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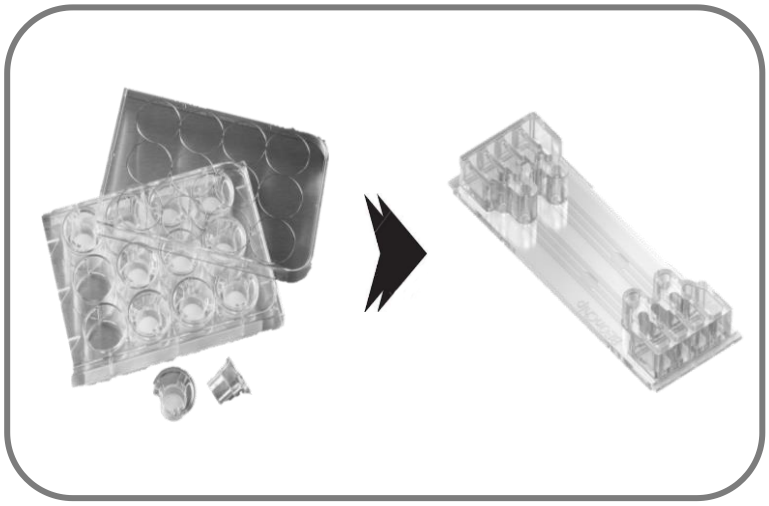
TRABAJO FIN DE MÁSTER

DEVELOPMENT OF IN VITRO BLOOD-BRAIN BARRIER MODELS: FROM TRADITIONAL 2D MODELS TO MICROFLUIDICS



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ABSTRACT

The blood-brain barrier (BBB) is a highly specialised complex structure, and is directly responsible for substances and drugs entering the central nervous system from the blood. Traditional in vitro models have problems to mimic the properties of the barrier in vivo. Therefore, the aim of this project is to work on the development of an in vitro model of the blood-brain barrier based on a microfluidic chip device. By studying the transition from a traditional model using transwell inserts to a three-dimensional model using a microfluidic chip device, which has features that better mimic the barrier in vivo. Progress was made in the development of the two-dimensional model in the inserts and the first steps for implementation in the three-dimensional model were taken.

The two-dimensional model on transwell inserts was being developed by a researcher of the research group, so in collaboration with her, integrity, permeability and immunostaining assays were performed on this model.

RESUMEN

La barrera hematoencefálica es una estructura altamente especializada y compleja, y es responsable directa de la entrada de sustancias y medicamentos en el sistema nervioso central desde la sangre. Los modelos in vitro tradicionales tienen problemas para imitar las propiedades de la barrera in vivo. Por lo tanto, el objetivo de este proyecto es trabajar en el desarrollo de un modelo in vitro de la barrera hematoencefálica basado en un dispositivo de microfluídica. Estudiando la transición desde un modelo tradicional que utiliza insertos de transwell a un modelo tridimensional que utiliza un dispositivo de microfluídica, el cual tiene características que imitan mejor la barrera in vivo. Se progresó en el desarrollo del modelo bidimensional en los insertos y se dieron los primeros pasos para la implementación del modelo tridimensional.

El modelo bidimensional en los insertos de transwell estaba siendo desarrollado por una investigadora del grupo de investigación, por lo que se colaboró con ella para realizar ensayos de integridad, permeabilidad e inmunotinción en este modelo.

LABURPENA

Hesi hematoentzefalikoa oso egitura espezializatua eta konplexua da, eta zuzenean arduratzen da odoletik nerbio-sistema zentralean substantziak eta botikak sartzeaz. In vitro eredu tradizionalek arazoak dituzte in vivo hesiaren propietateak imitatzeko. Beraz, mikrofluidika-gailu batean oinarritutako hesi hematoentzefalikoaren in vitro eredua garatzeko lan egitea da proiektu honen helburua. Transwell-en putzuak erabiltzen dituen eredu tradizional batetik hiru dimentsioko eredu baterako trantsizioa aztertzen ari da, azken hau hesiaren ezaugarriak hobeki imitatzen dituen mikrofluidika-gailu bat erabiltzen du. Bi dimentsioko ereduaren garapenean aurrera egin zen eta hiru dimentsioko eredua ezartzeko lehen urratsak eman ziren.

Transwell putzuen bi dimentsioko eredua ikerketa-taldeko ikertzaile batek garatzen are zuenez, eredu horretan integritate-, iragazkortasun- eta immunotindaketa-saiakuntzak egiten kolaboratu zen.

