PERSPECTIVE

Blood-brain barrier modeling: challenges and perspectives

The blood-brain barrier (BBB) forms a highly selective interface between blood and brain. Extensive research efforts have completely changed our view of the BBB in the last years, from a static, impermeable barrier to a dynamic, highly regulated and specific cellular system. The BBB ultrastructurally consists of specialized brain microvascular endothelial cells (BMEC) characterized by low pinocytic activity, absent fenestrations and expression of specific and polarized transcellular transport systems. BMECs are interconnected by protein complexes consisting of tight and adherens junctions leading to high electrical resistance and low paracellular permeability. Attached pericytes and astrocytic end feet processes are critically involved in induction, sustaining and regulation of the BBB properties. Together with smooth muscle cells, neurons and circulating blood cells, they form the complex system of the neurovascular unit (NVU) (Neuwelt et al., 2011). The NVU controls the transition of fluids, molecules and cells between blood vessels and the central nervous system (CNS) integrating physiological and pathological signals such as metabolic needs, rheological disturbances and inflammatory stimuli. BBB dysfunction or disruption is a hallmark in the pathogenesis of primary or secondary (caused by systemic diseases) neurovascular, infectious, inflammatory or degenerative diseases of the CNS (Ruck et al., 2014). Hence, BBB research is of interest for multiple disciplines including neuroscience/neurology, (patho)physiology, pharmacology, internal medicine and many more.

Given the importance of BBB research, there is a constantly increasing need for new and advanced model systems and techniques. Since human studies are restricted to post mortem investigations or imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) with limited resolution, nowadays most research on the BBB still involves laboratory animals. Vital dyes like Trypan Blue or Evans Blueto study *in vivo* permeability of the BBB have been used since the 19th century. Pharmacodynamic and pharmacokinetic properties of brain targeting drugs are still mostly studied with *in vivo*/*in situ* methods such as internal carotid perfusion or intracerebral dialysis fibers (Partridge, 1998). However, high financial and labour costs, limited transferability and overstraining complexity are in favour of parallel *in vitro* approaches. Advantages and disadvantages of the most important current and perspective *in vitro* models are discussed; an overview is presented in **Figure 1** and **Table 1**.

Isolated brain microvessels were the first approach of an *in vitro* BBB model (Joo, 1985). Microvessels are isolated from brain tissue by mechanic homogenization, enzymatic digestion and a variety of filtration and/or density gradient centrifugation techniques. This model system is easily accessible, well-established and maintains many structural and functional properties of the BBB. However, isolation of microvessels is labour intensive and difficult, viability of microvessels is often poor and it is not possible to study directional transport mechanisms or cellular transmigration.

Isolation and cultivation techniques of brain-derived endothelial cells were developed in the early 1980s and remarkably facilitated *in vitro* BBB research. Cell-based approaches enable simplified, cost effective, highly flexible and easy controllable *in vitro* models of the BBB. Endothelial cells can be tapped from many different sources such as human or rodent brain tissue or cell-line- and stem-cell-derivatives (Lippmann et al., 2014). However, primary cells derived from humans or genetically modified animals are of limited availability, critically affecting cost- and time-effectiveness. On the other hand, endothelial cell lines cannot fully substitute for primary cells as important cell compromising translation of results properties are altered to the *in vivo* situation. Moreover, the isolation of endothelial cells from transgenic animals or from patients allows for the reproduction and investigation of mechanisms involved in initiation and/or progression of several CNS diseases. An advanced understanding of these mechanisms is essential for the development of new therapeutic strategies. **Transwell-systems** are the most common and widely used cell-based *in vitro* models of the BBB.

