IN VITRO TRI-CULTURE BLOOD-BRAIN BARRIER (BBB) MODEL ENABLING DIRECT INTERCELLULAR CONTACT AT A SUSPENDED LAYER

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ABSTRACT

This paper reports the development of an in vitro tri-culture BBB model that forms suspended BBB layers where direct intercellular connections among BBB cells are established. The in vitro BBB model consists of top and bottom culture chambers and a porous membrane with 30 µm holes, whose hole diameter is 2~3 times larger than the size of cells to allow direct cell-to-cell communication. The porous membrane was coated with hydrogel and a mixture of collagen IV and fibronectin, and the three types of BBB cells of the human brain microvascular endothelial cells, pericytes, and astrocytes were cultured on the membrane. The establishment of the tri-cultured BBB layers was validated by immunostaining. Immunostaining images provided a clear expression of three different identification proteins of the BBB cells confirming the establishment. Then, the successful culture of cells on the large-hole membrane was validated by the measurement of high TEER values of 67 $\Omega \cdot cm^{-2}$ and low permeability coefficient values of 2.9×10^{-7} $cm \cdot s^{-1}$. In summary, the development of the in vitro tri-culture BBB model was successfully delivered.

KEYWORDS

blood-brain-barrier (BBB), in-vitro model, direct cell-to-cell contact, intercellular interaction, SHH signaling pathway, tight-junction, tri-culture, suspended layers

INTRODUCTION

The blood-brain barrier (BBB) is a key structure in preserving homeostasis of the central nervous system (CNS) [1]. It is located between the blood and the brain, is composed of tightly bound cell layers, and provides selective chemical transportation. The BBB helps to prevent undesirable damage and malfunction of the CNS by clearing harmful substances [1]. Meanwhile, the BBB also inhibits drug transport to the brain in some cases. Thus, the BBB has been of critical interest to drug delivery in neuroscience research and the pharmaceutical industry in the past few decades.

Chemical transportation utilizes mainly two pathways through the BBB: the paracellular pathway and the transcellular pathway [1]. The paracellular pathway allows the transportation of hydrophilic chemicals smaller than intercellular tight-junction gaps. The transcellular pathway allows the transportation of lipophilic chemicals absorbed on the blood (luminal) side of the endothelial cells and conveyed to the brain (abluminal) side through the cells.

Interestingly, the expression of the sonic hedgehog (SHH) signaling pathway was reported to determine the tightness of the paracellular pathway [2]. This signaling pathway induces the synthesis of tight junction proteins such as claudin and occludin. However, the pathway becomes more activated when the distance between endothelial cells and astrocytes is small. Thus, the establishment of direct intercellular connection could lead to enhanced BBB integrity. Enhanced BBB integrity was observed by 10% higher trans-endothelial electrical resistance (TEER) values and 40% lower permeability coefficient values at the promoted SHH signaling pathway [3].

Current in vitro models, unfortunately, fail to provide direct intercellular connection. Despite advancements of multi-chamber structure and co-/tri-cultured BBB layers [4-9], membranes with sub-3 µm pore diameters and 10~30 µm thicknesses prevent direct intercellular communication and lower the probability that the signaling proteins of the SHH pathway reach their corresponding receptors. Recently, the development of an in vitro BBB model incorporating a thin silicon nitride (Si_xN_y) membrane was reported [10]. Despite the significantly reduced thickness of the membrane, direct cell-to-cell communication was limited due to the very small 0.5-µm diameter pore size.

To overcome such limitations, a novel in vitro BBB model incorporating larger-than-a-cell-sized holes and a thin hydrogel layer with an already validated polycarbonate membrane was developed. The membrane had 30-µm diameter large holes that allow BBB cells to form layers with an almost-zero distance between them. The hydrogel layer provides an initial extracellular matrix for seeding astrocytes, leads the astrocytes to be connected across the pores in the lateral direction, and is removed before the