KEY WORDS

Blood-Brain Barrier, In vitro BBB model, BBB on chip devices, microfluidic chip devices.

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LIST OF ABBREVIATIONS

Blood-Brain Barrier	BBB
Tight junctions	TJ
Zonula Occluden	ZO
Phosphate Buffered Saline	PBS
Glucono-Delta-Lactone	GDL
Dulbecco's Modified Eagle's Medium	DMEM
Fetal Bovine Serum	FBS
Trans-Endothelial Electrical Resistance	TEER
Zonula Occludens 1	ZO1
Bovine Serum Albumin	BSA

PROJECT CONTEXT

The following document is the memory of the master's thesis entitled "Development of in vitro blood-brain barrier models: from traditional 2D models to microfluidics" from the master's degree in Advanced Materials Engineering. This has been carried out in the Group in Science and Engineering of Polymeric Biomaterials, ZIBIO group, of the Department of Mining-Metallurgy Engineering and Materials Science of the University of the Basque Country (UPV/EHU).

This work is part of a European project that proposes a new strategy based on nanoencapsulated biomacromolecules with potential therapeutic application for the treatment of glioblastoma. It is based on polymeric nanogels to transport and protect a monoclonal antibody through the blood-brain barrier (BBB), making it effective against the tumour. This European project consists of two main parts: i) the development of polymeric nanovehicles that transport monoclonal antibodies, and ii) the development of an in vitro model based on microfluidics of the blood-brain barrier.

The present master´s thesis focuses on the second objective of developing the blood-brain barrier in vitro model, by studying the transition from a traditional transwell (2D) cell culture system to a three-dimensional system based on microfluidic chip devices, to better simulate the characteristics and microenvironment of biological blood-brain barrier. For this, the barrier was first modelled on the transwell inserts and tested for integrity and permeability, using transendothelial resistance tests, dextran permeability essays and immunostaining of binding proteins. Then, the first steps have been taken in order to recreate this model on microfluidic chips, performing cell cultures on them and combining cell cultures with hydrogel to achieve barrier structuring.

This is crucial to allow the evaluation of the nanovehicles and to analyse the effectiveness of the treatment developed in the other phase of the European project.

OBJECTIVES AND SCOPE

The main objective of the work is to study the development of an in vitro model of the blood-brain barrier by studying the transition from a 2D traditional model to a 3D model, using a microfluidic chip device. In order to achieve it, this has been divided into several sub-objectives which were fulfilled throughout the work. These sub-objectives are the following:

A. Culture a 2D model of the BBB using transwell inserts.
B. Perform integrity and permeability tests on the 2D model.
C. Grow cells on the chip device.
D. Create a structured model with hydrogel and cells on the chip device.
E. Immunostain of the 2D model tight junctions.
F. Create a co-culture model of cells using hydrogel on the chip device.

As outlined in this paper, it has been achieved to complete all sub-objectives up to and including E., which is currently under development. Objective F. remains outstanding. As explained in the Project Context section above, this research is part of a larger European project with a longer timeframe. Therefore, the objective of creating an in vitro model of the blood-brain barrier, both the 2D and 3D model, will be further developed.

BENEFITS

The development of an in vitro model of the blood-brain barrier based on a microfluidic chip device has several benefits. The BBB plays a crucial role in protecting the central nervous system from harmful substances in the blood, including drugs. By creating a model that better mimics the properties of the barrier in vivo, researchers can better predict how drugs and other substances will interact with the BBB, and thus improve drug delivery and therapies.

By studying the transition from a traditional two-dimensional model using transwell inserts to a three-dimensional model using a microfluidic chip device, researchers can identify the benefits of this technology and compare them with the technology used to date. Thus, improving the development of in vitro models of the BBB.

In addition, working with this type of technology will provide knowledge and experience to the research group on its use. This will be useful for future development of other models, whether or not they are BBB models.

