A MULTI-LAYERED MICROFLUIDIC DEVICE FOR IN VITRO BLOOD-BRAIN BARRIER PERMEABILITY STUDIES

Ross Booth[2*] and Hanseup Kim[1]
[1]Electrical and Computer Engineering, [2]Bioengineering
University of Utah, Salt Lake City, UT

ABSTRACT

The blood-brain barrier (BBB) blocks most compounds from entering the central nervous system (CNS), limiting the success rate of new treatments of CNS disease. Insight needed to overcome these limitations can be gained through innovation of more representative models of the BBB. We present the first multi-layered microfluidic device (MMD) for dynamic modeling of the BBB. Brain endothelial cell monolayers cultured in the device exhibited the following BBB properties: Tight junction formation as indicated by immunostaining, trans-endothelial electrical resistance (TEER) values of over 250Ωcm^2 by day 3 of culture, and size-selective permeability of a range of test permeates.

KEYWORDS: Blood-brain barrier, Culture models, Microfluidics, Permeability, Central nervous system

INTRODUCTION

The BBB, a unique trait of the CNS, prohibits most compounds from entering the brain. Due to a lack of knowledge about the role the BBB plays in disease progression and treatment strategies, a demand exists for reliable models **(Fig 1A)** to study the BBB [1]. Animal models are subject to time, cost, and ethical constraints, while *in vitro* models are comparatively low-cost, repeatable, and high-throughput **(Fig 1B)**. The conventional transwell *in vitro* BBB model lacks a dynamic microenvironment [2], while fluid shear stress enhances endothelial phenotype of cultured cells [3]. The use of microfluidics allows dynamic control over cellular microenvironment and controlled delivery of test compounds. In an effective *in vitro* model, a physiologically relevant microenvironment should be employed **(Fig 1C)**. The key structural component of the BBB is the endothelial cell layer, and its confluence and tight junction expression are prerequisites for permeability studies. Barrier function can be quantified by measuring permeability of inert solutes across the membrane. As a precursor to permeability studies, TEER can be measured electrically, and has an inverse relationship with permeability.

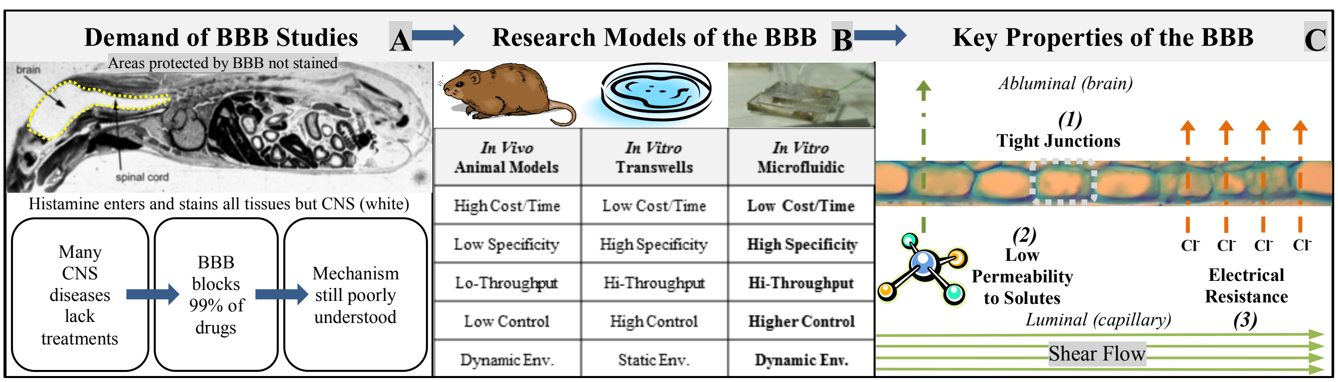

Figure 1: Modeling the BBB with fluidic devices to further treatment of CNS disease

***A.** Due to selectivity of the BBB as demonstrated by a histamine stain of an adult mouse [4], development is slow for treatments of CNS. **B.** Improved experimental models of the BBB will contribute to development of CNS disease treatments. **C.** Properties of an effective BBB model: Physiologically relevant microenvironment (shear flow); Tight junction expression in endothelial cell layer (Fig3); Monolayer confluence indicated by TEER (Fig4); Measurable permeability to solutes (Fig5).*

The conventional dynamic *in vitro* BBB (DIV-BBB) [5] has utilized hollow fiber technology to mimic the neurovascular unit; however, the system exhibited some limitations: very high functional media volume (1.3ml) was required; fiber walls were significantly thicker (150µm) than transwell membranes (10µm) preventing physical contact between co-cultured cell layers; and electrodes lacked optimal geometry resulting in ill-defined ion flow paths. To address such limitations, we present a micro-scale BBB module that uses significantly lower (12µl) functional media volume, encloses a thin (10µm) transwell membrane, and comprises thin-film TEER electrodes with optimal surface area vs. culture area **(Fig 2)**.