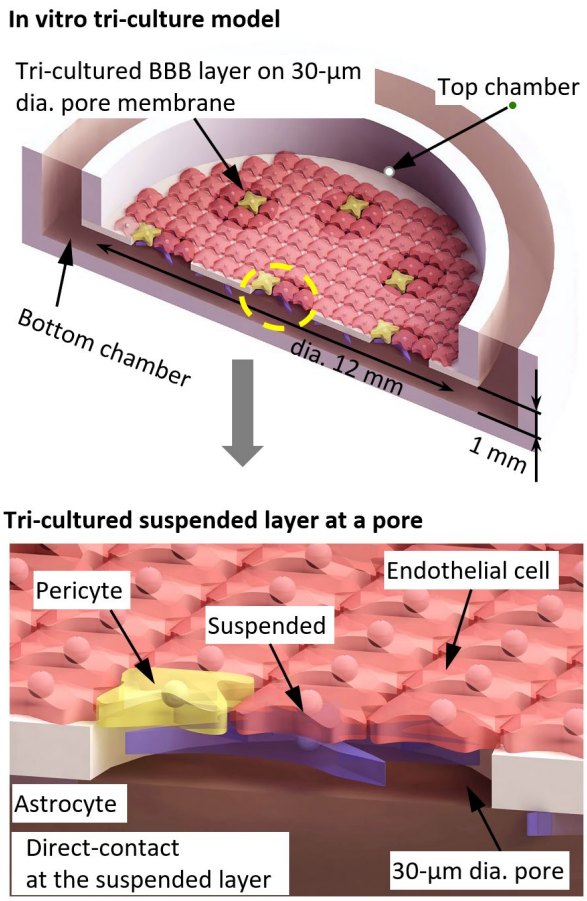

Figure 1. Illustrations of the in vitro tri-culture BBB model at the multi-chamber structure and the detailed suspended cell layers at a pore.

other BBB cells of endothelial cells and pericytes are seeded. Thus, direct cell-to-cell contact between endothelial cells and astrocytes is achieved as shown in Fig.1. Additionally, pericytes are included in this in vitro BBB model to help the proliferation and differentiation of the BBB cells [11].

This paper reports a novel in vitro tri-culture BBB model that is capable of providing suspended cell layers with direct cell-to-cell contact. The fabrication of the in vitro model, tri-culture of BBB cells, and evaluation of the suspended cultured BBB layers and the direct cell-to-cell contact are described.

METHODS

Structure & Fabrication

The tri-culture platform consisted of one porous membrane separating top and bottom chambers. A commercially validated transwell (3413, Corning, NY) was utilized to provide the multi-chamber structure. The existing membrane was replaced with a sheet of porous polycarbonate membrane using adhesive (SILASTIC, Medical Adhesive Silicone, Type A, Dow-Corning, MI) in Fig.2(b). The new polycarbonate membrane had 30-µm diameter pores at 1×10^4 pores·cm^{-2} pore density and 7% porosity (hydrophilic polycarbonate membrane, Sterlitech, WA). The 30-µm diameter pores were larger than the cell sizes of 10~15 µm [1]. The membrane was cut in an 8-mm diameter circle using surgical scissors. The top side of the membrane was then coated with a degradable hydrogel (TrueGel3D, SigmaAldrich) and cured under the pressure of 7.0×10^1 kPa with a 1 kg mass overnight at room temperature.

Spin-Coating Hydrogel Layer

A thin hydrogel layer was spin-coated to form a suspended extracellular matrix on the porous membrane. The hydrogel layer (a) was made by mixing 4 different components of a degradable polymer, cell-degradable solution, buffer solution, and water following the product information [12]. The degradable polymer was mixed with the buffer solution at 300 rpm using a vortex mixer (97043-562, VWR), and the degradable polymer mixture solution was incubated for 1 hour at room temperature. Separately, the cell-degradable solution was diluted with water using the vortex mixer. This cell-degradable solution was incubated for 5 min at room temperature. Then, a final hydrogel solution was prepared by mixing 10 µl culture medium, 36 µl degradable polymer mixture solution, and 4 µl cell-degradable solution. Hydrogel solution (50 µl) was then applied on the top surface of the 30 µm hole membrane on a Si-wafer, and the Si-wafer was spun at 1000 rpm for 40 seconds (WS-400A, Laurell Technologies). The hydrogel-coated membrane was incubated at 37 °C for 20 minutes in Fig.2(b).

Cell culture

Tri-cell culture was performed with human brain microvascular endothelial cells (HBMECs), human brain perivascular cells (pericytes), and human brain astrocytes.

