In Vitro Blood–Brain Barrier Models: Current and Perspective Technologies

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Abstract

Even in the 21st century, studies aimed at characterizing the pathological paradigms associated with the development and progression of central nervous system diseases are primarily performed in laboratory animals. However, limited translational significance, high cost, and labor to develop the appropriate model (e.g., transgenic or inbred strains) have favored parallel *in vitro* approaches. *In vitro* models are of particular interest for cerebrovascular studies of the blood–brain barrier (BBB), which plays a critical role in maintaining the brain homeostasis and neuronal functions. Because the BBB dynamically responds to many events associated with rheological and systemic impairments (e.g., hypoperfusion), including the exposure of potentially harmful xenobiotics, the development of more sophisticated artificial systems capable of replicating the vascular properties of the brain microcapillaries are becoming a major focus in basic, translational, and pharmaceutical research. *In vitro* BBB models are valuable and easy to use supporting tools that can precede and complement animal and human studies. In this article, we provide a detailed review and analysis of currently available *in vitro* BBB models ranging from static culture systems to the most advanced flow-based and three-dimensional coculture apparatus. We also discuss recent and perspective developments in this ever expanding research field.

Keywords

in vitro models; blood–brain barrier; cell culture; CNS; endothelial; passive diffusion/transport; tight junction; alternative research

INTRODUCTION

The burgeoning field of cerebrovascular research has created new and exciting opportunities in both basic and clinical studies. Reproducing the physiological characteristics and response of the human brain microcapillaries *in vitro* represent a critical biotechnological breakthrough. Such a model may significantly help scientists to understand the mechanisms involved in the cerebrovascular response to a number of physiological and pathological stimuli. This, in turn, will provide new strategies to accelerate the development of novel central nervous system (CNS) drug therapies and reduce the burden of major neurological disorders. Many scientific evidences that the blood–brain barrier (BBB) dynamically responds to physiological and pathophysiological events (e.g., acute brain injury,[1,2]

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rheological disturbances,[3,4] and proinflammatory stimuli)[4–6] leading to the pathogenesis and progression of many neurological disorders (such as epilepsy, multiple sclerosis, Alzheimer, and so on, mostly associated with increased BBB permeability)[7–17] have shifted the current view of the BBB. This is now considered a dynamic and extremely complex vascular interface capable of exerting a vast array of specialized functions.[18]

The research community recognizes that mimicking the physiology and the functional response of the BBB *in vitro* is a challenging task. Initial *in vivo* studies provided critical insights on the pharmacodynamic and pharmacokinetic behavior of many different classes of drugs targeting the brain. These same studies also helped discover specific BBB transport systems by Oldendorf et al.[19–22] in the early 1970s, which were then characterized in the late 1980s using a more sensitive *in vivo* method: the *in situ* brain perfusion technique.[23,24]

However, a new approach was necessary to simplify the overwhelming complexity posed by the *in vivo* experimental conditions and to expedite the characterization of the BBB at the cellular and molecular levels. The development of new cell culture techniques and improved *in vitro* technologies provided the necessary tools to overcome these complexities and advance our understanding of the physiology and functions of the BBB.[25–35]

STRUCTURE AND FUNCTIONS OF THE BBB

The BBB consists of microvascular endothelial cells (ECs) lining the brain microvessels. Closely associated astrocytic end-feet processes[36] contribute to modulate ECs differentiation and the induction and maintenance of the BBB properties.[32–34,37–40] The BBB ECs are characterized by scarce pinocytotic activity, lack of fenestrations (i.e., openings), and unique expression patterns of trans-membrane transport systems to regulate the traffic of substances into and out of the brain parenchyma.[35] Interendothelial tight junctions (zonulae occludentes) between adjacent ECs form a physical barrier (gate function) that impedes blood-borne (including electrolytes and other water-soluble compounds)[35,41] and xenobiotic substances in the systemic circulation from entering the brain through paracellular routes. Therefore, transit across the BBB involves translocation through the capillary endothelium by asymmetrically expressed carrier-mediated transport systems.[35,42–44] These are responsible for the passage of certain water-soluble but biologically important substances (e.g., D-glucose, mono-carboxylic acids, amino acids, etc.)[32,35,44] from the peripheral circulation to neurons in the parenchyma.

Specific efflux transport systems at the BBB [e.g., P-glycoprotein (P-gp),[42] multidrug resistance-associate protein 4 (MRP4),[44] and breast cancer resistance protein][45,46] protect the brain from potentially harmful amphipathic and hydrophobic substances.[42,47] However, given their wide substrate specificity, these efflux transport systems may contribute to multiple drug resistance during pharmacological treatment of several CNS disorders such as epilepsy and brain tumors.[31,48–53] Furthermore, specific cytochrome P450 enzymes (e.g., P450 3A4) expressed at the BBB endothelial level under pathological conditions (e.g., drug-resistant epilepsy)[54,55] can catalyze the biotransformation of drugs targeting the brain (in addition to lipids and steroidal hormones),[56] thus further reducing their bioavailability (see Fig. 1).

IN VITRO BBBs: WHAT DO WE EXPECT FROM AN IDEAL MODEL?

The advancements occurred in the past two decades in the BBB modeling field were primarily dictated by two factors: (1) the need to advance our understanding of the complex molecular mechanisms regulating the brain environment and (2) facilitate the development of alternative and more effective pharmacological strategies aimed at CNS targets. Mimicking the functional properties of this very complex organ interfacing the brain with

the systemic vascular environment is critical for the development of novel CNS drugs and is an extremely challenging task. Even though it is still beyond our ability to reproduce all the complex functions of the BBB *ex situ*, there are several major requirements that an ideal *in vitro* BBB model should meet (see Table 1). These include (1) enabling the expression of interendothelial tight junctions between adjacent ECs, which facilitate the formation of a very stringent and selective barrier (limited paracellular diffusion); (2) *in vivo*-like asymmetric distribution (basal vs. luminal) of relevant transporters (e.g., Pgp, glucose transporter 1/Glut-1, etc.),[44] which confers polarization of the ECs[35,57–60]; (3) the expression of functional efflux mechanisms (e.g., P-gp, MRPs)[61–63] and drug metabolism[30,55,56,64]; (4) the ability to discriminate the permeability of substances (in the absence of specific transport systems) according to their specific oil/water partition coefficient and molecular weight.[65] Furthermore, an ideal BBB model should also be able to reproduce the effects of a large variety of hemodynamic (e.g., hypertension, flow cessation/ stroke, etc.) and systemic/inflammatory insults on the BBB that have been observed *in vivo* and can lead to the pathogenesis and progression of major CNS disorders (epilepsy,[12–14] Alzheimer's disease,[9,10,66] inflammation, brain edema, brain trauma, multiple sclerosis,[2,5,67–71] etc.).