The BBB device is comprised of five layers: two acrylic layers on which thin-film electrodes are deposited, two PDMS layers for definition of channel dimensions, and a porous polycarbonate membrane providing the culture surface. The luminal channel has high aspect ratio (2mm/0.2mm) for flow uniformity, and a wider abluminal channel (5mm/0.2mm) to minimize shear on co-cultured glial cells. Two sets of thin-film AgCl electrodes are placed opposite the 0.1cm^2 channel junction, with a total gap of 400 µm.

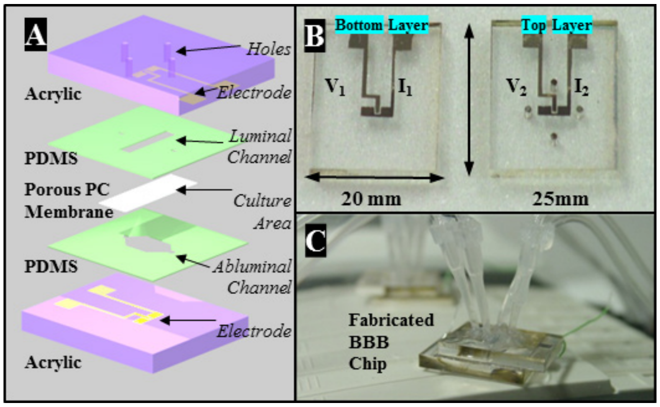

Figure 2: Device Components & Fabricated Prototypes ***A.*** *Scaled schematic of 5 layers of the MMD.* ***B.*** *Top and bottom acrylic layers. I_1, I_2 and V_1, V_2 are current and voltage electrodes.* ***C.*** *Full device assembly with pump connections and soldered wires.*

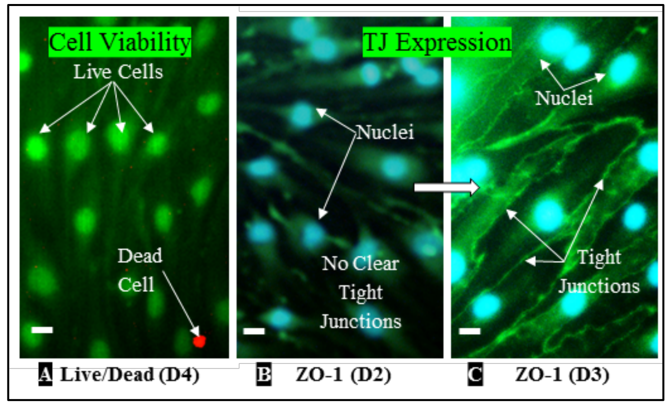

Figure 3. Microscope Images of bEnd.3 cells in BBB Device ***A.*** *Live/Dead stain on day 4 indicates high cell viability. (Green: Live, Red: Dead)* ***B.-C.*** *Immunofluorescent staining of Zonal Occludin (ZO-1) and DAPI indicates tight junction formation from day 2 (B) to day 3 (C) after seeding. (Green: ZO-1, Blue: DAPI, nuclei) Scale bar 10µm.*

FABRICATION & TESTING

PDMS layers are spin-coated at 288 RPM for 2 minutes to produce 200 µm sheets. The 3mm acrylic, 200µm PDMS layers, and Instachange (3M) sputter mask are patterned by laser cutting. Acrylic layers are cleaned in a sonicator bath, and deposited with 20nm Cr, 150nm Au, and 800nm Ag using sputter deposition. The Ag is chemically oxidized at room temperature with 30mM $FeCl_3$ for 60s. Acrylic layers are bonded to PDMS layers with silicone sealant (DC 734), and polycarbonate sheets (400nm pores, 10µm thick) are cut from transwells (Corning) and bonded between PDMS layers using spin-coated 50/50 toluene/PDMS prepolymer as previously described [6]. Copper wire is silver-epoxied to bond-pads for easy connection to an EVOM2 epithelial voltohmeter (WPI) for TEER measurements. Silicone manifold tubing (0.25mm ID) is sealed to input holes with silicone sealant and used with a 205S cartridge pump (Watson-Marlow).

For culture testing, devices are sterilized with 70% ethanol, membranes are coated with fibronectin (10µg/ml, 2h), then seeded with brain endothelial cells (bEnd.3) [7] at $6e^4/cm^2$ density for 2h, then DMEM:F12 growth medium is circulated at 1.3µl/min for 1 day, followed by 2.6µl/min subsequently. Immunostaining of TJ component ZO-1 was done by fixing with 4% PFA, blocking with 10% BSA, and incubation with mouse anti-ZO-1 primary antibody overnight (4ºC), AlexaFluor-488 goat anti-mouse for 2 hours, then counter-stained with DAPI . Live/Dead (MGT) solution was incubated 90 minutes to check viability. TEER was measured daily, calculated by subtracting background and normalizing for area. Flux of FITC-dextrans (4kD, 20kD, 70kD) and propidium iodide across the membrane was measured (BioRad Synergy) and permeability coefficients were calculated using the conventional equation for permeability [8].