1. INTRODUCTION

1.1 Blood Brain Barrier

Background

The blood-brain barrier (BBB) was first theorised in 1898, when A. Biedl and R.Kraus performed an experiment where they injected bile salts into the bloodstream of animals and they failed to penetrate the brain. But it was not until 2 years later that the term blood-brain barrier was coined by Max Lewandowsky. Meanwhile, P. Ehrlic was working on staining procedures in which chemical dyes are used to make microscopic biological structures visible, and when he injected the dyes into animals he saw that all organs except the brain were stained. This event was not attributed to the blood-brain barrier at first. But in 1918 when E. Goldmann, one of his students, performed the same experiment but this time injecting the dyes directly into the cerebrospinal fluid, it was observed that the other organs were not stained. It was at this moment that the evidence of a barrier between the central nervous system and the bloodstream was demonstrated [Fig 1]. At the time, this barrier was attributed to the blood vessels, as no specific membrane could be identified. [1]

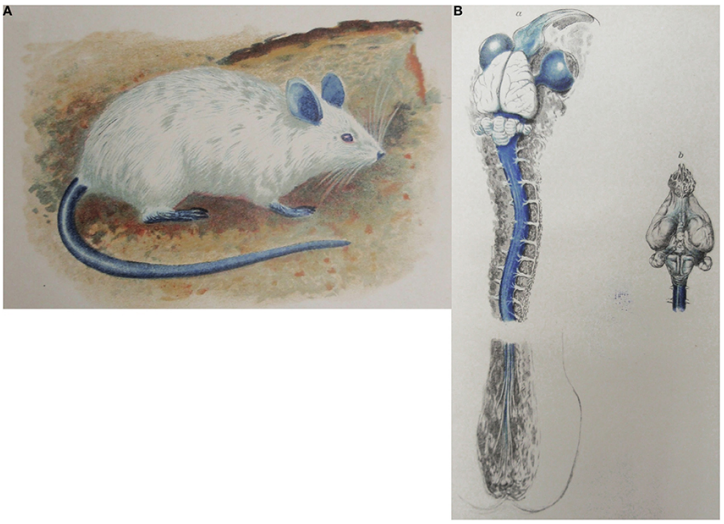


Figure 1: illustrations of Goldman's experiments injecting trypan blue. Left, Adult rat after systemic injection of staining solution. Right, Brain and spinal cord of adult rat after lumbar injections of staining, the spinal cord was stained while the brain was not

The BBB is the largest interface for the exchange of compounds between the blood and the brain, composed of endothelial cells coating the capillaries that penetrate into the brain and spinal cord. Its main function is to protect brain tissue from harmful agents in the blood while allowing the passage of substances necessary for the metabolic activity of the brain. Therefore, it plays a fundamental role in maintaining the homeostasis of the brain microenvironment. For this reason, it is a major obstacle to the entry of drugs and exogenous substances into the central nervous system. [2]

These characteristics are a result of the interaction between the different cell types of the neurovascular unit [Fig 2], mainly endothelial cells, astrocytes and pericytes.

