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Guidelines for Blood-Brain Barrier on-Chip Models for Drug Delivery Testing

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Foreword

This CEN Workshop Agreement (CWA 18315:2025) has been developed in accordance with the CEN-CENELEC Guide 29 “CEN/CENELEC Workshop Agreements – A rapid way to standardization” and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN/WS “BiSCUIT - Guidelines for Blood-Brain Barrier on-Chip Models for Drug Delivery Testing”, the secretariat of which is held by UNI Ente Italiano di Normazione consisting of representatives of interested parties on 2025-11-26, the constitution of which was supported by CEN following the public call for participation made on 2025-06-30. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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Introduction

The increasing demand for predictive, reproducible, and ethically responsible *in vitro* models in biomedical research has led to the development of microphysiological systems such as BBB-on-chip platforms. These models aim to accurately replicate the structure and function of the human BBB. For this reason, a highly selective and dynamic interface, formed by endothelial cells, astrocytes, and pericytes, controls the passage of substances between the bloodstream and the CNS [1]. Traditional methods, like static Transwell assays or 2D monocultures, do not fully capture the BBB's complexity and physiological behavior. In contrast, BBB-on-chip technologies involve microfluidics and biomimetic design to better simulate *in vivo*-like conditions, offering significant potential for neuroscience research, drug development, and toxicity screening [2].

Despite their promise, the current landscape of BBB-on-chip systems remains fragmented. Variations in chip design, cell sources, culture conditions, flow dynamics, and readout methods significantly hinder reproducibility, comparability, and broader adoption, especially in regulatory contexts [3]. To address this issue, a CWA is being developed under CEN, with support from national standardization bodies like UNI (Italy). While not legally binding, a CWA serves as a consensus-based "pre-standard" that promotes coordinated progress in emerging technologies, supporting scientific innovation, regulatory alignment, and ethical compliance.

The development of this CWA is directly influenced by the Horizon Europe-funded *BiSCUIT* project, which aims to commercialize a next-generation, sensor-integrated, 1:1 scale BBB-on-chip model. The main goal is to establish harmonized, scientifically rigorous guidelines for designing, fabricating, validating, and applying BBB-on-chip systems, focusing on five key areas: cell sources and culture conditions, drug permeability testing, microfluidic parameters, reference compound validation, data reporting and quality control.

By offering a unified framework, the CWA aims to bridge the gap between academic research and industrial application, ensuring interoperability across platforms and facilitating the integration of BBB-on-chip systems into pharmaceutical pipelines and regulatory evaluations. Harmonization enables more straightforward comparison between different in vitro BBB models, as well as between *in vitro* outcomes and *in vivo* human data, ultimately improving the reliability of experimental interpretations and the predictive power of these systems. This is particularly relevant given the high failure rate of CNS-targeting therapeutics, which is largely attributed to poor penetration across the blood–brain barrier, a major bottleneck in neurotherapeutic development. Establishing validated, human-relevant in vitro BBB systems could significantly enhance early-stage screening, neurotoxicity assessment, and therapeutic optimization.

Furthermore, this initiative strongly supports the ethical obligation to reduce animal testing, following the 3Rs principle and the EU Directive 2010/63/EU. BBB-on-chip models not only decrease the use of animals but also deliver more clinically relevant human data [4]. The CWA will incorporate integration with broader regulatory frameworks, including those for medical devices and ATMPs, thereby improving the translational potential of these technologies. The CWA extends the efforts of the CEN/CENELEC Focus Group on Organ-on-Chip, which has recognized the vital need for consensus terminology, reference materials, standardized operating procedures, and validation strategies [4]. The BBB-on-chip CWA is a clear step toward implementing these goals in a specific and highly impactful use case.

In conclusion, the CEN Workshop Agreement on BBB-on-chip models is a timely and strategic effort that tackles both scientific and regulatory challenges in the organ-on-chip field. The CWA, by encouraging collaboration among researchers and regulators, builds the foundation for interoperable, validated, and ethically sound BBB models that can speed up drug discovery, enhance understanding of neurological diseases, and lessen reliance on animal models. Through consensus and coordination, the CWA will not only promote technological progress but also support a more ethical and efficient future in brain health research.

1 Scope

This CEN Workshop Agreement establishes standardized guidelines for developing and using BBB-on-chip models as a uniform platform for evaluating how drugs pass into the brain. By offering a common framework, the CWA seeks to decrease dependence on animal testing while enhancing the reproducibility, reliability, and comparability of results across different laboratories and research institutions. The document highlights five key areas essential for the successful deployment of BBB-on-chip technologies.

— **Microfluidic design and operation:** Setting parameters such as flow rate, shear stress, and perfusion conditions to maintain physiologically relevant barrier function.

— **Cellular sources and culture strategies:** Providing guidance on the use of primary versus iPSC-derived cells, along with best practices for endothelial co-culture systems, to enhance model robustness and biological relevance.

— **Drug permeability assessment:** Standardizing testing protocols, including thresholds for TEER and the calculation of permeability coefficients, to ensure consistent criteria for evaluating drug transport across the BBB.

— **Model validation:** Recommending the use of reference drugs and benchmarking methods based on established human BBB permeability data, ensuring that models can be compared and validated against known outcomes.

— **Data management and reporting:** Establishing clear guidelines for documenting experimental design, methodology, and results, thereby enhancing transparency, reproducibility, and inter-laboratory comparability.

Through these guidelines, the CWA aims to speed up the adoption of BBB-on-chip models in research, regulatory, and industrial environments, while endorsing the principles of the 3Rs in biomedical research.

2 Normative references

There are no normative references in this document.

3 Terms, definitions and abbreviations

3.1 Terms and definitions

No terms and definitions are listed in this document.

3.2 Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
3Rs	Replacement, Reduction, and Refinement
AQP4	Aquaporin-4
ANG-1	Angiopoietin-1
ATMPs	Advanced Therapy Medicinal Products
BBB	Blood Brain Barrier

BCH	(2-aminobicyclo[2.2.1]heptane-2-carboxylic acid)
BCRP	Breast Cancer Resistance Protein
BMECs	Brain Microvascular Endothelial Cells
CD31	Cluster of Differentiation 31
CNS	Central Nervous System
CO_2	Carbon Dioxide
COC	Cyclic Olefin Copolymer
CWA	CEN Workshop Agreement
DMEM	Dulbecco's Modified Eagle Medium
EAAT	Excitatory Amino Acid Transporter
ECM	Extracellular Matrix
EIS	Electrochemical impedance spectroscopy
F12	Ham's F-12 Nutrient Mixture
FITC	Fluorescein isothiocyanate
GLUT-1	Glucose Transporter-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-Performance Liquid Chromatography
IL-1β	Interleukin-1-beta
iPSC	Induced Pluripotent Stem Cell
ISSCR	International Society for Stem Cells Research
IVIVC	*In vitro–in vivo* correlations
LAT1	Large Neutral Amino acid Transporter
LC–MS/MS	Liquid Chromatography coupled with tandem Mass Spectrometry
L-DOPA	Levodopa
P_{app}	Apparent Permeability Coefficient
PBPK	Physiologically Based Pharmacokinetic
PC	Polycarbonate
PDMS	Polydimethylsilixane
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PEG	Polyethylene Glycol
P-gp	P-glycoprotein
PK	Pharmacokinetic
PMMA	Polymethyl Methacrylate
PS	Polystyrene
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid

TEER	Transendothelial Electrical Resistance
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor Necrosis Factor-alpha
ZO-1	Zonula Occludens-1
γGTP	γ-Glutamyl Transpeptidase

4 Overview of the BBB-on-Chip Context

4.1 Overview of Static and Dynamic Systems

When working with BBB models, one of the first key distinctions comes from the presence or absence of fluid flow, classifying models as either **static** or **dynamic**.

Static systems are typically based on transwell plates or microfluidic chambers where cells are cultured without perfusion. These models are easy to design and operate, making them suitable for preliminary studies, resource-limited settings, or proof-of-concept studies. However, they lack the mechanical stimuli present *in vivo* and may create artifacts in drug testing. Therefore, when using these systems, limitations should be clearly acknowledged, and additional validation steps are recommended.