MODULATORY ROLE OF SHEAR STRESS AND GLIA IN ENDOTHELIAL PHYSIOLOGY AT THE BBB

It is well accepted by the scientific community that at least two key factors must be available to the ECs to properly differentiate and express a barrier phenotype *in vitro*: (1) physiological shear stress (SS), which is a frictional force generated by the exposure of the apical membrane of the ECs to flow; (2) cell to cell interactions including the relative exposure to "permissive" or "promoting" factors released by the surrounding cellular environment (in particular, abluminal glia).[3,33,34]

The exposure to physiological SS plays a critical role in modulating ECs' morphology. ECs grown in presence of flow are larger in volume than those grown under static conditions. They are also flattened and show an abundance of endocytic vesicles, microfilaments, and clathrin-coated pits,[72] thus exhibiting a level of differentiation[3,73,74] that more closely resembles that observed in BBB ECs *in situ*.[75]

Flow across the apical surface of the vascular endothelium activates a number of mechanosensors (e.g., integrins, caveolae, G proteins, and ion channels),[4,76] which transduce physical stimuli into biochemical signals (see Fig. 2). Despite the variety of potential mechanosensors present on the luminal side of the EC membrane, one major common downstream effect is the activation of extracellular-signal-regulated kinases 1/2.[4,77] These are pleiotropic modulators of the cell physiology and play an important role in the control of the expression of genes involved in the regulation of cell division, apoptosis, cell differentiation, and cell migration.[3] They affect multiple endothelial functions including (1) the production of vasoactive substances[78–80] and cell adhesion,[72,73,81] (2) expression of tight junctions,[3,82,83] (3) cell survival, (4) energy metabolism,[3] and (5) the expression of asymmetrically localized enzymes and carrier-mediated transport systems that engender a truly "polarized" BBB endothelium phenotype.[74,84] Turbulent, but not laminar, SS stimulates ECs' turnover[85] and accordingly, ECs' DNA synthesis (cell division) *in vivo* occurs preferentially at branch orifices with low turbulent flow rates where atherosclerotic processes are generally initiated.

Astrocytes–endothelial interaction is also of critical importance for the induction and maintenance of BBB properties.[34,37] Astrocytes with their closely associated astrocytic end-feet processes share the basal lamina enveloping more than 99% of the BBB endothelium.

Intercellular adhesion between astrocytes in the BBB have been observed in the form of gap junctions and adherens junctions.[86]

Initial observation by Davson and Oldendorf[87] as well as subsequent studies by others have clearly shown that astrocytes' interaction with the cerebral endothelium help modulate BBB function.[34,35,88–90] This includes upregulation of tight junctional proteins and tightness[91,92]; specific transport systems including the glucose transporter 1 (GLUT1), the large neutral amino acid carrier (L-system), and the A-system amino acid carriers.[32] Furthermore, astrocytes were shown to downregulate the expression of tissue plasminogen activator (tPA) and thrombomodulin in brain ECs *in vitro*.[93] More recently, a novel "BBB protective" role of astrocytes has been reported. This includes the nitric oxide (NO)-mediated release of interleukin-6 (IL-6). IL-6 has been shown to trigger production of matrix metalloproteinase (MMP) inhibitors such as $a2$-macroglobulin, which is released by perivascular astrocytes.[94–96] Astrocytes also serve as scaffolds, guiding neurons to their proper place during development and directing vessels of the BBB. Other studies also support the hypothesis of a neural induction of the cerebral microvasculature and BBB formation.[97]

The first try at reproducing *in vitro* the physiological characteristics and response of the human brain microcapillaries was attempted using isolated brain microvessels dissociated from the surrounding brain tissue.

ISOLATED BRAIN MICROVESSELS

Since the initial method was established by Ferenc Joó 40 years ago [182]; several other methods have been developed to obtain purified, functionally intact cerebral microvessels free of contamination by other brain tissue. Brain capillaries from the cerebral cortical gray matter are initially separated from the remaining brain tissue (which is usually discarded). Purification of the isolated brain microvessels is then obtained by combinations of mechanical homogenization, enzymatic dissociation, filtration, density gradient centrifugation, and column filtration.[98–100] Purity of microvessel preparation can be determined by visual inspection using light microscopy, scanning electron microscopy, and transmission electron microscopy[98] in combination with the concurrent expression of multiple brain microvascular endothelial markers such us Glut-1, transferrin receptor (OX-26), ZO-1, claudin-5, and claudin-12,[101–103] etc.). The lack of typical smooth muscle cells (such as calponin and desmin[104,105]) can be used to determine the absence of distal postcapillary venule fragments (and/or other larger vessels) in the preparation. However, this does not guarantee that BBB function and integrity are preserved. Isolated brain microvessels have been successfully used for the identification of mechanisms and biochemical signals that play a role in regulating BBB functions[106–109] in health and diseased conditions (e.g., brain tumors).[110] The major advantages of using isolated brain microvessels include the maintenance of the structural and cellular characteristics and properties (cell differentiation) in *ex vivo* experimentations. Additional advantages include the availability and an extensive number of well documented methods to obtain purified brain capillaries. On the contrary, there are a number of disadvantages that need to be taken into consideration: (1) the isolation procedures are quite difficult and labor intensive and despite the advancement in the purification techniques, the potential presence of neuronal and/or other contaminants is not negligible; (2) limited viability of the vascular endothelium possibly caused by metabolic deficiencies during vessel isolation. The use of alternative brain vessels such as pial microvessels have been considered but at the expense of lacking a number of peculiar characteristics proper of the BBB microcapillaries. These include (1) highly selective permeability to blood-borne and xenobiotic substances; (2) vascular polarization (the presence of asymmetrically distributed transporters including efflux

systems such as P-gp and MRPs); (3) metabolic transformation of endogenous and exogenous substances, which has been shown in BBB endothelial and glial cells.[54,111–113]

Because of the technical and functional limitations related to the use of isolated brain microvessels, different approaches based on computational models, artificial membranes, and *in vitro* cell culture BBB systems have been pursued.

IN SILICO MODELS: ADVANTAGES AND LIMITATIONS

Methods based on computer simulation (computer-assisted structure-based drug design) also known as "*in silico*" modeling are gaining more and more interest in the research field as the incredible development of computer technology and advance algorithms are becoming available. The major appeal of *in silico* research in medicine is its potential to accelerate drug discovery by predicting the efficacy and bioavailability of novel drugs in relation to BBB permeability. This is achieved with respect to passive diffusion and identified active transport mechanisms[114] using computational models that rely on known physicochemical parameters (e.g., solubility, lipophilicity, molecular size, hydrogen-bonding capacity, and charge). Using this technology, compounds are synthesized, prescreened, and virtually tested to predict how they will penetrate across the BBB into the brain. This allows for rapid and inexpensive screening of novel lead drugs, which limit the need for labor-intensive laboratory experiments and expensive clinical trials. Recently, this technique was used to discover potential inhibitors to an enzyme associated with cancer activity,[115] which was then confirmed *in vitro* and *in vivo*.[116] Several pharmaceutical companies (e.g., Roche) now use this technique as a routine part of the drug discovery process. However, this technology has limitations, which are related to the acquisition and reliability of the data sets necessary to elaborate the correct relationship between the three-dimensional (3D) structure of a molecule and its biological activity (structure–activity relationship, SAR). This is a necessary step to then determine the quantitative SAR.[117–119] Progress in understanding the role of the physicochemical properties relevant to important processes in membrane permeability (e.g., BBB) brings the rational design of novel effective drugs more within reach. In this contest, the use of computational *in silico* models can help in initial screening and effectively accelerate the drug development process but they still need to be supported by *in vitro* and *in vivo* studies to gather a number of crucial physicochemical measurements (e.g., those necessary to assess the contributions of passive and active transport).[120,121] Furthermore, at this stage, with respect to BBB, *in silico* models cannot be reliably used to predict brain distribution of a compound. *In silico* models still hold the promise for future breakthrough in the pharmaceutical drug development (at least for the foreseeable future) but cell-based and more importantly *in vivo* experimentations still will remain the "must to have" tools to support clinical studies and advancements in drug discovery.