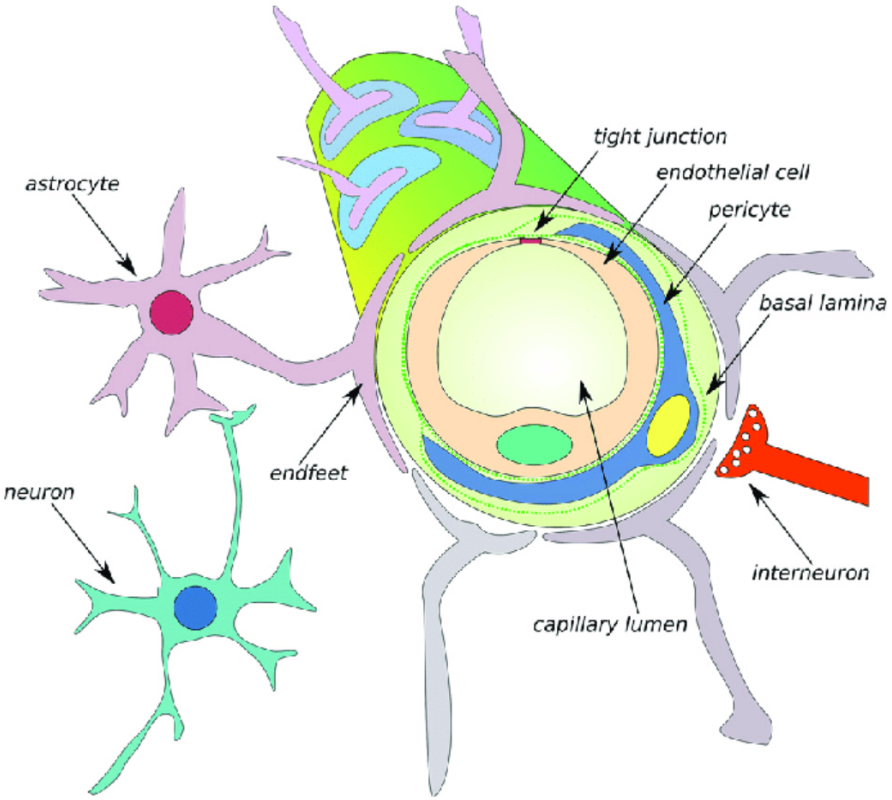


Figure 2: Neurovascular unit cells structure

Main BBB cells

Endothelial cells

BBB endothelial cells have different characteristics from other endothelial cells in the body, being phenotypically different in morphology, structure and function. These differences are: the presence of tight junctions (TJ), the absence of pores (fenestrations) and the use of active transport mechanisms to regulate the passage of molecules. They are also characterised by a more flattened appearance and a greater number of mitochondria than other endothelial cells. [3]

The tight junctions formed between the endothelial cells avoid water-soluble polar molecules and hydrophilic molecules from being able to cross the barrier, thus preventing the entry of unregulated molecules from the blood into the brain. These junctions also make the separation of the luminal portion clearer.

Molecules such as glucose, amino acids and other nutrients pass through endothelial cells via carrier-mediated transporters, while the entry of larger molecules such as insulin or leptin occurs via receptor-mediated endocytosis. Regarding small lipophilic molecules (< 400 Da), they can pass through the BBB, and other molecules like oxygen and carbon dioxide pass through by rapid diffusion in order to maintain the metabolism and pH of the brain microenvironment. [2]

Pericytes

Pericytes are cells that surround the endothelial cells of the capillaries [Fig 2]; they are mural cells with contractile capabilities that are positioned on the basement membrane in direct contact with the endothelial cells via synaptic connections. Their contractile capabilities play an important role in regulating capillary blood flow and blood-brain barrier permeability. [4]

They are especially abundant in the central nervous system, and the interaction between pericytes and endothelial cells increases their resistance to apoptosis, thus, being of vital relevance for maintaining the integrity and stability of the blood-brain barrier. Pericytes are pluripotent cells and have the ability to differentiate into endothelial or smooth muscle cells, which also contributes to the integrity of the BBB. [2]

Astrocytes

Astrocytes, also known as astroglia, are star-shaped glial cells found in the brain and spinal cord. Being the most abundant cells in the central nervous system, they have diverse functions and are involved in biochemical and physiological processes. The most relevant of these are: [5]

- ➢ Structural function, they support the neurons and other cells in the brain parenchyma.
- ➢ Maintain the ion balance of the extracellular space. Thus regulating ionic homeostasis.
- ➢ Uptake and processing of neurotransmitters.
- ➢ They function as mediators for signals from neurons to the vasculature.
- ➢ Biochemical control of endothelial cells forming the blood-brain barrier.
- ➢ Supply of nutrients to nerve tissue.

They do not contribute directly to the physical integrity of the barrier, but they do influence the functionality of the endothelial cells which are directly responsible for it. They also play a fundamental role in the maturation of the barrier during development, where it is necessary that the endothelial cells are in direct contact with the astrocytes in the basal lamina. In both cases the processes that occur in the terminal/distal part of the astrocytes that connect to the endothelial cells are critical, these are called endfeet processes. These are also particularly relevant in the control of cerebral blood flow, and in central nervous system repair processes. [2]

Tight Junctions

One of the key components of the BBB are the tight junctions, which are specialised structures that are formed between adjacent cells, sealing off the spaces between them and creating a continuous barrier [Fig 3]. Tight junctions in the BBB consist of transmembrane proteins, such as occludin, claudins and junction adhesion molecules (JAMS), which interact with each other and with the cytoskeleton of the cell to form a tight seal, which prevent the free flow of substances between the bloodstream and the central nervous system. [6]

Zonula occludens proteins, also known as ZO proteins, are a family of cytoplasmic accessory proteins that play a crucial role in the formation and maintenance of tight junctions. There are several different types of ZO proteins, including ZO-1, ZO-2, and ZO-3. These proteins are important for the proper assembly and functioning of tight junctions because they help to link the actin cytoskeleton of the cells with the tight junction complex. In addition to their role in maintaining the integrity of tight junctions, ZO proteins have also been shown to play a role in regulating the permeability of the BBB. This is important because changes in BBB permeability can have significant implications for brain function, including the development of certain neurological disorders. [2]