Dynamic systems, in contrast, incorporate continuous or pulsatile flow to mimic the hemodynamic forces experienced by the BBB *in vivo*. This promotes more physiologically relevant endothelial barrier properties, including improved tight junction expression and overall barrier integrity. Dynamic flow can also enhance nutrient delivery, waste removal, and the distribution of drugs and nanoparticles. Nevertheless, fluid conditions should be properly calibrated to remain within physiological shear stress ranges, as further discussed in clause 4.5.

When selecting between static and dynamic systems, it should be considered the research objective, required physiological relevance, technical complexity, and available infrastructure.

Dynamic models should be used for the following applications:

— **Testing of Nanoparticles, Colloidal Systems, or Unstable Lipophilic Drugs**

  In static systems, nanoparticles tend to precipitate or sediment, leading to their accumulation on the endothelial layer. This can result in overestimation of uptake or transcytosis, and non-physiological interactions due to aggregation. Continuous perfusion prevents accumulation and more accurately reflects *in vivo* exposure conditions.

— **Modeling Physiological Shear Stress**

  Shear stress promotes tight junction formation, correct endothelial polarization, and expression of transporters. These properties cannot be replicated in static systems but are stimulated by dynamic flow mimicking capillary-level forces, which enhances *in vivo*-like responses.

— **Performing Quantitative PK or Comparative Studies**

  Dynamic models provide precise control over exposure time, flow rate, and concentration profiles, which are critical for PK modelling. They allow real-time or repeated sampling from the vascular and brain compartments, enabling calculation of permeability coefficients (P_{app}), clearance rates, and transcytosis kinetics under flow conditions that mimic the *in vivo* environment. These features make dynamic systems particularly suitable for bridging *in vitro* to *in vivo* data or supporting PBPK modelling.

— **Testing Biologics with Rapid Degradation or Binding Kinetics**

For antibodies, peptides, and RNA therapeutics, flow helps maintaining compound stability and mimics *in vivo* pharmacodynamics. In static models, rapid degradation or non-specific binding to cell surfaces or materials may misrepresent true BBB permeability.

4.2 Materials Selection

The selection of material for BBB-on-chip devices is critical for the establishment of relevant and robust models. The ideal material should possess the following properties:

- **Biocompatibility** – to support cell viability, adhesion, and function without inducing toxicity;
- **Sterilizability** – for effective cleaning and sterilization without material degradation;
- **Low ad-/absorption of small molecules** – to ensure accurate drug concentration and permeability measurements. While adsorption is usually seen as a limitation, it can sometimes be useful for studying ECM–molecule interactions **or** drug entrapment that mimics physiological processes. Conducting experiments both with and without cells can help differentiate material-related effects from biological interactions.

Additionally, depending on the application, design, or analytical needs, the following properties can also be considered:

— **Optical transparency** – to enable high-resolution imaging and optical readouts, such as fluorescence microscopy;

— **Gas permeability** – for proper oxygen and CO_2 exchange in closed designs;

— **Elasticity** – to mimic soft tissue mechanics and accommodate microfluidic design needs;

— **Surface modifiability** – for functionalization or coating with ECM proteins when needed.

Despite continuous efforts from the research community, a material possessing all these properties is still not available, so compromises should be accepted between the following:

— **PDMS** is a commonly used material due to its alignment with most of the ideal properties, including biocompatibility, sterilizability, elasticity, and optical transparency. However, it has a high affinity for absorbing and adsorbing small hydrophobic molecules, therefore affecting drug quantification [5].

— **Thermoplastics** like PS, PMMA, PC, and COC offer improved chemical resistance and lower adsorption, and they are particularly suited for mass production as they can be moulded via injection moulding. Nevertheless, they are poorly permeable and rigid, and not adequate for complex designs.

— **Glass** is particularly suited for high-resolution imaging; however, fully glass devices are not commonly used because of their fragility and technical complexity of fabrication. Nevertheless, glass slides and coverslips are widely used as inert and biocompatible bottom support for PDMS devices.

— **Photocurable resins** used for 3D printing allow for rapid and precise fabrication of complex structures. However, their use for biological experiments is still limited due to their opacity and biocompatibility concerns [6]. Their use should be limited to cases where resin formulations and surface treatments have been optimized for biological compatibility.

While none of these materials fulfil all the desired criteria for BBB-on-chip models, specific **experimental needs** can guide optimal material selection. Below are practical recommendations for choosing materials based on the nature of the test or drug:

— **Hydrophobic or Lipophilic Drugs** (e.g., cannabinoids, paclitaxel, doxorubicin)

  Materials with high absorption/adsorption of small hydrophobic molecules should be avoided when working with hydrophobic or lipophilic drugs because they can lead to underestimation of the actual concentration available for BBB transport. In such cases, materials such as COC, PC, or PMMA are the most suitable due to their chemical resistance and low interaction with hydrophobic compounds. If PDMS cannot be avoided, surface treatments like plasma activation, PEG-coating, or working in slightly alkaline conditions can help reduce drug loss. Increasing flow rate and reducing surface roughness are also advisable strategies to mitigate absorption effects [5].

— **Nanoparticles, Micelles, or Liposomes**

  For studies involving nanoparticles, micelles, or liposomes, dynamic systems fabricated with low-affinity materials are strongly recommended to prevent interaction with the device. For instance, COC and PC are preferred over PDMS. Additionally, the compatibility of the material with the nanoparticle formulation, including surfactants or solvents, should be verified in advance.

— **Long-Term Experiments (>48 hours)**

  When the experiment duration exceeds 48 hours, gas permeability becomes a critical parameter. PDMS provides excellent oxygen and CO_2 exchange, making it suitable when maintaining cell viability over extended culture periods is essential. However, if less permeable materials are used, design considerations can be adopted to ensure adequate aeration or perfusion.

— **Real-Time Imaging and Optical Assays**

  Optical transparency is essential for visual inspection and microscopy analysis. PDMS bonded to glass coverslips remains a gold standard for high-resolution optical access, while COC and photocurable resins may also be used if transparent enough.

— **Co-culture Systems**

  When including different cell types, surface properties play a significant role in supporting adhesion and differentiation. Materials should allow for ECM coating and support the formation of complex cell-cell interactions. PDMS remains suitable for this purpose when functionalized with ECM proteins like fibronectin, collagen IV, or laminin.

— **Unstable Compounds (mRNA, peptides, enzymes)**

  When unstable compounds are introduced in the system, materials with chemically inert surfaces such as glass or COC should be prioritized to avoid unwanted degradation or denaturation. PDMS and photocurable resins should only be used if thoroughly validated for compound stability and inertness, including potential leachables or pH shifts they can induce.

In summary, no single material meets all ideal criteria, and trade-offs are often necessary. The material(s) used, any surface modifications applied, and any known interactions or limitations relevant to their experiment should always be explicitly reported. Pre-validating material compatibility with the tested compounds is strongly encouraged, especially when working in regulated environments or with sensitive analytes.

Finally, other components, such as membranes, adhesives, and tubing, should also be non-toxic, chemically inert, and stable under flow conditions. Whenever possible, the use of standardized, sterilizable, and reproducible materials is strongly encouraged to improve inter-laboratory consistency and facilitate future regulatory acceptance.

4.3 Appropriate Cell Types and Co-cultures

The physiological relevance of a BBB-on-chip model heavily depends on its biological components, whose core is represented by BMECs. These cells form the inner lining of brain capillaries, which separate the blood from the brain parenchyma, providing a physical barrier to molecules trying to reach the brain.

When selecting which BMECs to include in the model, it is important to assess their **species origin**. Human cells should be preferred for translational relevance, especially when studying human drug responses, testing antibodies, or other targeted molecules. While animal-derived cells can facilitate early-stage or comparative studies due to easier availability and alignment with *in vivo* animal models, they present significant translational limitations and can yield misleading results due to interspecies differences.

Another aspect that should be considered is whether the cells are immortalized, primary cells, or iPSC-derived, since they possess different properties:

— **Immortalized cell lines** are easy to culture and maintain, making them suitable for method development, high-throughput screening, and preliminary testing. However, they generally exhibit weaker barrier properties due to the immortalization process [7]. Some commonly used immortalized BMECs are:

  - **bEnd.3 (mouse):** they are widely used, robust and easy to maintain, but exhibit very low TEER and limited BBB phenotype. As explained above, their animal origin is not ideal for human studies.

