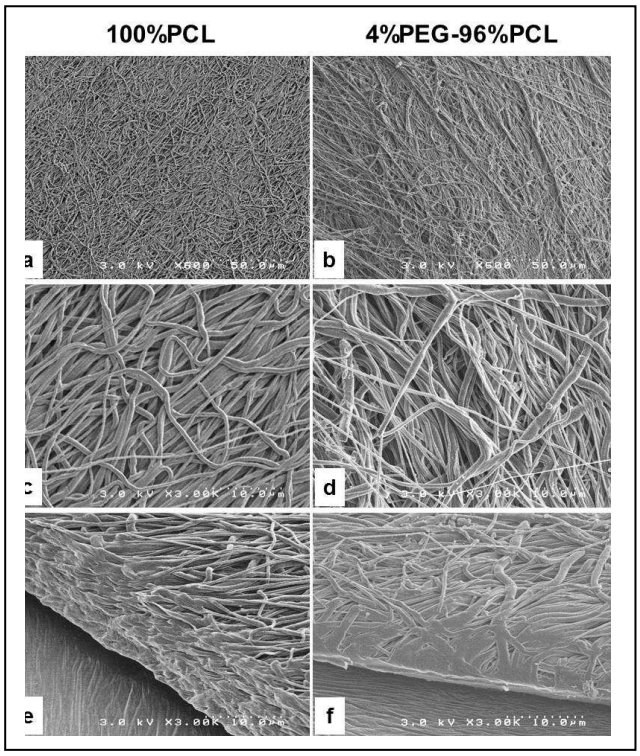


Figure 1. SEM images of the 100%PCL (left column) and 4%PEG-96%PCL membranes (right column) at 600x (a,b) and 3000x (c,d) magnification. The thickness of the membranes was measured by tilting the stage (e,f, 3000x magnification).

To evaluate permeability of the membranes, diffusion of dextran with two different molecular weights and inulin was measured. Both copolymers allowed diffusion of dextrans and inulin, with a decreased permeability when the membranes were coated with type I collagen (Fig. 2).

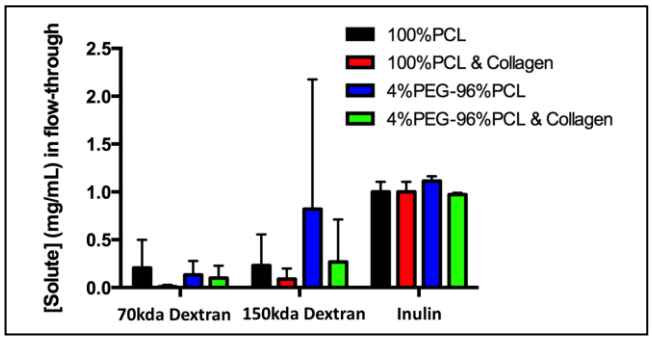


Figure 2. Diffusion of fluorescent molecules through copolymer membranes. Values are expressed as average +/- standard deviation of N=3 replicates.

Upon closer inspection, the permeability of both copolymers allowed for similar diffusion profiles for both dextran bead sizes. Only when inulin was used a molecular weight cut off could be seen. Of note, 4%PEG-96%PCL membranes yielded relatively less homogeneous when not coated with collagen.

Adhesion, morphology and proliferation of the neurovascular unit cell types were evaluated after 7 days from seeding (Fig. 3).