IMMOBILIZED ARTIFICIAL MEMBRANE CHROMATOGRAPHY

Immobilized artificial membrane (IAM) chromatography phases attempt to mimic the membrane lipid environment found in living cells. This technology is more commonly used for purification of many biological substances but found other more specific applications in the study of drug–membrane interactions and drug permeability across the BBB.[122] Originally developed at Purdue University by Charles Pidgeon, the IAM stationary phase consists of a monolayer of carboxyl phospholipids that bear interfacial functional groups identical to the membrane phospholipids forming the cell membranes (e.g., phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, phosphatidylethanolamine, and phosphatidylserine) covalently immobilized on an inert silica support. The resulting IAM surface is a chemically stable chromatographic material, which mimics the lipid outer layer of a biological cell membrane. Because solute partitioning between the IAM bonded

and aqueous mobile phases is similar to that observed between a liposome and the aqueous phase, IAM chromatography can be used for the non-covalent immobilization as well as analytical and preparative separation of membrane-associated proteins. IAM chromatography has also gained acceptance as a method to estimate membrane permeability of small drugs and to measure their specific phospholipophilicity.[123–125] However, there are several limitations that hinder the validity of this technique as a method to assess drug permeability: (1) the IAM surface has limited ability to reproduce lateral membrane diffusion, which occurs at the cellular level *in vivo*; (2) the IAM surface is a lipid monolayer, thus using this model to extrapolate solute diffusion across a physiological membrane bilayer is extremely difficult; (3) IAM surface cannot mimic active efflux or drug metabolism, which normally occurs at the BBB level.[30,55,61,62] Currently, IAM chromatography is primarily used as a single-step purification method for membrane proteins (e.g., aldolase B).[126]

PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAYS

Parallel artificial membrane permeability assays (PAMPAs) have become a quite useful and versatile *in vitro* tool for predicting drug permeability across specific physiological membranes *in vivo*. The system is based on the use of specially designed artificial lipid-impregnated membrane established on a solid filter support (e.g., polycarbonate) between a donor and an acceptor compartment. In this system, multiwell microtiter plate is placed at the bottom for the donor compartment, whereas the artificial lipid membrane/acceptor compartment is placed on top in a "sandwich" like configuration. Initially, the test drug is added in the donor compartment and allowed to diffuse across the membrane. The drug diffused in the acceptor compartment is then measured and quantified (generally by ultraviolet–visible spectroscopy or liquid chromatography–mass spectrometry). PAMPAs have been developed to allow for a high degree of versatility, automation compatibility, and assay reproducibility. The components of the simplified model can be varied to mimic, with high accuracy and reproducibility, the *in vivo* biological membrane of interest (e.g., gastrointestinal tract,[127,128] skin,[129–131] and BBB).[132,133] Furthermore, the system can be modified to assess the effect of a wide range of pH on the permeability of the test drug of interest. The effect of physiological pH or pH gradient[134] to which the specific organ/membrane is exposed *in vivo* (e.g., gastric pH range 1.0–2.5, intestine pH range 6.6–7.0, etc.) can thus be tested. The donor and/or acceptor compartments can be modified to emulate *in vivo* conditions by incorporating solubilizing agents or additives that bind the compounds as they diffuse across the lipid membrane. PAMPAs rely on passive diffusion for permeability across membrane. This is one of the main entry routes for drugs absorption across the majority of physiological membranes and thus, PAMPAs are considered suitable early stage absorption screening tool alternatives to more demanding and expensive cell-based assays. However, a major limitation of PAMPA systems versus cell-based and *in vivo* studies is its the inability to reproduce active transport mechanisms (influx and/or efflux) or metabolic transformations that a compound may incur during transcellular routing across some physiological membranes (e.g., BBB endothelium[51,54,55,135] etc.). This may significantly affect predictability of bioavailability of the drug on the targeted site and thus use of PAMPAs alone would be insufficient. Their combination with *in silico* (described above)[51,55,135,136] and the development of more complex algorithms that better reflect the behavior of highly dynamic physiological membranes (e.g., BBB) can further extend the use of this broadly adopted technology beyond an early stage screening tool.

IN VITRO CELL-BASED BBB MODELS

In vitro models of the BBB started to emerge in the early 1990s as potential new research tools complementary to *in vivo* and human studies in basic, translational, and clinical/

pharmaceutical research bearing a number of desirable advantages: (1) cost effective—they are relatively inexpensive when compared with animal experimentation with a significantly higher throughput for drug permeability testing (a quality most desirable in the pharmaceutical industry); (2) simplified working environment—the artificial environment provided by these *in vitro* models allows studying and manipulating the BBB without the cumbersome number of additional variables to sort out when working with an entire organism; (3) versatility—they provide highly controllable and in some case quasi-physiological environments [cell-based and extracellular matrix (ECM) dynamic systems] outside of a living organism in which cells or tissue can be manipulated to study and dissect out the response of a specific tissue/organ to a broad range of physiological and experimental stimuli otherwise difficult to reproduce *in vivo*.

Noteworthy is the fact that cell-based BBB models can be established with any type of cell source (human, animal, or cell line derivative) including the availability of BBB ECs and astrocytes freshly isolated from human brain tissue (e.g., from patients undergoing temporal lobectomies for drug refractory epilepsy, brain tumor resections, aneurisms, etc.). This allows to some degree to reproduce *in vitro* pathological abnormalities (e.g., overexpression of drug efflux systems such as P-gp) that may facilitate the study of BBB dysfunctions in relation to the pathogenesis and progression of CNS disorders (e.g., drug-resistant epilepsy, brain tumor, etc.). However, the price for a simplified working environment and cost effectiveness is not something to take superficially. There are a number of serious drawbacks associated with the use of any *in vitro* systems including *in vitro* cell-based BBB models. Cells cultured *ex situ* in an artificially environment undergo dedifferentiation because of lack of exposure to physiological factors (whether diffusible from surrounding cells/tissue or dependent upon mechanical and physical stimuli such as SS and cell to cell contact). This can affect the expression (deregulation) of relevant cell biological features (e.g., transporters, ligands, enzymes, etc.), thus potentially altering the BBB physiology *in vitro* as well as its response to endogenous and exogenous stimuli. Therefore, validation of *in vitro* findings in *in vivo* conditions is necessary.

STATIC MODELS OF THE BBB: EC MONOCULTURES

The most common and widely utilized BBB model was developed based on a rather simplified view of the BBB, which was depicted as a monolayer of highly specialized brain microvascular ECs. This system, known as the Transwell apparatus (e.g., Corning, Lowell, MA), is a vertical side-by-side diffusion system across a microporous semipermeable membrane that separates the luminal (vascular) and the abluminal (parenchymal side) compartments. Brain vascular ECs (from various sources: bovine, rodent, porcine, non-human primate, and human) are grown to confluence on the upper (luminal) surface of the membrane immersed in their specific growth media. The advantages of cultured endothelium include the potential for using pure cell populations as well as their relative viability compared with isolated arterioles *ex situ*. The microporous membrane interface allows for nutrient exchange and the passage of cell-derived and exogenous substances but does not allow for cell movement across the two compartments.