When the cells were fully cultured in a flask at conditions of 37 °C and 5% CO_2 in an incubator (NU-4750, Nuaire), they were seeded into the in vitro tri-culture BBB model. A mixture of mouse collagen type IV (354233, Corning, NY) and human fibronectin (354008, Corning, NY) at a concentration of 100 µg·ml^{-1} in Fig.2(c) was prepared. The 200 µl mixture was applied to the bottom surface of the membrane and left in the incubator at 37 °C for 30 minutes. The astrocytes were seeded to the bottom surface of the top chamber at the concentration of 5.0×10^4 cells·cm^{-2}, and cultured in the incubator for 2 hours in Fig.2(d). The top chamber was flipped and placed into the culture well plate, and the astrocytes were cultured for 3~4 days in Fig.2(e). When the astrocytes were successfully

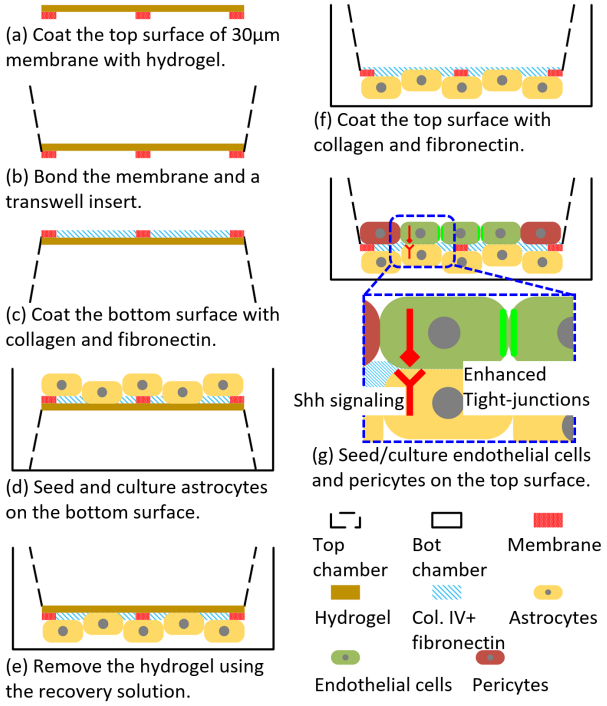

Figure 2. Processes of fabrication, coating, and cell-culture. Top chamber was fabricated with a transwell insert and 30 µm membrane, the coating was performed with biodegradable hydrogel, collagen and fibronectin, and the brain cells were cultured on the suspended layer.

cultured, the hydrogel on the top surface of the membrane was removed by applying a cell recovery solution of the hydrogel product [12]. Cell recovery solution (600-µl) was applied to the top side of the chamber where the hydrogel was present. The culture chamber was incubated at 37 °C for 30 minutes. After the hydrogel layers were removed, the top surface of the membrane was coated with the mixture of collagen type IV and human fibronectin at the same concentration in Fig.2(f). The pre-cultured HBMECs and pericytes were seeded onto the top surface of the membrane at the concentration of 10.0×10^4 cells·cm^{-2} and 1.0×10^4 cells·cm^{-2}, respectively. Finally, the BBB cells were cultured for ≥5 days, and the culture media was changed daily in Fig.2(g).

Suspended Tri-Cultured Cell Layers

The suspended tri-cultured cell layers were validated by optical and immunostaining images and fluidic movement testing. The porous membrane coated with the hydrogel and the mixture of collagen type IV and fibronectin was imaged using a color microscope (EVOS FL Auto, Thermo Fisher). The image was taken before the astrocytes seeding. Immunostaining images were taken with a confocal microscope (FV1000, Olympus), after the cells were successfully cultured. Immunostaining imaging using the confocal microscope proved the presence of identical biomarkers indicating three different cells on the culture membrane. Primary antibodies (abcam, MA) of zonula occludens (ZO-1) for HBMECs, platelet-derived growth factor receptor (PDGFR) beta for pericytes, and glial fibrillary acidic protein (GFAP) for astrocytes were selected as biomarkers, and they were visualized using the secondary antibodies (Thermo Fisher Scientific, MA). The nuclei of the cells were stained with DAPI (Enzo, NY) in phosphate-buffered saline (PBS) for 20 minutes. Immunostaining images showing direct

contact between BBB cells were taken every 1 µm thickness using the confocal microscope. Stacked images (2D and 3D) from the optical and confocal microscopes were then post-processed using ImageJ (version 1.53f51; National Institutes of Health) and analyzed.

Additionally, the successful formation of the suspended cell layers was verified with a measurement of hydraulic flow at the media application of 200 µl to the top chamber and 600 µl to the bottom chamber. After 30 minutes, the height difference between the top and bottom chambers was recorded.