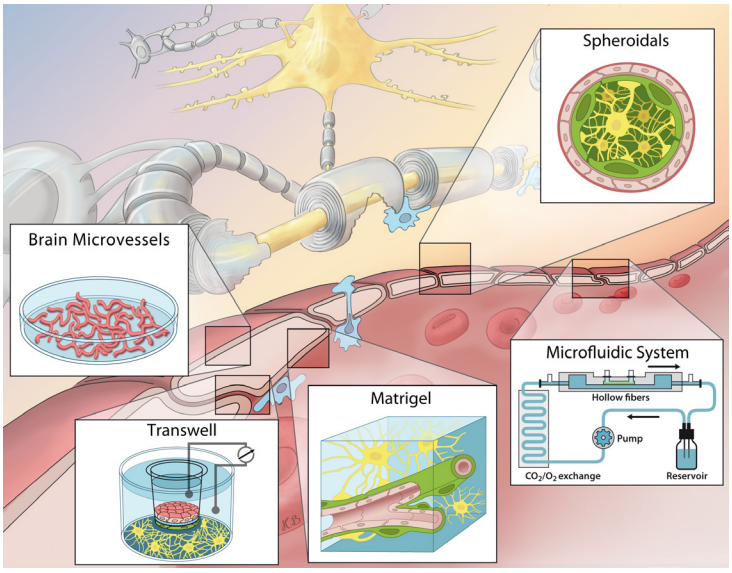

Figure 1 Schematic overview of current *in vitro* blood-brain barrier (BBB) models.
Brain microvessels are isolated from brain-tissue by mechanic homogenization, enzymatic digestion and further purification techniques. Transwell systems are vertical side by side diffusion systems. Brain endothelial cells (BMEC, red) are grown on a semipermeable membrane separating a luminal and abluminal compartment. Differentiation status and barrier properties of endothelial cells are improved by coculture with pericytes (green) in juxta-position and astrocytes (yellow) in the abluminal compartment. In matrigel and other three-dimensional extracellular matrix (ECM)-based models, BMECs (red) form tube-like structures surrounded by pericytes (green) and astrocytes (yellow) in gel matrices containing ECM proteins. In spheroidal models, BMECs (red), pericytes (green) and astrocytes (yellow) are able to freely self-assemble to ball-shaped cellular aggregates without any scaffolding material. Microfluidic systems provide physiologic shear stress to BMECs producing laminar flow by a computer-controlled pumping mechanism. In these systems, BMECs are cultured in the lumen of microporous, glass or plastic hollow fibers coated with ECM proteins. A bundle of hollow fibers is placed into a sealed chamber, where different ports allow accessing the luminal and abluminal compartments. The hollow fibers are connected to a medium reservoir through a continuous tubing system enabling gas exchange.

Monolayers of endothelial cells are grown on a semipermeable membrane separating a luminal and abluminal compartment (Bittner et al., 2013). This model allows to study directional transport mechanisms, cellular transmigration processes and transendothelial resistance (TEER). It is ideal for permeability testing and binding affinity measurements. Moreover, Transwell systems are currently the best suited models to access BBB - immune cell interactions, especially to investigate transmigratory processes. On the other side, loss of BMEC characteristics such as polarization and barrier function have to be considered since cell-cell interactions and shear-stress being critical for endothelial differentiation are missing (Naik and Cucullo, 2012). In the last years, many approaches have been undertaken to overcome these shortcomings. In **two-dimensional (2D)-Transwell systems,** endothelial cells are cocultured with astrocytes grown in the abluminal compartment of the Transwell system. Herein endothelial cell-astrocyte interactions have been shown to increase expression of transport systems and tight junction proteins. The addition of pericytes grown on the opposite site of the semipermeable membrane further improves differentiation status and barrier properties of BMECs. Another strategy is to modulate differentiation status of BMECs by signalling molecules. Recently retinoic acid has been shown as a soluble factor significantly enhancing the BBB phenotype of BMECs (Lippmann et al., 2014). However, 2D models still lack the 3D *in vivo* organization and direct cell-cell interactions leading to a "phenotypic drift" of BMECs acquiring more generic endothelial cell properties; especially receptor-mediated transport systems are impaired (Urich et al., 2013). This issue is addressed by **three-dimensional (3D) extracellular matrix (ECM)-based and spheroidal models**. In ECM-based settings like the matrigel system cells are grown in gel matrices allowing for free migration and assembling (Davis et al., 2007). In the matrigel system, BMECs build up tube-like structures surrounded by pericytes and astrocytes. In spheroidal models, cells are able to freely self-assemble without a scaffolding material forming ball-shaped cellular aggregates. BMEC form an outer cell monolayer lined-up by pericytes on the inside; astrocytes are located in the core of the spheroids (Urich et al., 2013). Despite the evident advantages of 3D models, they are more expensive and more difficult to set up and control. The artificial matrix provided in ECM-based models only sparsely resembles the *in vivo* composition and organization of proteins critically affecting transferability of *in vitro* findings. Moreover, some aspects of BBB physiology such as cell-migration and directed transport mechanisms need even more sophisticated techniques and devices, such as recently developed **3D microfluidic systems** where cells are seeded in arrays of micropillars thinly layered with ECM matrix proteins (Toh et al., 2007). Advanced imaging techniques (*e.g.*, two-photon imaging, confocal microscopy) allow for the assessment of dynamic processes in these microfluidic systems. These models also imitate physiologic shear stress by providing laminar flow being essential for BMEC differentiation and function. Former microfluidic channel-based systems utilized 2D growth substrates such as glass or plastic surfaces coated with ECM proteins, however associated with the deficiencies of 2D systems. Moreover, microfluidic systems are expensive, difficult to set up and use and high cell numbers are needed to establish the model.

Overall, none of these currently available models can be appraised as being ideal. Such a model should provide physiologic expression and organization of tight junction proteins with very limited paracellular diffusion. BMECs should be polarized with properties of *in vivo* luminal and abluminal transport systems, exert functional efflux, metabolic and catalytic mechanisms. The ideal model would reproduce *in vivo* barrier properties such as transendothelial resistance and permeability coefficients according to hydrophilic properties and molecule size. Transmigratory processes would be tightly regulated and dependent on cell-cell interactions. The model should represent a dynamic system responding to hemodynamic, nutritional, inflammatory and pharmacological stimuli. The 3D organization of the BBB and laminar shear stress should be included in this model, too. Last but not least, it is expected to be user friendly, cost and time effective, customizable and scalable.