Because of its intrinsic simplicity, this BBB model allows for high-throughput drug permeability testing and binding affinity (receptor–ligand) measurement.[137] In particular, they are ideal systems to study Michaelis–Menten kinetics of transport because of fixed volumes in each compartment.[137]

However, this rather simplistic reconstruction of the BBB lacks a number of critical features that are necessary for the development of true BBB properties *in vitro*. The absence of natural physiological stimuli such as cell to cell interaction with perivascular astrocytes and

other parenchymal cells (e.g., neurons), the exposure to intraluminal SS and circulating blood cells significantly limit the ability of the vascular endothelium to develop and/or maintain the intrinsic BBB properties and functions observed *in vivo*.[138–140] Furthermore, this nonphysiological culture condition may accelerate endothelial dedifferentiation and further enhance the loss of the BBB characteristics with serial cell passage (e.g., cellular polarity determined by the functional specialization of the apical and basolateral membranes).[57] The lack of antimitotic influences by laminin and flow shorten the life span of the endothelium and cause ECs to grow uncontrolled in a multilayer fashion[141,142] when cells start dedifferentiating. ECs grown in Transwell tend to have irregular patterns of cell adhesion, which prompt the so-called "edge effect." This term refers to a condition wherein the inability to form proper tight junctions between adjacent cells and between the endothelium alongside the perimeter of the membrane and the inner wall of the luminal chamber leads to artifactual paracellular diffusion, thus affecting the reliability of permeability measurements across the endothelial monolayer. This is of special concern for compounds that are highly hydrophilic and poorly cross the lipid membrane bilayer of the cells. In addition, EC monolayers are exposed to serum proteins on both sides (luminal and abluminal), whereas, *in situ*, this occurs only at luminal/vascular side and the abluminal side is either exposed to astrocytic/parenchymal released factors.

STATIC TWO-DIMENSIONAL MODELS OF THE BBB: COCULTURE OF ECS AND GLIA

The addition of abluminal astrocytes (in juxtaposition to the endothelial monolayer) have been shown to facilitate the formation of more stringent interendothelial tight junctions and the overall expression of proprietary BBB features,[143] that more closely resemble that of the BBB *in situ* (see Fig. 3).

There is significant body of evidence, both *in vitro* and *in vivo*, to indicate that astrocyte interaction with the cerebral endothelium helps determine BBB function, morphology (i.e., tightness), and protein expression.[89,90,144] Intercellular adhesion between astrocytes in the BBB has been observed in the form of gap and adherens junctions.[86,102,103] The presence of glia and the establishment of glial–endothelial interactions have been shown to increase the expression of transporters (e.g., Glut-1 and MRPs such as P-gp) and tight junctions, and have also helped to induce a phenotype more closely mimicking that found *in vivo*. A limited cell polarity is inducible in ECs when cocultured with glia or in the presence of glial-conditioned medium.[40,145] The exposure to glia promotes the expression of brain endothelial marker enzymes (e.g., OX-26, alkaline phosphatase, acetyle-holinesterase, Na^+–K^+-ATPase) as well as that of a number of specialized transport and efflux systems (e.g., facilitative Glut-1, γ-glutamyltransferase, and Pgp)[32,34,57,59,146,147] typically observed in BBB ECs *in vivo*. Transendothelial electrical resistance (TEER), which measures the ionic conductance across the BBB, is typically higher in EC–glia coculture systems than EC monocultures. The difference in the measurement indicates formation of a more stringent and selective vascular bed than monocultures.

However, despite the clear advancement in BBB modeling provided by the use of abluminal endothelial–glial cocultures, static systems lack the ability to allow for endothelial exposure to physiological SS. Given the unique vascular modulatory role of shear forces as well as that of abluminal astrocytes (and perhaps pericytes),[88] it is not surprising that attempts have been made to develop more sophisticated dynamic (flow-based) coculture BBB models.

DYNAMIC *IN VITRO* BBB MODELS

Cone–Plate Apparatus

The use of a purpose-built cone and plate viscometer was the first attempt to enable the endothelial exposure to flow *in vitro*.[148] In this system, the ECs monolayer seeded on the bottom of the plate is exposed to SS generated by a rotating cone. The shear force is transmitted to the vascular endothelium through the culture media spinning in the direction of the rotating cone (see Fig. 4). The angular velocity and the cone angle determine the level of SS generated, which is not entirely uniform along the radius of the plate supporting the ECs monolayer. Reversing and pulsing the angular velocity of the cone can be used to achieve back-flow and pulsatile SS, respectively. The cone–plate apparatus described by Bussolari and coworkers[148,149] was designed to study the effects of laminar or turbulent fluid SS on cells and the relative contribution of fluid viscosity, time of exposure, and other physiological variables. Laminar SS (as detailed above) plays a major role in BBB endothelial physiology such as promoting BBB tightness, the expression of drug resistance mechanisms, ion channels, and other specialized transport systems. SS can modulate even the bioenergetic behavior on the BBB endothelium.[3] Previous studies have also shown that SS induces the production of vasoactive substances.[150] However, turbulent SS has quite a different effect of BBB endothelial physiology including increased cell proliferation, loss of BBB tightness, and increased transendothelial permeability. Therefore, the cone–plate apparatus represents the first attempt to introduce laminar or turbulent flow *in vitro* to study its effect on BBB physiology

This system *per se*, however, was not designed to reproduce precisely the flow and milieu experienced by vascular endothelium *in vivo*. Furthermore, lacking the ability to expose ECs to perivascular modulatory factors (from glia and other cells) does not allow full differentiation of ECs into a BBB phenotype, thus further diminishing the significance and reliability of the results.

However, the importance of flow as a major modulator of BBB physiological properties and the need for BBB models better capable of mimicking the physiological function and response of the brain microcapillaries led researchers to develop new generations of dynamic *in vitro* systems. To date, two designs based on different technologies are available: (1) the so-called "dynamic *in vitro* BBB model" (DIV-BBB), which makes use of artificial capillary-like structural supports (hollow fibers) for coculturing ECs and astrocytes and also allows for the presence of intraluminal flow; (2) collagen 3D matrix supports (3D ECM-based BBB models).

3D Dynamic *In Vitro* Models of the BBB

The use of artificial capillary-like structures such as hollow fibers (generally made of thermoplastic polymers such as polypropylene, polysulfone, etc.) has been initially exploited for the construction of bioreactors for mass production of antibodies (or other biologically active substances) or for the expansion of hybridoma cells. More recently, the same technology has been adapted to model hollow organ-like structures such as the BBB.[151] This newly developed *in vitro* system, which is referred to as the DIV-BBB model (see Fig. 5), allows brain microvascular ECs (animal or human, both primary cultures and cell lines) to be cocultured with abluminal astrocytes and under the exposure of quasi-physiological pulsatile laminar SS. This allows for the formation of a BBB that closely resembles the *in situ* both functionally and anatomically.[75,141,152]

The functional core of the DIV-BBB apparatus is represented by a bundle of microporous pronectin-coated polypropylene hollow fibers, which enable co-culturing of brain and non-brain vascular ECs from various origins [such as human umbilical vein endothelial cells

(HUVECs)] with glial cells. These glial cells are seeded in juxtaposition to the ECs on the outer surface (perivascular/parenchymal side of the brain microcapillaries) of the same hollow fibers. The artificial capillaries are connected to a media reservoir through a gas-permeable (e.g., silicon) tubing system for the exchange of O_2 and CO_2. A servo-controlled variable-speed pulsatile pump generates the intraluminal flow, which can be regulated to produce (based on the diameter of the hollow fibers and the viscosity of the medium) SS levels and intraluminal pressure physiologically comparable to that observed in capillaries *in vivo* (generally between 5 and 23 dynes/cm^2).[149,153] The same computer controlled pumping mechanism can be used to reproduce the rheological features of other vascular districts (e.g. venules and large arteries), thus further expanding the usability of this system in vascular research. The DIV-BBB allows reproduction of multiple functional properties and physiological responses observed at the BBB *in situ*. These include low permeability to intraluminal potassium and polar molecules (e.g., sucrose and other paracellular markers), high TEER,[154] negligible extravasation of proteins, the expression of specialized transporters, ion channels,[141,155] and efflux systems (e.g., Glut-1 and Pgp,).[155,156] In addition, this system allows for concomitant circulation of blood cells,[157] which enables to study a number of cerebrovascular/systemic pathologies related to rheological impairments and/or inflammation[158] including hypoperfusion, cerebral ischemia,[68] and brain edema.[71] On par with previously described BBB models, the DIV-BBB can be established using primary (commercially available or freshly isolated from brain and non-brain tissues)[75,156] or immortalized cell lines.[154] Furthermore, the structure and design of a variant of the original DIV-BBB model allows for the inclusion of other relevant cell types,[141] which might even include pericytes and neurons.