$$P = \frac{J_s}{A \cdot C_L} \tag{1}$$

Where P is the permeability coefficient, J_s is solute flux across the membrane, A is membrane area, and C_L is concentration on the luminal (source) side of the membrane. Epithelial coefficients P_e are calculated by subtracting the inverse of the overall P value by the inverse of coefficient P_b from a blank membrane, as in the following equation [9].

$$\frac{1}{P_e} = \frac{1}{P} - \frac{1}{P_b} \tag{2}$$

RESULTS AND DISCUSSION

Live/dead stains on day 4 were indicative of acceptably high cell viability **(Fig 3A)**. Selective staining of tight junction component Zonal Occludin-1 after day 3 after seeding indicated distinct tight junction formation **(Fig 3B-C)**, while stains on day 2 of culture exhibited comparatively weaker expression. This supports the practice of using day 3 as a minimum threshold for permeability studies. TEER is an indirect method of validating a contiguous cell layer before permeability experiments. TEER values typically exceeded $250\Omega cm^2$ by day 3 of culture **(Fig 4)**. For these reasons, day 3 was the chosen time point for permeability measurements, with a $250\Omega cm^2$ threshold as a precursor for permeability assays. Calculated permeability coefficients of a range of inert solutes fit an exponential relationship to stokes radii of inert permeates with R^2=0.97 **(Fig 5)**. A shift of this curve would be indicative of a quantifiable change in barrier function in future studies, while TEER measurements can be conducted at any time interval non-invasively. This robustness demonstrates a clear practical advantage over static transwell systems, which need to be removed from their culture environment to measure TEER.

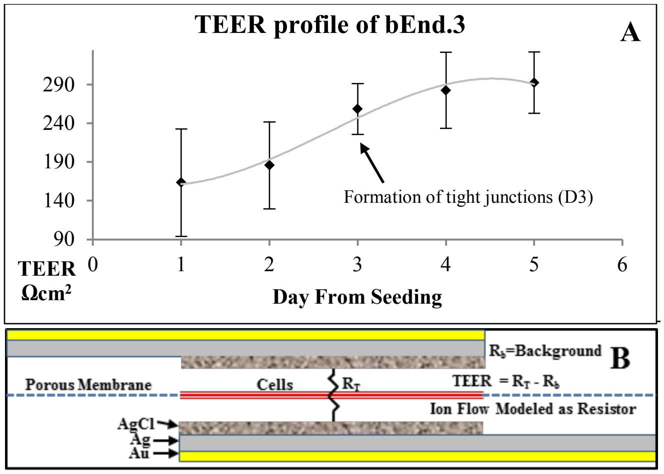

Figure 4: TEER of bEnd.3 Layers in Dynamic Culture
***A.** Measured TEER shows a taper over 250 Ωcm^2 by day 3 after seeding. n≥3 **B.** Cross section of the electrode interface. Resistive model of the membrane is used to calculate TEER.*

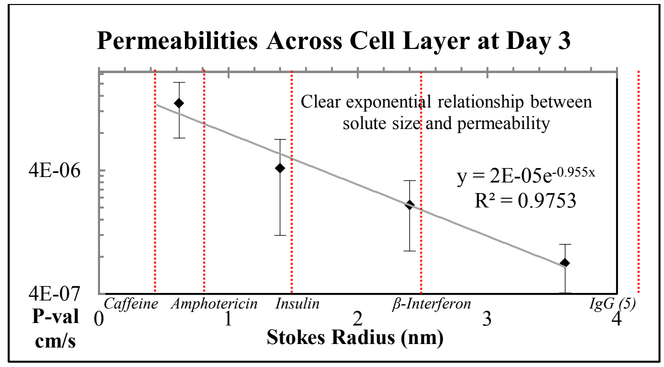

Figure 5: Permeability of Fluorescent Solutes
The exponential curve shows the measured permeability coefficients across bEnd.3 cells, day 3, of Propidium Iodide and FITC-Dextran (4kD, 20kD, 70kD) as a function of stokes radius. In future studies, a shift of this curve can quantitate changes in barrier function. R^2=0.97, n≥4. Stokes radii of most existing CNS drugs lie in the .5-1nm range.

CONCLUSION

The results demonstrate that our MMD is an effective vehicle for assessing BBB permeability demonstrating sufficient sensitivity to a wide array of molecular weights for quantitation of barrier function in future experiments with varying model parameters. Microscopic evaluation indicates sufficient endothelial structure for BBB studies, and microfluidics expose the cell layers to dynamic shear stress while allowing the researcher to carefully control delivery of test solutes through the system. Finally, integration of thin-film electrodes allow TEER measurements to be conducted at any interval non-invasively, while continuous flow allows fixation of solute concentration to keep solute flux constant during permeability assays. To further characterize the system, it needs to be tested under co-culture with glial cells in the abluminal chamber.

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CONTACT: *Ross Booth, tel: +1-208-8615597; Ross.Booth@utah.edu