The still unmet need for better *in vitro* BBB models fuels continuous efforts of the research community. Pharmaceutical and biotechnological researchers aim particularly for cost-effective and high-throughput systems, while basic and translational researchers try to develop more realistic models mimicking *in vivo* physiological or pathological conditions. Computer-based ***in silico* models** could be the best candidates to improve cost and time efficacy. ***In silico*** models are potentially best suited for the development of drugs predicting efficacy and bioavailability in the CNS. Many pharmaceutical companies have already implemented this technology in their early drug development programs helping to identify well-tolerated, safe and effective compounds (Wager et al., 2012). However, these models are just as good as the parameters provided. Many physicochemical parameters of a drug could be deceived from its chemical structure; while there is only sparse knowledge on transport processes and metabolic transformation at the BBB and their regulation. Further studies are needed to close this gap. In search for more realistic BBB models, recently the group around John Wikswo started an ambitious NIH funded (National Institutes of Health, USA) project (Alcendor et al., 2013). The group is working on a three-dimensional, multi-compartment, microfluidic organ-on-a-chip approach of the NVU. The **NVU-on-a-chip system** will include a CSF compartment, a neuronal compartment with neurons and glia cells, and a blood compartment formed by hollow fiber capillaries covered with BMEC on the luminal and with astrocytes and pericytes on the abluminal surface. "Arterial" and "venous" hollow fibers are connected to a realistic blood-surrogate supply incorporating circulating immune cells. Another hollow fiber recapitulates the choroid plexus. The neuronal and the CSF compartment are separated by an ependymal cell layer. Hence the model reproduces all critical barriers influencing CNS homeostasis between blood and brain, brain and CSF, and blood and CSF. Cells are seeded in ECM-matrices for a realistic three-dimensional organization. Laminar flow is produced by a computer-controlled microfluidic system. Microdialysis fibers in each compartment will enable monitoring of metabolites and signalling molecules, on-chip miniature electronic sensors will be able to measure TEER for instance. All parameters will be monitored and controlled by a computer-based system. If successful, this model will provide important insights into the regulation of CNS homeostasis under (patho)physiological conditions, thereby potentially promoting the development of new drugs. However, this system will have to prove user friendliness, cost and time efficacy and transferability.

In conclusion, the last years have brought impressive advancements in the field of *in vitro* models of the BBB. Nevertheless, these models are still no replacements for animal or human studies, but rather represent complementary methods

Table 1 Advantages, limitations and recommended applications of common *in vitro* BBB models

Model	Advantages	Limitations	Recommended application(s)
Isolated brain microvessels	- Easily accessible - Well-established - Structural/functional properties of the BBB	- Labour-intensive - Difficult isolation procedure - Poor viability - No investigation of directional transport and cell migration	Light, electron and fluorescence microscopy of morphologic characteristics
Transwell-systems	- Time- and cost-effective - Common and widely used - Easy to control - Best suited to study BBB - immune cell interactions	- Limited complexity - Rapid loss of BBB characteristics: "phenotypic drift" (improved by glia cell co-culture) - Better suited for short-term culture	Immune cell - BBB interaction, migration, permeability and affinity binding assays, TEER measurement
ECM-based models	- Three dimensional cell organization - Reduced "phenotypic drift" - Investigation of ECM and BBB cell interactions - Suitable for long-term studies	- Expensive - Difficult to set up - Limited control - No implementation of shear stress	ECM and BBB cell interactions, developmental studies on BBB formation
Spheroidal models	- Three dimensional cell organization - Reduced "phenotypic drift" - No scaffold material needed	- Limited control - No implementation of shear-stress - No ECM-BBB cell interactions	Expression studies of transport receptors, junctional proteins, adhesion molecules etc.
Microfluidic systems	- Implementation of shear stress - Three dimensional systems available - Computer-controlled parameters - Structural/functional properties of the BBB	- Expensive - Difficult to set up - Only for skilled users - High cell numbers required - Problematic cell harvesting - No visualization of the intraluminal compartment	Cell migration and adhesion studies, shear stress experiments
In silico models	- Time- and cost-effective - Highly reproducible - Highly quantitative	- Currently limited parameters, and consecutively - Limited complexity	Prediction of drug efficacy and bioavailability

BBB: Blood-brain barrier; ECM: extracellular matrix; TEER: transendothelial resistance.

facilitating the progress in pharmaceutical, biotechnological, basic and translational research. Moreover, a specific model should be carefully chosen according to the specific research question critically influencing the reliability and validity of the results.

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