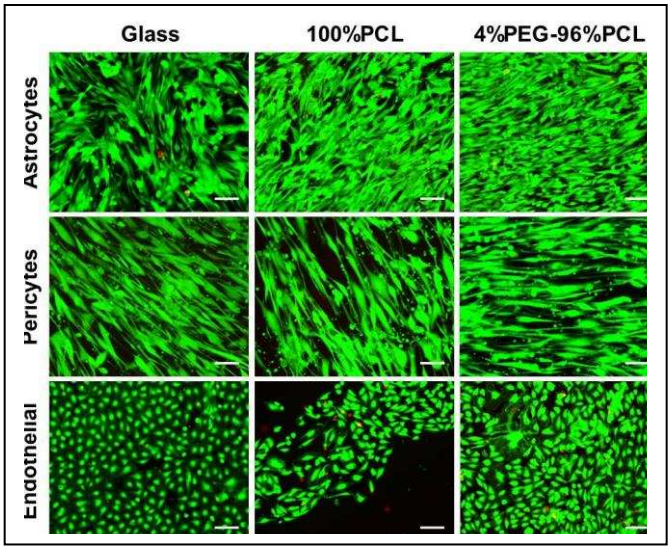


Figure 3. Live/Dead images of cells cultured on different polymeric membranes. Scale bar = 100 μm

Astrocytes and pericytes did not show significant differences in terms of shape and growth on the two polymers and compared to glass. Endothelial cells, which are generally more sensitive to chemistry and roughness of the substrate, did not grow uniformly on 100%PCL, but they did monolayers on the 4%PEG-96%PCL exhibiting morphology

comparable to glass. All cell types were viable on all the substrates. Higher density of the 3 cell types was observed onto the 4%PEG-96%PCL membranes, while endothelial cells exhibited lower proliferation on 100%PCL membranes.

We then investigated if the membranes with cells could recreate desired properties characteristic to the basal lamina of a complete BBB (e.g. with endothelial cells). To do this, endothelial cells were grown on the two membranes for a minimum of 7 days to form a confluent monolayer. The permeability of these monolayers to 70 kDa dextran was reduced when the cells grew on collagen-coated membranes and increased in DMSO-disrupted monolayers (Fig. 4) [13]. The 4PEG-96%PCL membranes, on the other hand, showed a lower permeability compared to the 100%PCL when collagen was used.

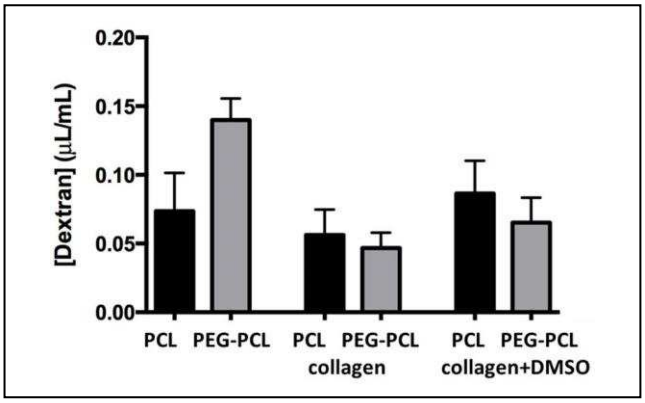


Figure 4: Permeability of endothelium growing on different fiber mats.

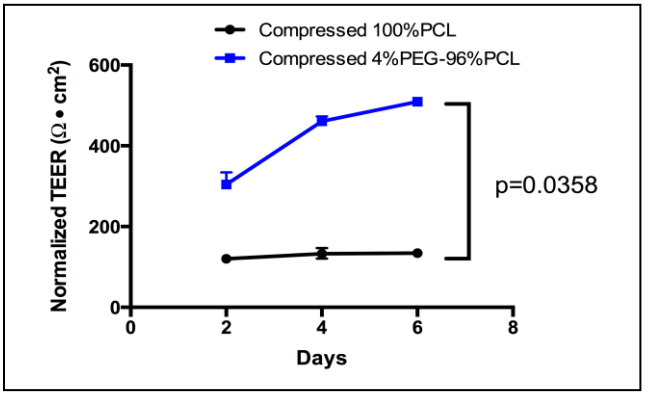


Figure 5: TEER of endothelial cell layers on collagen coated fiber mats

To evaluate the formation of a tight endothelial cell layer [14], TEER was measured for the collagen coated mats after 6 days of culture. Quantitatively higher as well as quantitatively increasing TEER was observed over time for endothelial cells grown on 4%PEG-96%PCL membranes compared to 100%PCL membranes.