  - **hCMEC/D3 (human):** they express many BBB markers but typically show low Trans endothelial Electrical Resistance.

  - **hBMEC (human):** they express several BBB markers and have proved to be the most suitable and promising cell line for a human *in vitro* BBB model in terms of barrier tightness and paracellular permeability [8].

— **Primary cells** better replicate barrier characteristics, but suffer from limited availability, donor variability, and a short lifespan in culture. Primary BMECs should ideally be used at passage 3 to 8, to preserve phenotype and barrier function. Beyond this threshold, increased variability and decreased tight junction integrity may compromise experimental reliability and inter-laboratory comparability [9].

— **iPSC-derived cells** represent a valid alternative due to easier access compared to primary cells, and improved barrier properties compared to immortalized cells. However, their vascular phenotype and barrier tightness can vary significantly between differentiation protocols [10]. Below are valuable examples that have been validated in the literature:

  - **Lippmann et al. (2012, 2014)** [11], [12] is one of the most widely cited protocols, which produces BMEC-like cells with high TEER (>1,000 $\Omega \cdot cm^2$) and robust tight junction expression. It is recommended for applications requiring strong barrier function and reproducibility, but it can require co-culture with pericytes or astrocytes to stabilize long-term barrier integrity.

  - **Hollmann et al. (2017)** [13]is a modification of Lippmann's protocol to improve scalability and reduce variability across iPSC lines. It is recommended for labs working with multiple iPSC donors or high-throughput applications.

- **Qian et al. (2017)** [14] uses small molecule-driven differentiation, aiming to reduce reliance on complex co-cultures or undefined components. It is recommended for simplified setups or screening models with fewer variables.

Moreover, to better mimic *in vivo* conditions, **co-cultures** of endothelial cells with other cell types found in the brain microenvironment are recommended. Mainly, **glial cells** such as astrocytes and pericytes are crucial for the modulation and maintenance of BBB integrity, promoting tight junction formation, regulating permeability, and supporting metabolic function. Therefore, their inclusion is especially suggested when modelling **physiological barrier function**, evaluating **drug permeability**, or investigating **BBB response to inflammatory or pathological stimuli**.

Depending on the intended application, neurons and microglia can also be included to extend the biological relevance of the model:

— **Neurons** are advisable in studies of neurotoxicity, neuroinflammation, or drug effects on synaptic function.

— **Microglia** should be included when investigating immune responses, neuroinflammation, or the effects of compounds with potential immunomodulatory activity.

In contrast, for high-throughput screening, device optimization, or basic permeability assessments, a monoculture of endothelial cells or a simplified co-culture with astrocytes can be sufficient, depending on the desired level of complexity and physiological accuracy.

All considerations concerning species origin and cell source (primary, immortalized, or iPSC-derived) apply equally to these supporting cell types. In all cases, the inclusion of additional cell types should be justified based on experimental goals and technical feasibility, and their identity and function should be validated where possible.

When using primary, non-commercial cells, all relevant donor information should be reported to ensure transparency and reproducibility. This includes at minimum the donor's age, sex, and health status (e.g., male or female, healthy or diseased), as these factors can significantly influence endothelial phenotype, gene expression, and barrier performance. Similarly, for iPSC-derived BBB models, it is recommended to provide a detailed characterization of the BBB-specific marker expression profile, confirming the acquisition of endothelial and barrier phenotypes. Key markers include tight junction proteins (Claudin-5, Occludin, ZO-1), endothelial transporters (GLUT-1, P-gp, BCRP), and receptor or adhesion molecules (VE-cadherin, PECAM-1). Reporting these parameters facilitates comparability across studies and laboratories, supporting the establishment of standardized criteria for BBB model validation.

In addition to validating cell identity and function, the users of this document should adhere to internationally recognized ethical and technical standards for stem cell research. The *ISSCR Guidelines for Stem Cell Research and Clinical Translation* (2021) highlight transparency, ethical procurement, and thorough documentation of stem cell provenance and quality.

4.4 Culture Conditions and Maintenance Procedures

Culture conditions play a critical role in the development and maintenance of barrier functions, since they directly affect cellular behaviour and function. Key factors include media composition, environmental conditions, seeding protocols, co-culture timing, and flow.

The **composition of culture medium**, including supplements and serum concentration, strongly influences endothelial behaviour, especially for the formation of tight junctions. Depending on the source and origin of cells, it is important to use consistent media composition and check for barrier function before proceeding with experiments (see clause 5). In co-culture systems, the media should support all cell types included in the model, so a compromise or dual-media strategy can be necessary to meet the nutritional and biochemical needs of both endothelial and glial cells. Some examples include:

— A **1:1 mix of endothelial medium and astrocyte medium** to support both BMECs and astrocytes.

— Use of a **common basal medium** (e.g., DMEM/F12 or Neurobasal) supplemented with low serum, **glutamine**, **B27**, and selective growth factors depending on the non-endothelial cell types involved.

Where possible, defined, serum-free, or low serum media should be preferred for reducing variability.

Environmental control is just as important. Temperature of 37°C, relative humidity of 95%, and CO_2 concentration of 5% should be maintained throughout the culture period, because they can heavily impact cell viability and function. Limitations in gas diffusion that can cause alteration of pH media should be considered when using some materials (see clause 4.2), and precautions such as HEPES addition to media should be considered. Additionally, supplementing the medium with HEPES should be considered during non-invasive imaging or measuring sessions that require long time, to limit alterations in cell function.

Seeding protocols and **co-culture timing** are among the most impactful parameters that can cause variability between models. Endothelial cells should be seeded to form a compact monolayer once confluent, and ECM coatings (mainly collagen IV and laminin) can be considered if limitations in cell attachment occur. Supporting cells (e.g., astrocytes or pericytes) should be introduced either simultaneously or sequentially, depending on the model design, and can be encapsulated in an ECM-like matrix to provide a physiological 3D structure. Commonly used matrices include collagen I, Matrigel, and fibrin, which are selected based on desired stiffness, biological activity, and compatibility with cell types [15].

For dynamic systems, the selection of the appropriate **flow rate** is pivotal for the correct development of the endothelial barrier properties (see clause 4.5).

Finally, **maintenance procedures** should include regular monitoring of barrier integrity, control of pH, and potential contamination. Media replacement schedules should be standardized: ideally, media should be replaced every two to three days, as for traditional cell cultures, or more often if visual inspection of phenol-red added media suggests so (e.g., media getting yellowish). Moreover, tubing and reservoirs should be cleaned or replaced according to the device's sterility protocol.

Harmonizing these procedures across labs and platforms is key to improving reproducibility and enabling cross-study comparisons. Detailed documentation of all culture and maintenance steps is strongly recommended.

4.5 Microfluidic Systems and Parameters

The microfluidic architecture and flow parameters of BBB-on-chip models are fundamental to replicate the hemodynamic conditions of the brain microvasculature. Many different designs can be found in the literature, which all have in common the presence of two different compartments: one representing the blood vessel and the other the brain parenchyma. These can be separated via **physical barriers**, such as porous membranes or structural walls within the device, or via **hydrogels**, which allow direct contact between cells [16].

Regardless of the specific design, the integration of **fluid flow** plays an important role in the formation of a physiological BBB, but it should be properly calibrated. Specifically, fluid flow should be selected depending on channel geometry to achieve physiological **shear stress** (6-15 dyn/cm^2 [17]). This is one of the most critical factors for barrier formation, as the tangential force exerted by fluid flow on the endothelial cell surface enhances tight junction formation and overall barrier properties. Flow conditions below or above this range can impair barrier function or lead to non-physiological responses, such as stress-induced permeability or detachment. The flow rate also influences how many molecules interact with the BBB, meaning that higher flow rates reduce the possible interactions and lower flow rates increase them. For this reason, during drug administration, it can be appropriate to adapt flow rates to match physiological **blood velocity** (0,5-2 mm/s [18], [19]), rather than shear stress, in order to better simulate realistic molecule-cell interactions.