Measurements of TEER and permeability

After cell seeding, cell growth was monitored by real-time TEER values (EVOM^2 epithelial voltmohmeter, WPI), every day from Day 0. TEER values were determined in $\Omega \cdot cm^2$ according to the equation (1), $TEER = (R - R_{blank}) \times A$ (1), where R is the total resistance (Ω), R_{blank} is the background resistance (Ω) on Day 0, and A is the cell culture area (cm^2) [4]. After the TEER values were saturated, permeability coefficients were measured with 100 µM FITC-4k dextran in the tri-culture platform. The amount of permeated compound collected from the bottom chamber was then measured using a spectrometer (495/525 nm, SpectraMax Plus 384 Microplate Reader). The permeability coefficients P were calculated using equation (2), $P = J_s \times A^{-1} \times C_L^{-1}$ (2), where P is the permeability coefficient ($cm \cdot s^{-1}$), J_s is solute flux across the cell layers ($mol \cdot s^{-1}$), A is the membrane area (mm^2), and C_L is the concentration ($mol \cdot m^{-3}$) on the top channel, as previously described and used in [4].

RESULTS

The in vitro tri-culture BBB model with 30-µm diameter pore membrane was successfully fabricated. Successful culture of the suspended tri-culture BBB cell layers was verified, and direct contact between BBB cells were established at the pores of the membrane. These results were validated by imaging and hydrostatic flow testing and were further supported by the measurement of TEER values and permeability coefficients in Fig. 3-6.

Verification of Suspended Tri-Cultured Cell layers

The optical image of the membrane shown in Fig. 3(a) proved the formation of the successfully spin-coated hydrogel layers at the membrane. The observed collagen and fibronectin indicated the suspended layer of the hydrogel. The nuclei-stained image shown in Fig. 3(b) proved the cells were cultured and present on top of the pores. Culture of the BBB cells successfully covered the pores, proven by the result of no fluid movement across the tri-cultured BBB layers in Fig.3(c). Finally, the successful culture of three different cells of HBMEC, pericytes, and astrocytes was validated by the immunostaining image in Fig. 3(d). Identical protein expressions (green: HBMEC [ZO-1], red: pericytes [PDGRF], yellow: astrocytes [GFAP], and blue: nuclei) proved the successful tri-culture across the 30-µm diameter pore membrane. The merged image shows that fully confluent cell layers were established across the suspended cell layers. Thus, Fig. 3 supports the establishment of the suspended tri-cultured cell layers.

Evaluation of Direct Cell-to-Cell Contacts

Direct contact between BBB cells was confirmed via imaging, as shown by the 3D stacked image in Fig. 4. Side views of the reconstructed image show cell connections through the 30-µm pores. The images imply that intercellular connections are unlikely using conventional 20~30 µm thick membranes.

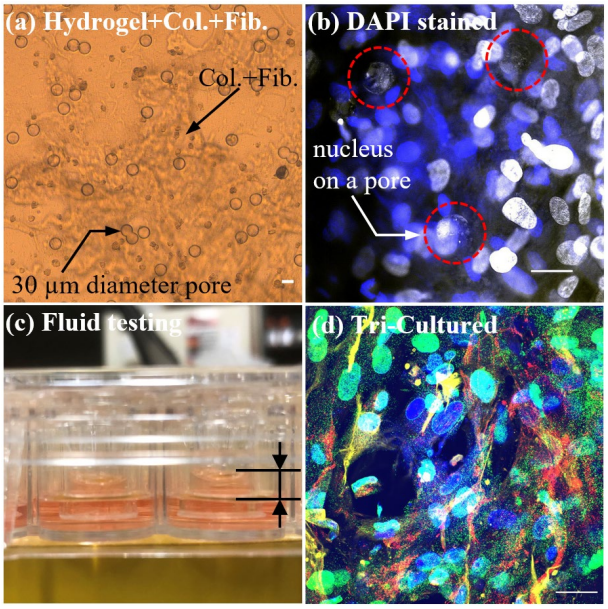

Figure 3. Images proving suspended cell layers and extracellular matrix. (a) optical image after coating with the hydrogel, collagen IV and fibronectin, (b) DAPI stained image showing nucleus existing on pores, (c) recorded photo showing a height difference between the top and bottom chambers after 30 min, and (d) merged image of the three different BBB cells. Scale bar: 30 µm.