## IV. Conclusion

Among the test copolymers, the 4%PEG-PCL demonstrated better support for growth of endothelial cells, pericytes and astrocytes likely because the chemistry and morphology of this fiber membrane mimics those of the BBB basal lamina. The successful selective permeability of this fiber membrane is a substantial advantage towards using them for in vitro BBB models. Without a physiologically comparable barrier, which critically defines cerebral vasculature, observed effects from paracellular and transcellular diffusion, as well as lower TEER findings, have limited applicability towards design and testing of neuro-pharmacological drugs in animal and human settings. In future work, we plan to include multiple types of neurons with the previously tested cell types and to co-culture them together in the transwell model. Finally, the integration of electrospun fiber membranes into a microfluidic device could better evaluate how test drugs and nutrients alter the BBB and the constitutive brain cells beyond it [14].

## Acknowledgment

The authors would like to thank Prof. D. Alcendor at Meharry Medical College, Nashville, for providing materials and advice for pericytes culture; Prof. John Daniels at Vanderbilt University and Prof. John Wikswo at Vanderbilt University, Nashville, for advices on TEER and microfabrication respectively and for lab availability.

## References

[1] P. Ballabh, A. Braun, M. Nedergaard, "The blood-brain barrier: an overview: structure, regulation, and clinical implications," Neurobiol Dis. 16(1):1-13, 2004.

[2] M. Ueno, "Molecular anatomy of the brain endothelial barrier: an overview of the distributional features," Curr Med Chem. 14 (11):1199-206, 2007.

[3] N.J. Abbott, A.A. Patabendige, D.E. Dolman, S.R. Yusof, D.J. Begley, "Structure and function of the blood-brain barrier," Neurobiol Dis, 37(1):13-25, 2010.

[4] G. Hurtado-Alvarado, A.M. Cabañas-Morales, and B. Gómez-Gónzalez "Pericytes: brain-immune interface modulators," Front Integr Neurosci., 7: 80, 2013.

[5] D.J. Alcendor, A.M. Charest, W.Q. Zhu, H.E. Vigil, S.M. Knobel, "Infection and upregulation of proinflammatory cytokines in human brain vascular pericytes by human cytomegalovirus," J. Neuroinflammation, 9 95, 2012.

[6] A. Reichenbach, H. Wolburg,. in Neuroglia 2nd edn (eds Kettemann, H. & Ransom, B. R.) 19–35 (Oxford Univ. Press, New York, 2004).

[7] L.L. Rubin,. et al. "A cell culture model of the blood– brain barrier," J. Cell Biol. 115, 1725–1735, 1991.

[8] V.R. Sinha, K. Bansal, R. Kaushik, R. Kumria, A. Trehan A, "Poly-ε-caprolactone microspheres and nanospheres: an overview," Int. J. Pharm/ 278: 1–23.

[9] H.J.Sung, A. Luk, N.S. Murthy, E. Liu, M. Jois, et al," Poly(ethylene glycol) as a sensitive regulator of cell survival fate on polymeric biomaterials: the interplay of cell adhesion and pro-oxidant signaling mechanisms," Soft Matter 6: 5196–5205, 2010.

[10] S.W. Crowder, et al.,"Poly(ϵ-caprolactone)-carbon nanotube composite scaffolds for enhanced cardiac differentiation of human mesenchymal stem cells," Nanomed., 8:1763-1776, 2013.

[11] M.K. Gupta, et al., "Combinatorial Polymer Electrospun Matrices Promote Physiologically-Relevant Cardiomyogenic Stem Cell Differentiation," Plos One, 6, 12, 2011.

[12] L. Cucullo, M. Hossain, W. Tierney, D. Janigro, "A new dynamic in vitro modular capillaries-venules modular system: Cerebrovascular physiology in a box" BMC Neuroscience, 14:18 (2013).

[13] B.K. Lal et al, "VEGF increases permeability of the endothelial cell monolayer by activation of PKB/akt, endothelial nitric-oxide synthase, and MAP kinase pathways," Microvascular Research 62:252-262, 2001.

[14] D.J. Alcendor et al., "Neurovascular Unit on a Chip: Implications for Translational Applications," Stem Cell Res. & Ther., 4(Suppl 1):S18, 2013.