Different setups have been adopted to allow media perfusion, ranging from active pumps to gravity-driven systems. The choice depends on multiple parameters:

— **Flow type** – it is possible to provide continuous flow (typically syringe or pressure pumps) or pulsatile flow. The latter better mimics the rhythmic nature of blood circulation, but depending on the application, shear stress fluctuations may not always be desirable. Specifically, pulsatile flow should be avoided in studies focused on quantitative drug permeability, PK, or standardized barrier integrity measurements because it introduces variability or non-linear flow conditions that are harder to model and reproduce.

— **Flow rates** – as previously stated, target flow should be adjusted to generate physiological shear stress. Gravity-driven systems can provide low flows suitable for simple barrier models, but they lack precision, whereas active systems offer greater control and can provide higher flows.

— **Recirculation** – some systems allow for the recirculation of medium, which can reduce product waste (especially in systems that require high flow), maintain soluble factor concentration, and enable intercellular communication. However, it can also increase the risk of contamination and accumulation of cellular waste, so periodic medium refresh and accurate sterilization are highly recommended.

Other operational considerations that should be taken into account when including flow in the system are pressure control, flow stability, and bubble trapping. Air bubbles, fluctuations in pressure, or clogging can disrupt flow and damage cell layers, so the use of bubble traps and sterile pressure control setups is highly recommended.

Unification of microfluidic parameters is essential to enable reproducibility and meaningful comparison between models. Each study should report the applied flow rate, the derived shear stress (with method and assumptions), and a description of the adopted flow system.

4.6 Sensors and Other Monitoring Features

Sensorization of BBB platforms is essential for improving model reliability through real-time monitoring of barrier functions and drug-induced responses. They reduce reliance on endpoint-only assays, which can disrupt the system and have a limited time resolution, not being able to analyse fast events or molecules with short degradation times.

Three types of sensors can be integrated into a BBB-on-chip:

— **Electrical sensors** – they are the most widely used, especially for TEER measurements, which quantify the ionic resistance across the endothelial monolayer. TEER is strongly linked with tight junction integrity, so it provides an easy, non-invasive measurement of barrier integrity (see clause 5). Additionally, microelectrodes integrated into the parenchymal compartment can be used to record neuronal electrical activity.

— **Electrochemical sensors** – they are based on the same principle as electrical sensors, but the working electrode is modified to selectively bind a specific target molecule, thus affecting the measured electrical signal. They can be used to monitor oxygen level, especially when using materials that are poorly permeable to gases (see clause 4.2), or to evaluate molecule fluctuations depending on the application.

— **Optical sensors** – they exploit different features of light to detect different molecules, such as neurotransmitters, calcium, and other cellular signals. Detection can be based on labelling agents, genetically encoded fluorescent signals, or interactions between specific analytes and a transducer.

The choice of sensors should align with the application of the model and desired read-outs, and be compatible with the device's materials, geometry, and flow system. Sensor integration should also

consider sterility, signal stability, and cross-interference (e.g., autofluorescence or electrochemical noise).

Finally, these elements should be tested for their long-term stability within the specific cell culture environment, and interaction with specific drugs or compounds should be validated. Sensor calibration and performance data should be documented and reported alongside biological results.

5 Procedures for Testing the BBB Integrity

5.1 Dextran permeability

5.1.1 General

Evaluating **dextran permeability** continues to be among the most prevalent functional assessments for determining the paracellular integrity of BBB models. This assay directly measures the capability of macromolecules to cross the endothelial monolayer, providing an experimental indication of tight junction competency and overall barrier quality [20]. In both static Transwell systems and dynamic BBB-on-chip platforms, fluorescently labeled dextrans of specific molecular weights are used to quantify transport across the barrier.

Dextrans, typically labeled with **FITC**, are selected based on size to assess how well the barrier restricts molecules with varying hydrodynamic radii. Common variants include **3, 10, 40, and 70 kDa dextrans,** which help evaluate tight junction integrity and identify possible barrier breaches. The movement of these large molecules across the endothelial layer is tracked over time, and the $\boldsymbol{P_{app}}$ is calculated using Fick's law of diffusion, which allows normalization for surface area and concentration gradients, facilitating comparison across models and laboratories. Fick's law is:

$$P_{app} = \frac{\frac{dQ}{dt}}{A \times C_0}$$

where:

dQ/dt = rate of appearance of solute in the receiver (brain-side) compartment (it is usually reported in µmol/s);

A = surface area available for diffusion (it is usually reported in cm^2);

C_0 = initial concentration in the donor (luminal) compartment (it is usually reported in $\mu mol/cm^3$).

This value, expressed in cm/s, reflects the rate of diffusive transport adjusted for surface area and concentration gradient, with lower P_{app} indicating stronger barrier integrity.

When possible, P_{app} should be calculated using the porous area (A_{porous}) of the device, which is the total open pore area effectively available for solute transfer. This approach directly reflects the transport geometry and provides a physiologically meaningful permeability that is independent of the scaffold design. Also, the total device area (A_{total}) can be used. However, in this case, to allow cross-model and inter-laboratory comparison, it is important to also determine and report the apparent permeability of the device without cells (P_{mem}) under the same experimental conditions. This control value accounts for passive diffusion or leakage through the support material, therefore allowing comparisons with other devices tested in different experimental conditions (*e.g.*, with different C_0, dQ/dt, A). To isolate the endothelial contribution to permeability and correct for the device background, we suggest applying the resistance-in-series approach:

$$\frac{1}{P_{\text{total}}} = \frac{1}{P_{\text{mem}}} + \frac{1}{P_{\text{endo}}}$$

from which:

$$P_{\text{endo}} = \frac{1}{\frac{1}{P_{\text{total}}} - \frac{1}{P_{\text{mem}}}}$$

This relation avoids negative or non-physical permeability values and allows the calculation of the intrinsic endothelial permeability (P_{endo}) independent of the device characteristics.

In a typical protocol, a known concentration of FITC-dextran is introduced into the luminal (blood-side) compartment, while the fluorescence intensity in the abluminal (brain-side) compartment is monitored at set intervals [21]. Quantification involves comparing concentration profiles over time [22]. Dextran permeability testing is beneficial because it is **non-toxic,** compatible with live cells, and suitable for both **static and dynamic** (microfluidic) systems. It offers a **quantitative functional endpoint** that can be directly linked to other integrity parameters, such as TEER. To obtain reliable and comparable P_{app} results, we recommend the following experimental and calibration practices.

General preparation

— **Dextran molecular weight**: small (3–10 kDa) probes assess minor pore tightness, while large (40–70 kDa) dextrans reveal gross leakage.

— **Environmental stability**: temperature, pH, and flow rate should remain consistent during testing.

— Use phenol red–free medium during the assay to avoid interference with fluorescence readings.

— Use matched medium for all standards, controls, and samples (same serum/supplement composition) to avoid matrix effects.

5.1.2 Calibration curve

— Prepare a calibration (standard) curve of FITC-dextran using the same medium as the assay. Do not prepare standards in water or a different buffer.

— Cover the expected concentration range of the assay with multiple concentration points (e.g., 6) plus a blank (zero).

— Include at least one low-concentration standard near the lower limit of detection and one at the expected maximum sample concentration.

— Fit the calibration points with linear regression and require a high goodness of fit (e.g., $R^2 \geq 0{,}99$). Report the regression equation and range.

— Rebuild the calibration curve for each experiment or each plate/reader session.

5.1.3 Stability and blank controls

— Test FITC-dextran stability in the assay medium under assay conditions (temperature, light exposure, incubation time). Prepare time-course samples of a known standard incubated in medium and measure fluorescence at intervals matching your assay. If the signal decays, modify assay conditions (e.g., reduce exposure time, protect from light) or use a shorter assay window. As an

alternative, prepare the calibration curve at different time points, corresponding to those of the experimental test, to have similar signal decays.

— Include a blank (medium only) to measure background fluorescence. Subtract blank values from all sample and standard readings.

— Include a device blank (cell-free device) to assess autofluorescence and material background; subtract device blank where appropriate.

5.1.4 Instrument settings and photobleaching

— Keep plate reader or microscope instrument settings constant for all measurements in a given experiment (gain, excitation and emission wavelengths, integration time). Record these settings in the report.