Recently revised DIV-BBB systems enable cell trafficking across the BBB, which further extend the usability of this model to study and characterize the role of the BBB in the pathogenesis of major neuroinflammatory diseases (e.g., meningitis,[159] multiple sclerosis,[160] Alzheimer, etc.). The DIV-BBB provides a number of appealing advantages over conventional static systems. However, considering recent trends in the pharmaceutical field and clinical interests in the study of the CNS, there are a number of limitations that need to be addressed to expand the adoption of this BBB model. More specifically, its design does not allow for visualization of the intraluminal compartment to assess morphological and/or phenotypic changes of the vascular endothelium. Also, in contrast to conventional static BBB models (e.g., Transwell), this apparatus is not designed for a high-throughput pharmaceutical study, and the technical skills/time required to establish the system is significantly higher. Cell characterization is possible but limited because it requires harvesting the cells from either compartment. This is an invasive procedure that can alter (to various degrees) a number of morphological and physiological characteristics of the cells. Additionally, the initial cell load requirement to establish the DIV-BBB model is quite significant ($>1 \times 10^6$ cells), especially if primary cultures are to be used. Limited availability of manufacturers could be considered relatively inconvenient. However, increased awareness about the DIV-BBB and larger adoption of this model can help solve this issue in the near future.

3D ECM-Based BBB Models

Despite the low awareness of 3D tissue culturing systems in several research fields (including neurobiology and cerebrovascular research), the potential benefits of culturing cells using 3D artificial supports (even though more expensive and perhaps less convenient than current static two-dimensional models) is self-evident when one considers that the *in situ* microenvironment plays a critical role in modulating/regulating the functional properties of that specific organ (e.g., the BBB).[161,162] 3D *in vitro* tissue models are already available for drug discovery and transport studies related to a variety of organs (e.g., liver) and tissues

(e.g., cardiac, muscle, bone, etc.) as well as malignancy in solid tumors.[163] Similar advancements in tissue culture technology are now making the appearance in cerebrovascular and BBB research.

In vitro BBB models grown on self-polymerizing ECM protein scaffolds provide an alternative to the use of artificial capillary-like structures supports (e.g., DIV-BBB).

Three-dimensional ECM-based BBB culture systems enable close interactions between cells (and the exposure to the corresponding trophic and differentiating released factors) and the formation of quasi-physiological biochemical gradient exposure is necessary for cell-to-cell communication and cross-signaling (see Fig. 6). With recent advancement in the development of microfluidic systems, it is now possible to monitor cell migration in a 3D environment[164,165] with a direct application for cancer metastasis and angiogenesis studies. These models can thus study the effect of physiological and pathological stimuli in 3D BBB microenvironments. Furthermore, several high-resolution 3D imaging techniques based on the use of confocal microscopy, multiphoton microscopy, and optical coherence tomography available today, which can allow repeated assessment of dynamic changes to cells cultured in the 3D ECM microenvironments.

However, the development of an *in vivo*-like matrix architecture is a complex issue to address. Although being convenient to study specific roles of various ECM proteins (e.g., laminin, collagen, fibronectin, etc.) in cell differentiation, self-polymerizing ECM models (cross-linked ECM) can only provide a uniform matrix lacking the discontinuities and gaps that may normally be present *in vivo* and allow for cell migration. This problem was partially addressed by using cell-derived matrices, which maintain some of the original gaps that accommodate the cells.[164,166] The omission of minor ECM constituents potentially significant in the architectural assembly and matrix property is another concern until more sophisticated 3D matrix models become available in the future.

CELL LINES OR PRIMARY CULTURES?

Several cell lines (both animal and human derived) have been developed as biological surrogates for BBB modeling. These cell lines bear many advantages over primary cultures, which include being easy to grow and retain their differentiating properties even after repetitive passages, reducing cost and labor. Only a few immortalized human endothelial cell lines (HMEC-1,[167] HCMEC/D3,[154,168] and TY08[169]) have been developed and reportedly used for the establishment of *in vitro* BBB models. Animal-derived immortalized cell lines are more commonly used. Immortalized rat brain endothelial 4 cells are probably the most extensively animal-derived cell line used for BBB modeling,[170–173] although their use is only limited to static coculture conditions. These cells functionally express a variety of BBB transporters, drug-metabolizing enzyme activities, and mechanisms of active extrusion including Pgp and multidrug resistance-associated protein (Mrp1).[111,147,174–178] The use of intestine-derived cell line Caco-2 in the establishment of an *in vitro* BBB has also been taken into consideration.[179] Differences in drug permeability between the intestine and brain vascular-derived cells are too significant and limit the use of Caco-2 cells for BBB modeling.[180,181] However, genetic manipulation by the use of retroviruses and the introduction of immortalizing genes can affect a wide variety of cellular functions, potentially altering the physiological response to endogenous and exogenous stimuli, to which BBB ECs *in vivo* are normally subjected.

Highly purified populations of cultured human brain (*human brain microvascular endothelial* cells, HBMECs) and non-brain (e.g., *HUVECs*) vascular cells exhibiting excellent characteristics for the study of the developmental and pathophysiological processes of the BBB became available in the early 1980s. Unfortunately, the high level of

technical skill necessary to isolate these cells from the native tissue (especially brain tissue) as well as their rapid senescence, evident just after few cycles of cell division, made their use quite costly and time consuming. Today, they are more easily accessible through a number of companies that have made them commercially available, although still for a premium price. Among primary BBB cells, those of human origin provide the most accurate representation for studying neurological diseases involving BBB dysfunction (e.g., drug-resistant epilepsy).[55,156] Human specimens offer a variety of neurological etiologies that otherwise would be near impossible to recapitulate in cell lines or animal-brain-derived (rodent, porcine, or bovine) primary cultures. Even though HBMECs are not yet a realistic (cost-effective) alternative to the use of cell lines for industrial (pharmaceutical) screening (such as testing permeability, toxicity, etc.) and testing of novel drugs, they have unmatched value for basic and translational research.

PERSPECTIVE DEVELOPMENT IN BBB MODELING: CONCLUSIONS

The need to limit the increasing costs of experimental studies, prescreening, and validating novel CNS therapeutics is continuously pushing researchers to develop BBB *in vitro* models capable of high-throughput testing to accelerate drug design. On the contrary, basic and translational researchers are instead pushing toward more realistic BBB models wherein multiple determining factors (e.g., cell–cell interactions, cell migration, flow, microenvironment influences, etc.) can be reproduced, dissected out, and characterized. Requirements such as "higher predictability," "fully scalable," "customizable," and so on are becoming a frequent conundrum in pharmaceutical and biotech industries that researchers/developers will have to tackle. New artificial BBB systems will be required to incorporate a number of automatisms (e.g., control/adjust oxygen and CO_2 levels in the culture medium, real-time monitoring of BBB integrity, medium sampling, etc.) including an array of computer-controlled sensors to monitor a variety of physiological parameters (e.g. NO, glucose, lactate, etc.). This automatism will greatly minimize time-consuming routine maintenance and monitoring and facilitate operator efforts. Additional advancements will come with the introduction of new cell culture support materials. For example, incorporating the desired anchoring molecules (for cell adhesion or to stimulate specific cellular responses) in their matrix construction would allow for a higher degree of control over cell differentiation, cell interaction, and cell response. However, despite all the advancements in this field, it is important to understand that *in vitro* BBB models are not a replacement for *in vivo* and human studies but rather a complementary set of tools to facilitate drug discovery and to aid in the acquisition of additional knowledge (see Table 2).