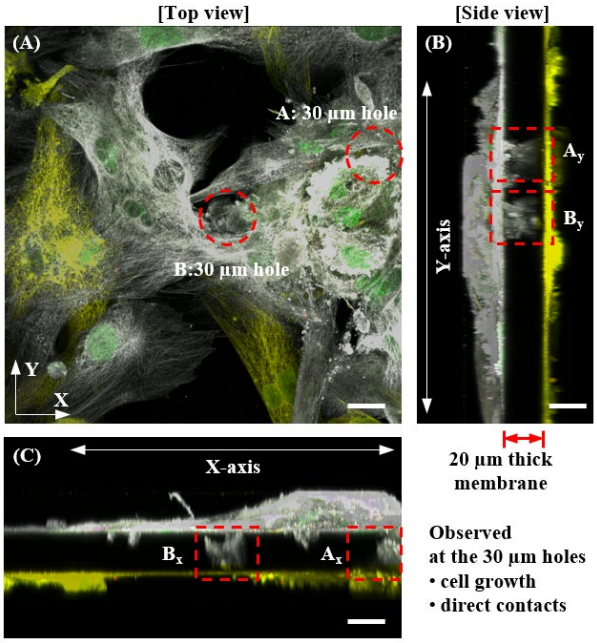

Figure 4. Reconstructed 3D stack images of the BBB cell layers (a) 3D stacked image from the immunostaining images taken every 1 µm, (b) Side-view image along Y-axis, and (c) side-view image along X-axis. Endothelial cells and pericytes are shown as gray, and astrocytes are shown as yellow. Scale bar: 20 µm

Evaluation of TEER and permeability

Higher TEER and lower permeability coefficient values prove the enhanced BBB integrity at the suspended tri-cultured BBB layers in Fig.5. TEER measurement showed the saturated

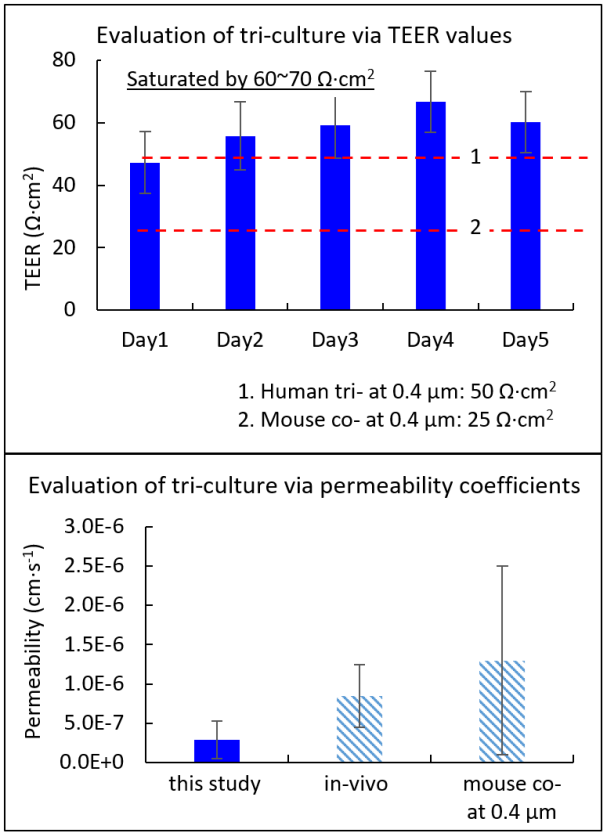

Figure 5: Evaluation of the established tri-culture cell layers via (a) measurement of TEER values for 5 days and (b) measurement of permeability coefficients on day 5.

TEER value of 66 Ω·cm^2 after 5 days of culture, which was 164% higher than the previous measured TEER values of the co-culture cell layers of mouse BBB cells seeded on a conventional membrane [4,13,14]. The tight BBB integrity was also proven with the measured permeability coefficient values, which were 66% and 81% lower than the previously measured in-vivo mouse test and in-vitro mouse co-culture test [14,15]. Thus, it is concluded that there is a strong correlation between the establishment of direct intercellular contact and BBB integrity.

CONCLUSION

This study reported the development of a tri-culture platform that enables building suspended BBB layers by incorporating 30 µm pores and an extracellular matrix consisting of biodegradable hydrogel, collagen IV and fibronectin. The HBMECs, pericytes, and astrocytes were successfully cultured, and direct contact between cells was successfully established. Finally, evaluations of the established BBB layers were performed by optical and immunostaining imaging, and measurement of TEER values and permeability coefficient values. The evaluations indicate that the suspended tri-culture BBB layers would be able to provide an in vitro testing tool of the brain microvascular system that is physiologically and mechanically more representative of the human BBB system.

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