— Minimize photobleaching by reducing exposure and using protective covers; test for photobleaching by repeated reads of the same standard. If photobleaching is significant, reduce read frequency or use lower excitation intensity.

5.1.5 Sampling procedure

— Define sampling time points before the experiment and use the same schedule across replicates. For static systems, typical intervals may be every 10–30 min for short assays or hourly for longer protocols; for dynamic systems, use continuous sensors if available or frequent timed sampling.

— Sampling with replacement (recommended when feasible). When removing aliquots for measurement, record and correct for the removed volume. Replace sampled volume with pre-warmed medium (blank) to maintain volume and concentration balance. Apply cumulative flux calculations that include sample removal corrections. This is the preferred approach because receiver volume remains constant, and straightforward mass accounting is possible.

— Sampling without replacement. In setups where the abluminal volume cannot be replenished after sampling, each measurement permanently removes part of the receiver medium, progressively reducing total volume and potentially altering concentration dynamics. This approach should be used only when technically unavoidable. The cumulative mass transfer should account for decreasing volume at each time point, and the number of samples should be minimized to prevent significant depletion. When possible, distribute measurements across replicate devices (staggered endpoint design) rather than repeated sampling of a single one, to preserve accuracy and avoid dilution or non-linear diffusion artifacts.

— Keep collected samples protected from light and analyze promptly. If storage is necessary, freeze at −20 °C or −80 °C and avoid repeated freeze–thaw cycles; validate stability under storage conditions.

5.1.6 Donor concentration stability

For valid P_{app} estimation, the donor (luminal) concentration should remain effectively constant during the time window used to estimate dQ/dt. As a practical criterion, the cumulative mass transferred during that window should not exceed 5% of the initial donor mass (i.e., $Q_{\text{crossed, total}} / \left(V_{\text{donor}} C_0\right) \leq 0.05$). If this is not achievable, laboratories should either maintain donor concentration *via* perfusion or use a corrected calculation that considers the time-dependent donor concentration (average or instantaneous) for normalization.

5.1.7 Data analysis and quality checks

— Convert fluorescence readings to concentration using the experiment-specific calibration curve after blank subtraction.

— Calculate dQ / dt from the linear portion of the cumulative transport *vs* time plot.

— Provide the device control (cell-free) curve and the medium blank with the reported dataset.

5.1.8 Reporting

— Report the calibration curve equation, R^2, standard points, instrument settings, sampling schedule, replacement volumes, and any sample storage conditions.

— Report donor volume, any perfusion parameters, donor concentration time course, and the method used for P_{app} normalization.

— Include post-assay imaging (e.g., fluorescence or confocal) to confirm monolayer continuity and rule out cell detachment or multilayering over the porous area.

5.2 TEER

5.2.1 General

TEER quantifies the electrical resistance across an endothelial monolayer and serves as a direct indicator of the functional integrity of tight junctions regulating paracellular transport. While permeability assays measure solute flux, TEER reflects ionic permeability, providing a rapid and sensitive, non-destructive measure of barrier tightness.

The principle is based on Ohm's law, where a small alternating voltage is applied across the barrier and the resulting current is measured to calculate resistance:

$$V = I \times R$$

Where:

V = voltage (in volts, V)

I = current (in amperes, A)

R = resistance (in ohms, Ω)

The total resistance includes contributions from the cell monolayer, the support membrane, and the culture medium. To isolate the true endothelial component, background resistance from a blank insert or empty device (without cells) should be subtracted.

5.2.2 Calculation and Area Considerations

To allow consistent comparison between devices and laboratories, it should be clearly defined which surface area is used for normalization and how the background correction is applied.

— The blank (device without cells) should be measured and subtracted to remove the contribution of the membrane and medium:

$$\mathrm{TEER} = \left(R_{\mathrm{cells}} - R_{\mathrm{blank}} \right) \times A_{\mathrm{total}}$$

— When the porous area of the membrane (the actual surface available for molecular exchange) can be determined or estimated, the TEER can also be calculated as:

$$\mathrm{TEER} = R_{\mathrm{cells}} \times A_{\mathrm{porous}}$$

Both approaches are valid, but for comparability across different platforms, the first one, using total area with blank correction, is preferred. This ensures that variations in membrane thickness, or material geometry (in case it is complex) do not artificially affect the reported TEER.

In all cases, the method used (porous *vs*. total area) and the correction applied (with or without blank) should be explicitly reported in publications and interlaboratory studies.

5.2.3 Practical notes

— TEER values obtained with different calculation methods (porous area *vs*. total area with blank subtraction) can differ numerically, but both can be compared if the correction approach is transparent.

— Periodic blank measurements (empty devices) help monitor membrane degradation, protein deposition from medium, or electrode drift over time.

The calculated resistance reflects the tightness of the cell junctions forming the barrier [23].

A high TEER value indicates that tight junctions are well-formed and paracellular permeability is low, reflecting the overall integrity of the barrier. TEER correlates inversely with permeability, meaning that as TEER increases, solute diffusion decreases exponentially. In microfluidic BBB-on-chip systems, electrodes can be integrated into the device as thin-film or interdigitated structures, enabling continuous, automated, and real-time impedance monitoring under dynamic flow. This configuration reduces errors from manual probe placement and allows the detection of transient barrier changes during exposure to drugs or cytokines.

Environmental parameters should be tightly controlled:

— Temperature (37 °C) and CO_2 (5%) should remain stable to avoid fluctuations.

— For experiments requiring prolonged measurements outside the incubator, use HEPES-buffered medium to maintain a stable pH and prevent CO_2-dependent acidification. This is particularly important during repeated TEER monitoring or time-lapse impedance spectroscopy.

5.2.4 Factors influencing TEER

Several biological and environmental factors strongly influence TEER. Fully confluent and mature monolayers exhibit higher resistance, whereas incomplete coverage or cell stress reduces it. Exposure to physiological shear stress enhances tight junction formation and cell polarization, while interactions with astrocytes and pericytes, mediated through soluble factors such as TGF-β or ANG-1, further reinforce barrier tightness [23]. Conversely, inflammatory cytokines (e.g., TNF-α, IL-1β) or oxidative stressors can sharply decrease TEER, mimicking pathological BBB disruption [23]. Experimental conditions such as temperature, medium conductivity, and osmolarity also affect readings, and the presence of air bubbles or electrode misalignment may introduce artifacts.

A stable TEER (±10% variation) over 24-48 h is generally accepted as an indicator of barrier maturity and functional stability. However, to fully characterize BBB performance, TEER data should be supported by additional assays, including **immunofluorescence** for junctional proteins (ZO-1, Claudin-5, Occludin) to confirm structural integrity and **cell viability** assessment using live/dead staining to ensure changes in TEER are not due to cytotoxicity. Together, these approaches link electrical, structural, and functional data for comprehensive barrier evaluation.

5.2.5 Electrochemical impedance spectroscopy

In addition to simple resistance measurements, **EIS** extends the TEER approach by recording impedance over a range of frequencies. This technique separates resistive and capacitive components, distinguishing paracellular junctional resistance from transcellular membrane capacitance. Therefore, such analysis provides a more detailed picture of barrier properties.

5.3 Expression of Specific Biological Markers

While TEER and permeability tests measure barrier performance, they do not verify whether the cells have a true brain endothelial phenotype**.** Therefore, characterizing **specific biological markers** is crucial to show that the model mimics the molecular structure of the in vivo BBB. These markers include **tight junction proteins, transporters, adhesion molecules, and astrocyte-induced factors,** each with a unique role in barrier function.

The major marker categories include [22], [24]:

1. **Tight Junction Proteins**: these define the paracellular seal of the BBB.
    a) **Claudin-5:** principal component determining ion selectivity and low paracellular permeability.
    b) **Occludin:** regulatory protein stabilizing junctional strands and signalling to the cytoskeleton.
    c) **ZO-1:** scaffolding protein linking junctions to actin filaments.

    Continuous, belt-like immunostaining of these proteins along cell borders indicates a mature, intact barrier. Fragmented or punctate localization suggests incomplete junction assembly or disruption due to stress.