Predictability of drug permeability across the BBB based on physicochemical parameters is not accurate because it lacks the contribution of active transport systems and metabolic transformation on drug bioavailability. Advanced cell-based models can to some extent reproduce the physiological behavior of the BBB *in situ*, thus providing more accurate results. However, validation *in vivo* is still required. Furthermore, *in vitro* cell-based models cannot provide information on drug distribution into the brain once the drug passes the BBB. It is possible that future and more advanced *in silico* models can partially address this issue. It is also important to understand that the goodness/reliability of a BBB model is not absolute but strongly depends on its specific use. This is a major determinant for the accuracy of the results as well as their translational/clinical significance.

Whether *in vitro*, *in vivo*, or clinical, each set of research tools and related technologies bring something unique on the table. The challenge for future breakthrough in CNS drug discovery is bringing together all these technologies as a team to facilitate the understanding of the pathogenesis of a disease, provide useful insights to modulate counteractive

physiological responses, and accelerate the development of novel clinical strategies and pharmacological treatments to reduce the burden of many CNS diseases.

Acknowledgments

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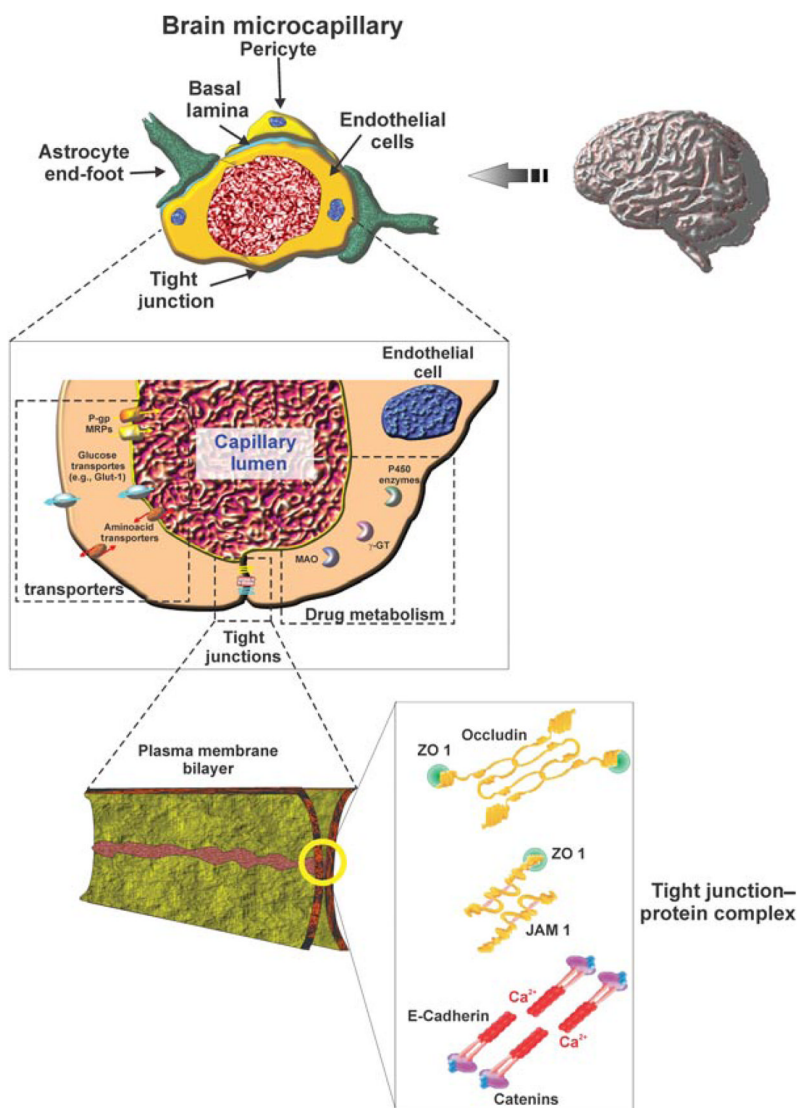
Figure 1. Schematic representation of a typical brain microcapillary
Note that the passage of substances across the BBB endothelium is controlled by a multimodal barrier system: (1) gating barrier (tight junctions), which prevent paracellular diffusion of polar molecules; (2) transport barrier, which includes number of active efflux systems (P-gp, MRPs, etc.) with affinity for lipophilic substances; (3) metabolic/enzymatic barrier (cytochrome P450 enzymes, MAO, etc.), which catalyze the oxidation/metabolism of organic substrates including xenobiotic substances such as drugs and other potentially toxic chemicals.

What happens to BBB endothelial cells when they are exposed to Flow?
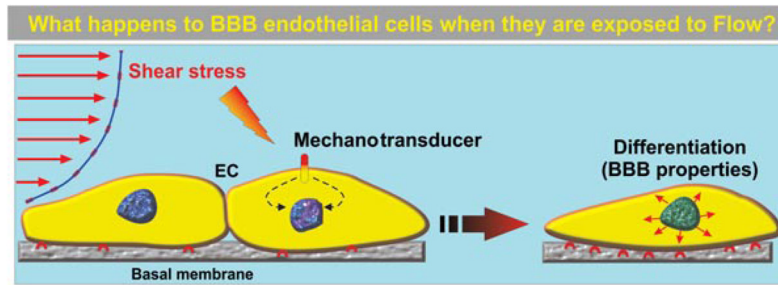
- ➢ Cell orientation changes with flow direction, distribution of cell cytoskeleton
- ➢ Production of vasoactive substances and improved cell adhesion
- ➢ Mitotic arrest
- ➢ Cell survival
- ➢ Glucose metabolism switch toward a more anaerobic pathway
- ➢ Tight junction formation
- ➢ The expression of asymmetrically localized enzymes and carrier-mediated transport systems that engender a truly "polarized" BBB endothelium phenotype