2. **Transport and Efflux Proteins:** these mediate the selective exchange of molecules across the BBB.
    a) **GLUT-1:** responsible for glucose uptake, a hallmark of CNS endothelial specialization.
    b) **P-gp** and **BCRP**: ATP-dependent efflux pumps that limit drug penetration.
    c) **EAAT1–3:** glutamate transporters maintaining neurotransmitter balance.

    Functional activity can be confirmed using fluorescent substrates and specific inhibitors (e.g., verapamil for P-gp).

3. **Adherens Junction and ECM Components:**
    a) **VE-cadherin** and **PECAM-1 (CD31):** mediate endothelial cohesion and signalling.
    b) **Laminin, agrin, and collagen IV:** define the basal lamina, facilitating endothelial–astrocyte communication.

4. **Astrocyte-Induced and Neurovascular Markers:**
    a) **γGTP:** upregulated through astrocyte interaction.
    b) **AQP4:** enriched in perivascular astrocyte endfeet, enabling ion and water regulation.

Analytical methods for detecting these markers include:

— **Immunofluorescence microscopy** to visualize localization patterns.

— **Western blotting and qPCR** for quantitative assessment of protein and mRNA levels.

— **Functional transporter assays** using labelled substrates and pharmacological inhibitors.

— **ELISA** for quantitative detection of soluble biomarkers (e.g. cytokines or transport proteins)

Interpretation requires a **multimodal approach** that links structural and functional evidence. For example, high TEER combined with continuous ZO-1 staining confirms the maturity of tight junctions, while reduced P-gp expression despite high TEER could suggest limited efflux capacity. Therefore, combining data from morphology, electrophysiology, and transport assays offers the most reliable validation.

5.4 Selection of BBB Development Stage for Drug Testing

5.4.1 General

The **developmental stage** of the BBB model significantly influences experimental results. Like *in vivo*, BBB formation *in vitro* is a slow process that involves the gradual development of tight junctions, transporter expression, and cellular polarization. Conducting permeability tests too early, before the BBB is fully matured, can result in overestimated permeability and false-positive drug penetration data; on the other hand, testing too late may overlap with decreased cell viability or senescence.

In **iPSC-derived BMEC cultures**, TEER typically increases over the first 48–72 h after seeding and stabilizes around **days 4–6**, coinciding with peak tight junction expression and consistent metabolic activity. In microfluidic platforms, the presence of flow and shear stress may accelerate this process. However, these trends can vary significantly depending on cell seeding density and adhesion quality. Low initial cell density, uneven seeding, or poor substrate coating can delay confluence and tight junction formation, resulting in markedly lower and more variable TEER readings. Ensuring optimal cell attachment and uniform coverage of the membrane surface is therefore essential for reproducible barrier establishment.

The optimal stage for drug testing should be determined based on:

— **Functional maturity:**

- TEER plateau maintained for at least 24-48 h.
- Low permeability to 4 kDa dextran ($<1\times10^{-6}$ cm/s).

— **Molecular confirmation:**

- Stable expression of claudin-5 and ZO-1.
- Consistent transporter levels (GLUT-1, P-gp).

— **Viability and morphology:**

- No detachment, apoptosis, or morphological abnormalities.
- No formation of cell multilayer.

The choice of maturity stage may also depend on the **experimental purpose** [25]:

1. **Early-stage or immature barriers**: useful for studying pathological conditions (e.g., inflammation, hypoxia, and tumours) or for testing macromolecules and nanoparticles that may target compromised BBBs.

2. **Mature, high-resistance barriers**: required for evaluating small molecules intended to cross an intact BBB or for screening neuroprotective compounds.

In co-culture systems, especially tri-cultures with astrocytes and pericytes, synchronization is essential. These systems might need an extra **24–48 h** to reach equilibrium as cross-cell signalling fully establishes the barrier phenotype.

5.4.2 Reporting

Experimental reports should specify:

— Cell type, passage number, and seeding density.

— Time post-seeding at which testing was performed.

— Co-culture configuration and maturation time.

— TEER and permeability values obtained immediately before the assay.

Providing these details supports data comparability across laboratories and facilitates the integration of results in pre-normative standardization efforts.

5.5 Validation using Drugs with Known BBB Permeability In Vivo

5.5.1 General

A validated BBB model should not only exhibit appropriate morphology and function but also replicate the known permeability behaviour of reference drugs *in vivo*. This step provides the ultimate confirmation of the model's predictive accuracy and translational reliability [25], [26].

5.5.2 Selection of Reference Compounds

To capture the full spectrum of transport behaviours, a panel of compounds with established *in vivo* characteristics should be used:

— **Low-permeability markers:** mannitol, sucrose (paracellular probes).

— **High-permeability small lipophilic molecules:** caffeine, propranolol, diazepam (lipophilic and transcellular diffusion).

— **Low-permeability hydrophilic drugs:** atenolol, inulin.

— **Actively transported substrates:** *digoxin* is a substrate of the efflux transporter P-gp, which pumps certain molecules from the brain side back into the bloodstream, limiting their accumulation in the CNS. *L-DOPA*, on the other hand, crosses the BBB through the LAT1, which facilitates the uptake of specific amino acid-like molecules into the brain.

5.5.3 Experimental Validation Procedure

Each reference compound is tested under identical conditions as experimental drugs:

— Determine P_{app} using the same permeability or microfluidic assay setup.

— Verify **active transport activity** using pharmacological inhibitors:

  - When *verapamil*, a known P-gp inhibitor, is introduced, the permeability of *digoxin* should increase. This confirms that the efflux activity of P-gp is operating correctly in the model.

  - Similarly, when BCH, an inhibitor of LAT1, is applied, the uptake of *L-DOPA* should decrease. This reduction demonstrates that the influx observed under control conditions is indeed mediated by the LAT1 transporter.

5.5.4 Operational Considerations and Analytical Determination

Experimental procedures for assessing drug permeability and transporter activity largely follow the same workflow described in the permeability section (see clause 5.1). Sampling frequency, compartment volume management, and data analysis (including cumulative flux or concentration-time–time curve calculations) should be consistent to ensure comparability across compounds. The same principles apply regarding sampling with or without replacement and corrections for volume removal.

For **quantification of drug passage across the BBB**, several analytical techniques can be employed depending on the physicochemical properties of the compound. Further details on this topic are addressed in clause 6.3.

To ensure reliable comparison between different compounds or devices, concentrations in the receiver compartment should always be reported as absolute values (µM or ng/mL) and converted to flux or P_{app} values according to the formulas provided in clause 5.1.

For transporter validation experiments, inhibitor and control conditions should be run in parallel and analysed using the same quantification method. The ratio of permeability (or apparent flux) in the presence *versus* absence of inhibitor provides a direct measure of transporter involvement.

Finally, all quantification methods should be validated for linearity, limit of detection, and limit of quantification. Consistency in calibration, sampling intervals, and data normalization is important to ensure that observed differences reflect true biological transport phenomena rather than analytical variability.

6 Guidelines for Drug/Compound Testing

6.1 Drug/Compound Properties and Handling Procedures

Once BBB integrity has been verified, compounds of interest may be tested in the device to assess their ability to cross the barrier and to analyse their effect on the system. However, prior to any experimentation, proper characterization and handling of the tested compounds should be ensured. The following parameters should be evaluated and reported:

— **Molecular weight and size** – small lipophilic molecules (<500 Da) typically cross the BBB by passive diffusion, while larger molecules (e.g. antibodies, peptides) may require active transport or exhibit limited penetration [27]. Reporting molecular size is critical for comparison across models.

— **Lipophilicity** – the rate at which a solute enters the CNS is strongly correlated to its lipid solubility: small lipophilic molecules can readily diffuse across the endothelium, whereas more hydrophilic compounds generally require transport mechanisms [27]. However, lipophilicity also increases the interactions with some device materials (see clause 6.2).

— **Solubility** – poorly soluble drugs risk precipitation, particularly in static cultures, which can lead to non-uniform exposure and overestimation of permeability. Solubility enhancers such as cyclodextrins or surfactants can be considered but should not compromise cell viability or barrier properties.

— **Chemical stability** – some compounds may degrade in physiological conditions, introducing bias in permeability measurements. Therefore, stability should always be verified under experimental conditions, including exposure to medium, pH, and device surfaces.

To minimize variability, increase reproducibility, and allow comparisons between models, the following handling procedures should be adopted:

— Recording detailed information regarding compound source, lot number, preparation method, solvent type, concentration ranges, and storage conditions.

— Preparing stock solutions in conditions that maintain compound stability (e.g., minimizing freeze–thaw cycles).

— Filtering or sterilizing compounds prior to introduction into the BBB-on-chip system to prevent contamination.

— For compounds prone to absorption or adsorption on device surfaces, performing pre-validation tests in cell-free devices to identify and mitigate losses due to non-specific binding.

— Regularly verifying compound concentration and homogeneity, and periodically test for degradation products, especially for compounds with known instability.

This approach may support harmonized data generation, reliability, and cross-platform comparability in BBB-on-chip drug testing.

6.2 Experimental Setup and Controls

Establishing a robust and reproducible experimental setup is essential for generating meaningful and comparable results when testing drugs on BBB-on-chip models. Careful attention should be given to device configuration, operational parameters, and the implementation of suitable controls.

All **parameters** regarding device design, composition (e.g., cells, ECM), and flow conditions should be documented and justified according to the study objectives (see clause 4) and the compound properties (see clause 6.1). Flow rates and shear stress should be adjusted to maintain physiological relevance, ensuring proper endothelial morphology and tight-junction formation [28].

Exposure conditions such as drug concentration and exposure time should be selected in accordance with both compound stability and physiological exposure levels: the experiment duration should be sufficient to allow for transcytosis, but not exceeding physiological exposure time, to avoid artificial accumulation that does not reflect the human bioavailability.

To distinguish biological effects from system-related artifacts, suitable **controls** should be implemented:

— **Positive and negative reference compounds** should be used to evaluate device performance and sensitivity. Some examples are caffeine or diazepam as high-permeability controls and sucrose, inulin, or mannitol as low-permeability controls (see clause 5.5). Reference compounds should be tested periodically to monitor inter-batch consistency and model stability over time.

— **System-related controls** in cell-free devices may help evaluate compound stability within the system, interactions with device material, and baseline barrier properties. When possible, passive diffusion assays using tracers of known molecular weight can serve as benchmarks for verifying the mechanical integrity of the membrane or channel system.

— **Replicates**, both biological and technical, should be included to assess intra- and inter-experimental variability. Statistical analysis should be applied to account for experimental variability (see clause 6.4). A minimum of three biological replicates per condition is generally recommended for statistical robustness, though this may vary with device throughput.

Environmental factors (e.g., temperature, CO_2 level, pH stability, and medium renewal) should be continuously monitored or controlled to avoid experimental drift during long-term assays.

Barrier integrity should be confirmed both before and after exposure (e.g., TEER or tracer assays), as some compounds may disrupt tight junctions or reduce cell viability. Intermediate integrity measurements during exposure can provide kinetic insight into transient barrier effects, particularly for compounds suspected of modulating tight-junction dynamics.

A practical workflow to integrate these principles should include the following steps:

1. Assess baseline barrier integrity (e.g., TEER, tracer assay).
2. Introduce reference compounds (high- and low-permeability) under the same conditions intended for the test drug.
3. Apply the compound of interest.
4. Monitor barrier integrity during and after exposure, since some compounds may disrupt tight junctions or reduce cell viability.
5. Collect samples systematically, accounting for the removed volume in cumulative drug quantification.
6. Limit experiment duration to the physiological exposure window to avoid non-representative accumulation.
7. Perform post-experiment validation in a cell-free setup to confirm compound stability and exclude device-related losses.

In addition, the experimental plan should specify data recording frequency, equipment calibration procedures, and acceptance criteria for key readouts (e.g., TEER values, cell viability thresholds, permeability ratios). Establishing these parameters a priori enhances reproducibility and facilitates cross-laboratory comparison.

6.3 Drug Detection Methods and Procedures

Selecting the appropriate detection technique is crucial for accurately quantifying drug permeability and distribution in a BBB-on-chip system. Different aspects should be considered when choosing an analytical approach [29], including:

— **Physicochemical properties of the compound** (e.g., molecular weight, polarity, stability, fluorescence) – these characteristics determine the suitability of different detection techniques (e.g., LC–MS for small polar compounds, fluorescence or radiolabelling for large biomolecules) and influence sample preparation steps.

— **Expected concentrations on the donor and receiver sides** – analytical methods should provide sufficient sensitivity to detect small concentration differences across the endothelial barrier, especially when drug transport occurs at low rates or when compound recovery from the receiver side is minimal.

— **Labelling feasibility (fluorescent or radioactive)** – labelling can facilitate tracking and quantification, but it should not alter the compound's properties or interaction with the BBB.

— **Sample volume requirements** – BBB-on-chip devices often yield limited sample volumes, so low-volume compatible methods, such as LC–MS/MS or microplate fluorescence assays, are preferred.

— **Compatibility with the device materials and cell-culture matrix** – interactions between the compound and materials (e.g., PDMS absorption) or medium components (e.g., proteins, serum) can affect recovery and detection. Method validation should include controls to account for potential adsorption, degradation, or signal interference (see clause 6.2).

Some examples of detection methods and their typical applications are provided below:

— **Label-free methods,** such as **LC–MS/MS**, are highly specific and suitable for both small molecules and biologics. They allow for multiplex analysis and are considered the gold standard for quantitative permeability studies. Calibration curves should be prepared in the same matrix as the samples to minimize matrix effects.

— **Fluorescent-based detection** can be used for compounds that are naturally fluorescent (e.g., fluorescein, rhodamine) or can be conjugated to fluorophores without altering transport properties. These assays are well-suited for real-time or high-throughput screening but require prior validation to exclude dye interference or altered compound behaviour. Calibration curves should be established in the same medium used for experiments to account for potential matrix effects, and background fluorescence from blank samples should always be subtracted.

— **UV–Vis spectrophotometry** may be applied for compounds with well-defined absorbance peaks, though its sensitivity is limited for low concentrations typically encountered in BBB assays.

— **HPLC**, optionally coupled with UV or fluorescence detection, provides accurate quantification for small molecules and allows separation of metabolites or degradation products

— **Radiolabelling** remains a sensitive and quantitative method for permeability studies, particularly for low-concentration analytes. However, it requires specialized facilities, strict safety protocols, and controls for isotope stability and adsorption.

— **Immunoassays**, such as ELISA, can be applied for biologics like antibodies, peptides, or cytokines. They offer high sensitivity and selectivity but should be validated for cross-reactivity and matrix effects.

— **Integrated biosensors** (see clause 4.6) may enable continuous measurement of compound concentration, barrier integrity, or cellular responses with minimal sample handling [30]. While these techniques are still undergoing validation, their use should be reported in detail and compared with established analytical workflows to ensure accurate interpretation and cross-study compatibility.

Finally, all analytical steps, calibration standards, validation data should be documented to ensure the reproducibility of permeability and transport measurements in BBB-on-chip systems.

Together, these considerations guide the selection and validation of sensitive, accurate, and reproducible analytical workflows for assessing the transport and metabolic behaviour of compounds in BBB-on-chip systems.

6.4 Data Acquisition and Interpretation

Accurate data acquisition and transparent interpretation are essential to derive meaningful conclusions from drug testing in BBB-on-chip systems. The reliability of the results depends not only on the detection method but also on how samples are collected, processed, and normalized.

During the experiment, **systematic sampling** from both the blood (donor) and brain (receiver) compartments should be performed at predefined intervals to capture the transport kinetics of the compound [31]. Sampling frequency and collected volume should be adjusted based on the expected rate of drug transport, compound stability, and available medium volume within the microfluidic system. It is

crucial to record the exact time and volume of each withdrawal, and to account for the removed volume when calculating cumulative transport.

To ensure accuracy, **data correction** for drug degradation, adsorption, or evaporation should be performed where applicable. In this context, the use of appropriate controls is crucial to help distinguish true biological transport from compound loss, instability, or technical variability within the system (see clause 6.2).

Quantitative evaluation typically involves calculating the apparent permeability coefficient (P_{app}) or related transport metrics (e.g., cumulative transport). For meaningful comparison between models or platforms, these values should be **normalized** to the device's membrane porous surface area and experimental conditions such as drug concentration, exposure time, and cell density. Moreover, results should include measures of variability (e.g., standard deviation or confidence intervals) derived from biological and technical replicates.