Figure 2. Effect of shear stress forces on BBB endothelial cells
Shear stress (SS) is a major pleiotropic modulator of the endothelial cell physiology by controlling gene involved in the regulation of cell division, differentiation, migration, and apoptosis. For example, the exposure to physiological SS promoted mitotic arrest by contact and the endothelium assumed the typical monolayer appearance observed *in vivo*.
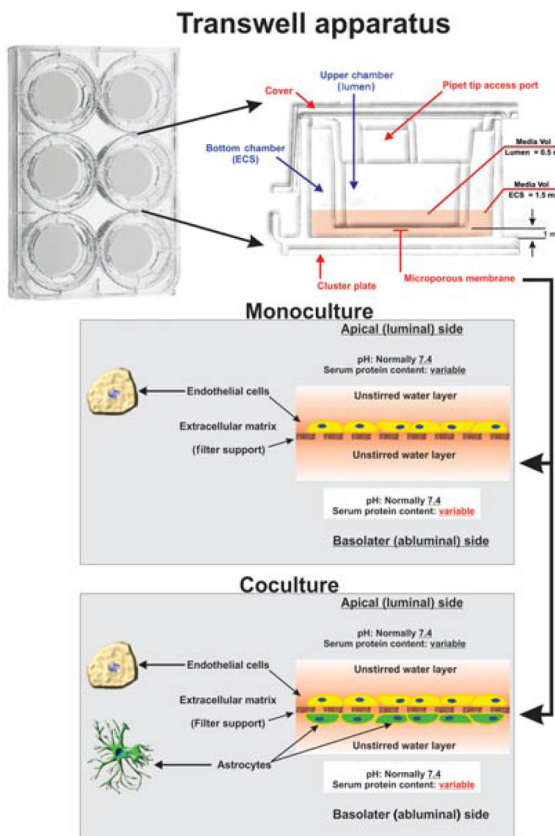
Figure 3. Schematic representation of a Transwell apparatus
The Transwell (e.g., Corning) is a vertical side by side diffusion system across a semipermeable microporous membrane, which allows for free passage of nutrients and diffusible factors between the luminal and abluminal compartments. Depending on the pore size of the membrane, cell trafficking across the compartments (generally lumen to albumen) can be enabled. Note the cellular layout in monoculture (endothelial cells only) and coculture (luminal endothelial cells, which juxtapose perivascular/abluminal astrocytes) *in vitro* BBB configurations.

Cone–plate shear apparatus

$$T_\alpha = \frac{\mu\omega}{\alpha}$$

$$\tilde{R} = \frac{r^2 \omega \alpha^2}{12\nu}$$

Unidirectional laminar shear

Oscillatory shear

α
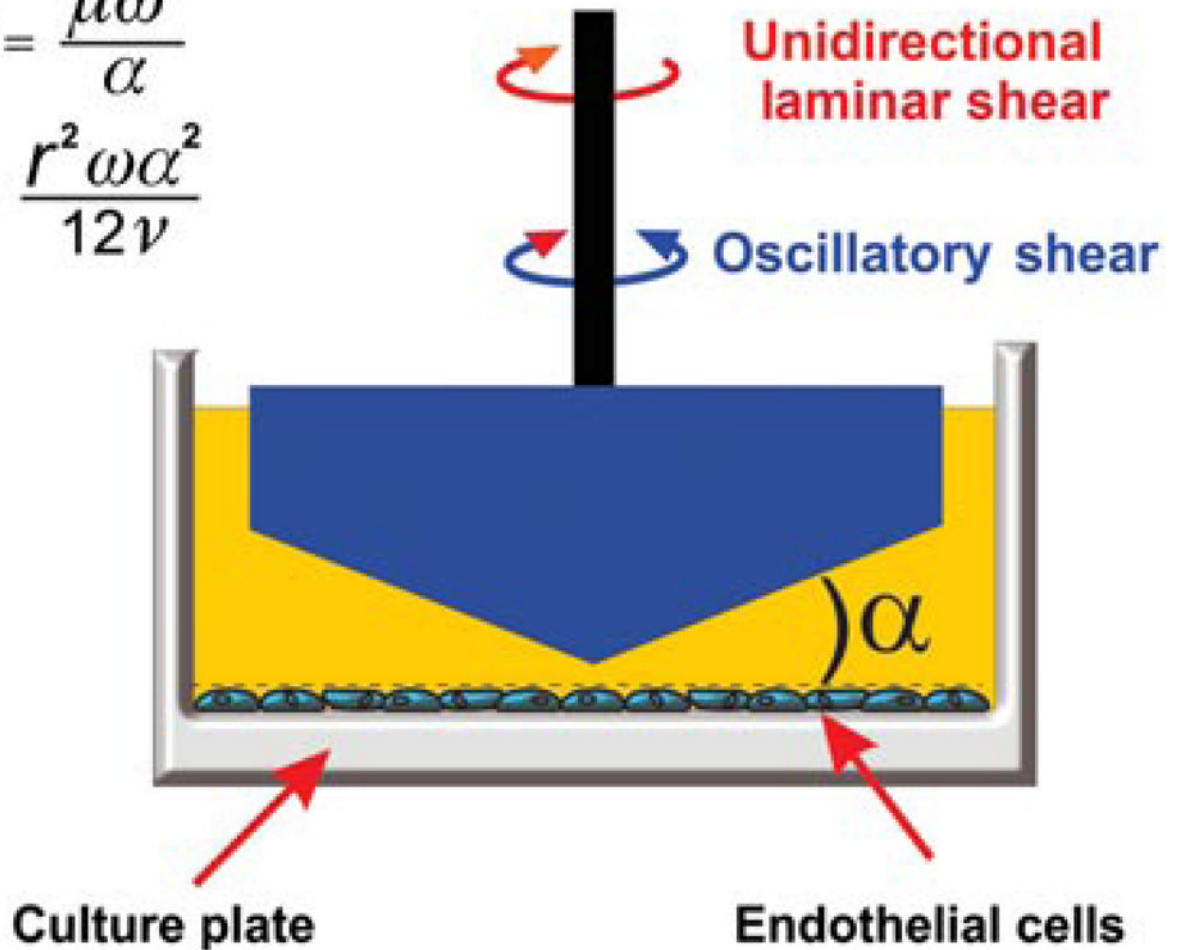
Culture plate

Endothelial cells

Figure 4. Schematic representation of a cone and plate viscometer
The endothelial monolayer can be exposed to a quasi/uniform laminar or pulsatile shear stress (SS) by the use of a purpose-built cone and plate viscometer. Note that μ is the viscosity, ν is kinematic viscosity, ω is angular velocity, and &agr; is the cone angle. The cone angle and the angular velocity determine the level of SS to which the endothelium is exposed.
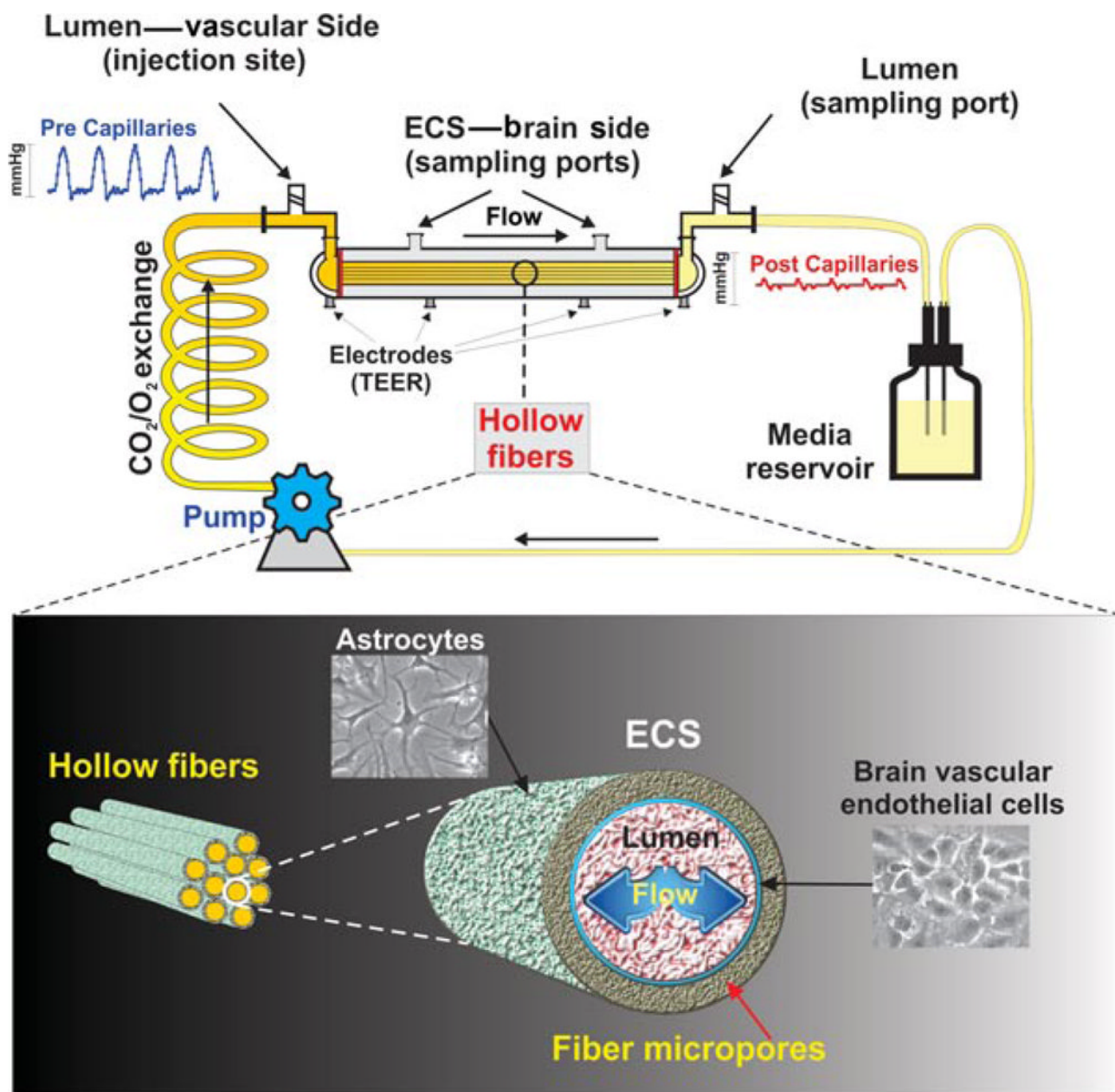
Figure 5. Dynamic *in vitro* BBB model (DIV-BBB)
The top panel shows a schematic representation of a DIV-BBB module. In this system, cerebral endothelial cells are cultured in the lumen of fibronectin-coated polypropylene microporous hollow fibers, in the presence of astrocytes cultured on poly-D-lysine-coated outer surface of the same. The bundle of hollow fibers is suspended inside a sealed chamber. The artificial capillaries are in continuity with a medium source through a flow path consisting of gas-permeable silicon tubing. Ports positioned on either side of the module allow access to the luminal and abluminal compartments. The capillaries are exposed to luminal pulsatile flow generated by a pumping mechanism. Note the pressure waveforms changes (pre-versus postcapillaries) mimicking the rheological changes observed *in vivo*.
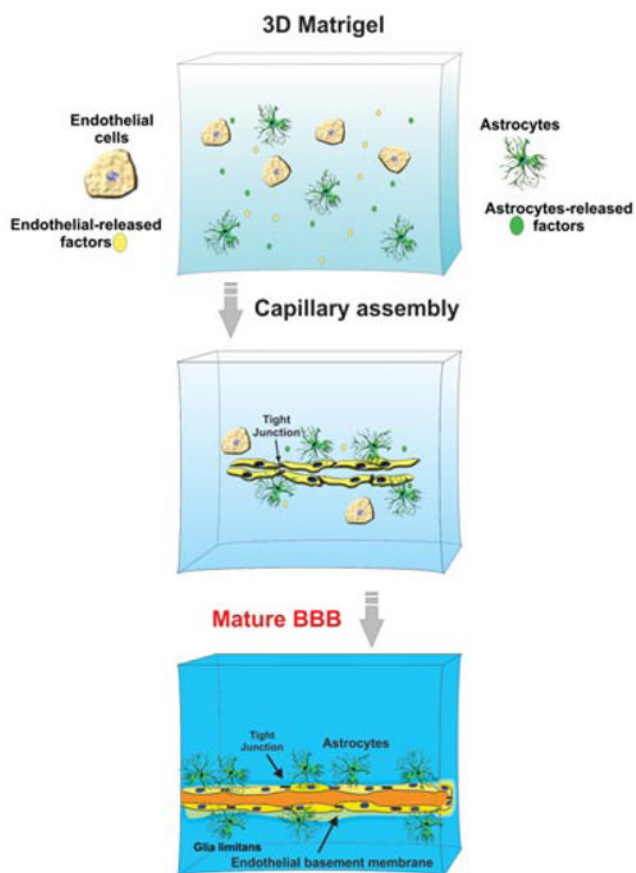
Figure 6. Schematic representation of a 3D ECM-based *in vitro* BBB model
Note the assembly of a microcapillary-like structure.

Table 1

Functional and Structural Requirements for An Ideal In Vitro BBB Model to Mimic the In Vivo BBB Model

Functional Features of An Ideal *In Vitro* BBB Model
Enable the expression of tight junctions between adjacent endothelial cells.
Negligible paracellular diffusion between endothelial cells.
Selective and asymmetric permeability to physiologically crucial ions (Na^+, K^+, Cl^-).
Functional expression of efflux systems and selective transport mechanisms (e.g., P-gp, MRPs, hexose, aminoacid, monocarboxylic acid, and other relevant transporters).
Expression of drug-metabolizing enzymes (P450s, MAO, etc.).
Exposure to laminal shear stress (apical membrane), glia (basal membrane), and other permissive factors that promotes growth inhibition and differentiation of endothelial cells.
Responsiveness to permeation modulators (e.g., hyperosmolar mannitol) as well as other stimuli (endogenous and exogenous) that can affect BBB integrity and function.
Ability to reproduce the effect of a wide range of physiological and pathophysiogical stimuli (hypertension, flow arrest, inflammation, etc.) that affect the BBB *in vivo*.
User friendly, scalable, and cost effective.

Functional requirements for an ideal in vitro BBB model. The following table includes the in vitro functional and structural requirements essential to mimic the in vivo BBB.

Table 2

Side by Side Comparison Between *In Vitro, In Vivo*, and Clinical Studies to Elucidate Differences, Overlaps, and Comlementaries Between Them

Measurable Endpoints	*In Vivo* Animal	Clinical Human	*In Vivo* Static and Dynamic
Brain imaging	Yes	Yes	No
Histology	Yes	Yes, but limited to tissue reactions from patients or post-mortem donors	No
Molecular biomarkers	Yes	Yes, but limited (e.g., serum urine)	Limited
Molecular mechanisms	Yes	No	Yes
Intravital microscopy	Yes	No	No
Monitoring clinical outcome (e.g., progression/regression)	Yes but correlation with patients can be difficult	Yes	No
Longitudinal BBB response	No	No	Yes
Cell isolation	Yes, but labour intensive	Mostly Limited to tissue reactions and blood cells	Yes
Cell manipulation	Limited	No	Yes
Control over physiological and experimental variables to dissect out specific effects	Limited	No	Yes
Drug permeability	Yes, but labour intensive	No	Yes
Drug distribution	Yes	No (limited to constrast enhancement for CT Scan and MRI)	No
Drug efficacy and safety	Yes	Limited to retrospective data analysis	No
Time course (acute BBB experimentation)	Limited and labour intensive	No	Yes
Clinical significance	Vary	Yes (with large data seta and mostly retrospective	Limited (require validation
Best suited for	Translational and Functional	Functional and Clinical studies	Basic and Translational

Side by side comparison between in vitro, in vivo and clinical studies. The table reports relevant examples to elucidate differences, overlaps, and complementarities between them.