All raw data, calculation methods, and result transformations should be documented in detail. For **data interpretation**, results should be discussed in relation to physiological BBB characteristics and known pharmacological properties: high permeability should correlate with small, lipophilic compounds or known CNS-active drugs, while low permeability may indicate size restrictions, hydrophilicity, or active efflux. Deviations from expected profiles may reveal model-specific limitations or biological insights, such as transporter overexpression or tight-junction heterogeneity.

Finally, transparent reporting of sampling strategy, detection parameters, normalization methods, and calculation formulas should be ensured to support reproducibility and facilitate cross-laboratory comparisons. By following these guidelines for data acquisition and interpretation, BBB-on-chip experiments may produce harmonized, reliable, and interpretable results, supporting both scientific and regulatory progress.

6.5 Comparison Between Results from Different Models

Inter-model comparison is essential to evaluate the predictive reliability and generalizability of BBB-on-chip systems. Results should be interpreted in the context of other in vitro, in silico, and ex vivo models to identify methodological biases and highlight model-specific strengths.

When comparing permeability or transport data across BBB-on-chip systems, it should be ensured that:

— **Experimental parameters** such as compound concentration, exposure duration, flow rate, temperature, and cell types are reported in detail and aligned as closely as possible between models.

— **Quantitative outputs** (e.g., P_{app}; clearance rates; efflux ratios) are normalized to the **membrane surface area, flow conditions, and exposure concentrations** to allow direct comparison.

— **Barrier integrity thresholds** (e.g., TEER values, tracer permeability) are considered, as differences in tight-junction tightness or co-culture complexity may strongly influence transport readouts.

— **Material composition and surface chemistry** of the devices are taken into account, since drug adsorption or binding may lead to under- or overestimation of permeability values.

— **Species origin and differentiation stage** of the employed cells (e.g., human iPSC-derived vs. primary animal endothelial cells) should be clearly specified, as these factors directly affect transporter expression and metabolic activity.

Whenever possible, data should be benchmarked against standardized reference compounds or datasets (see clause 6.2). Correlation analysis, such as plotting P_{app} values obtained from different models against each other, can help evaluate consistency, bias, and dynamic range.

To facilitate harmonization, it is recommended that all BBB-on-chip results are reported following a **minimum information standard**, including device geometry, shear stress, cell source, barrier integrity metrics, and detection method. Such transparency enables meta-analyses, inter-laboratory comparison, and long-term model validation.

Ultimately, model-to-model comparison should emphasize complementarity rather than competition: the goal is to determine which model best replicates specific aspects of BBB physiology, such as passive diffusion, transporter-mediated efflux, immune cell interaction, or metabolic coupling, and to clearly document each model's limitations and strengths. When comparing models, it may be helpful to consider details such as cell composition (endothelial only versus with pericytes/astrocytes/neurons/microglia), mechanical environment (static versus defined shear or pulsatile flow), substrate or ECM, cell source (primary, iPSC-derived, or immortalized), and assay conditions (media, temperature, sampling method, normalization to surface area) [32]. Direct comparisons between static and dynamic systems are possible, but it is suggested only when readouts are harmonized using the same permeability markers, standardized TEER measurements and units, consistent sampling and normalization procedures, and matched positive and negative controls (clause 5.1, 5.2) [33].

Specifically, the formulas are consistent across static and dynamic conditions; however, interpreting the resulting values should account for the different transport regimes described in clause 4.5. As summarized in clause 4.1, physiological shear stress tends to increase tight-junction organization and endothelial polarization, meaning that paracellular transport is usually reduced under flow, and static systems may overestimate passage through intercellular pores. Conversely, dynamic conditions often enhance receptor recruitment, transporter localization, and ligand accessibility at the luminal surface (see clause 5.5) [34], [35]. For this reason, a targeted compound or actively transported molecule can show higher transcellular or receptor-mediated transport in dynamic systems, even when paracellular permeability decreases. In other words, static assays tend to overrepresent paracellular diffusion, whereas flow-based assays are better suited to reveal transcellular or receptor-/transporter-mediated components for ligands designed to use these pathways, although the dominant route remains highly compound- and model-dependent.

6.6 Comparison with *in vivo* Data

Comparison with *in vivo* data is critical to assess the physiological and translational relevance of BBB-on-chip models. This step bridges preclinical *in vitro* findings with *in vivo* pharmacokinetic and pharmacodynamic observations, helping determine the model's predictive power for human brain exposure.

When performing IVIVC, the following aspects should be considered:

— **Parameter alignment:** *in vitro* transport metrics such as P_{app} or permeability-surface area product should be converted to units comparable with *in vivo* parameters (e.g., brain uptake rate, $K_{p,brain}$). Scaling factors based on surface area, flow, and volume can support this extrapolation.

— **Physiological scaling:** differences in shear stress, protein binding, and transporter expression between the in vitro and in vivo environments must be accounted for. Quantitative proteomics data can aid in normalizing transporter abundance for PBPK modelling.

— **Reference compound validation:** using drugs with well-documented *in vivo* BBB permeability (e.g., caffeine, diazepam, atenolol, sucrose) allows calibration of BBB-on-chip outputs against established benchmarks.

— **Pharmacokinetic context:** whenever possible, *in vitro* permeability data should be interpreted in relation to plasma concentration–time profiles, unbound drug fraction, and systemic clearance.

— **Species-specific differences:** caution should be applied when comparing in vitro models based on human-derived cells with in vivo data from animal models. Differences in transporter profiles and metabolism can significantly alter drug disposition.

Establishing quantitative IVIVC models may enable **translation of BBB-on-chip results into human-relevant predictions**, improving drug selection and reducing reliance on animal experiments. Integration with computational modelling (e.g., PBPK or QSAR approaches) can further enhance predictive capacity.

Finally, both **concordant and discordant findings** should be reported, as deviations from in vivo outcomes can reveal model limitations or uncover previously unrecognized biological mechanisms relevant to BBB transport and drug disposition.

Annex A
(informative)

Conclusion and outlook

This document provides a standardized framework for developing, validating, and applying BBB-on-chip models, supporting their integration into drug discovery, safety testing, and preclinical research. Especially, it has been provided guidance on microfluidic design, material selection, cell sourcing, barrier integrity evaluation, drug testing procedures, and analytical workflows, with the aim of decreasing variability across platforms and improving comparison between studies.

The document highlights the importance of transparent reporting, standardized reference compounds, and rigorous quality control as important steps toward wider regulatory acceptance. BBB-on-chip technologies can reduce animal testing, enhance human relevance, and speed up the development of CNS-targeting therapies. Their combination with computational tools, such as PBPK modelling and quantitative proteomics, further boosts their translational potential.

Looking ahead, implementing this CWA across research labs, industry, and regulatory bodies will gradually align methods and help establish criteria for future European Standards (EN), ultimately boosting reproducibility, interoperability, and innovation in the organ-on-chip field.

While this CWA offers basic guidance for BBB-on-chip systems, several limitations should be recognized.

— **Technological diversity:** BBB-on-chip platforms differ significantly in geometry, fabrication, biological complexity, and sensor integration. The document cannot address every design variation, and specific implementations may need customized procedures beyond this document's scope.

— **Evolving field:** Organ-on-chip technologies are advancing quickly. New materials, microfabrication methods, sensing strategies, and cell differentiation protocols may develop after publication, requiring regular updates.

— **No prescriptive performance thresholds:** Although common approaches and metrics are described, the document does not set fixed acceptance criteria for TEER, permeability, or transporter activity, as these are still model-dependent and subject to ongoing scientific debate.

— **Limited clinical correlation data:** The validation of BBB-on-chip results against human in vivo data remains incomplete, and IVIVC frameworks are still developing. As a result, this document cannot ensure accurate predictions for all compounds or uses.

— **Not a regulatory standard:** The document offers guidance but does not replace regulatory requirements. Additional validation, qualification, or certification steps might be necessary depending on the intended use (e.g., GLP studies, regulatory submissions).

Despite these limitations, the CWA marks a significant step toward harmonizing BBB-on-chip research and advancing the development of more robust, comparable, and human-relevant in vitro BBB models.

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