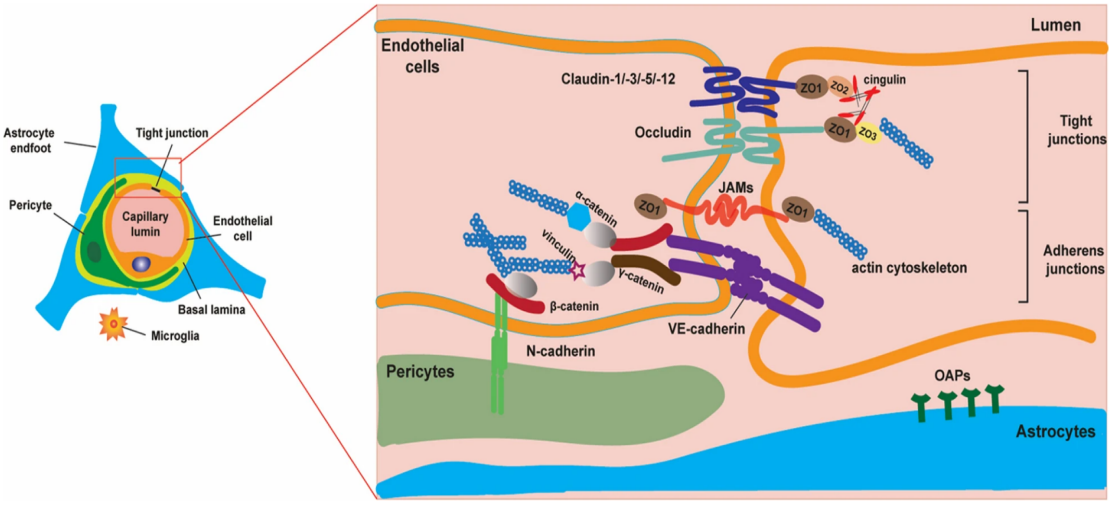


Figure 3: Left, neurovascular unit. Right, close up on the tight junction

The tight junctions of the BBB play several important roles in maintaining the stability and proper function of the central nervous system. Firstly, they prevent the passive diffusion of most ions, small molecules, and macromolecules between the bloodstream and the brain, thereby limiting the entry of harmful substances and pathogens. This helps to protect the delicate neural tissue from damage, and to maintain a stable and controlled environment for neural function.

In addition to preventing the entry of harmful substances, the tight junctions also play a role in actively transporting essential substances into the brain. For example, glucose and amino acids are transported into the brain through specialised transporters, which are expressed by the BBB endothelial cells. This ensures that the brain has access to the essential nutrients it needs to function properly.

It is also worth noting that the tight junctions of the BBB are dynamic, and can be regulated by various factors, including hormones, cytokines, and drugs. For example, certain substances, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), can increase the permeability of the BBB, allowing harmful substances to enter the central nervous system. Similarly, drugs such as ethanol can also alter the tight junctions of the BBB, increasing the permeability of the barrier. [2]

To conclude, tight junctions are a critical component of the blood-brain barrier, playing a crucial role in protecting the brain and regulating the exchange of substances between the bloodstream and the central nervous system.

Role of the shear stresses

Shear stress is an important factor that contributes to the formation and maintenance of the blood-brain barrier (BBB). Shear stress is the force generated by the flow of blood along the walls of the blood vessels. In the brain, this flow creates a shear stress on the endothelial cells that form the BBB. It has been shown that this shear stress specifically affects the formation and integrity of tight junctions, which are key components of the BBB as explained above.[7]

Shear stress has been shown to regulate the expression and localization of tight junction proteins, such as occludin and claudin, by activating signalling pathways

that control the formation and maintenance of tight junctions. This has also been proven by in vitro studies. [7], [8]. In addition, shear stress can also regulate the permeability of the BBB by controlling the activity of transporters and enzymes that regulate the transport of substances between the bloodstream and the central nervous system. For example, shear stress has been shown to modulate the activity of glucose transporters and efflux pumps, which control the transport of glucose into the brain. [2]

In conclusion, shear stress is an important factor that contributes to the formation and maintenance of the BBB, and plays a role in regulating the permeability and stability of the barrier. Understanding the role of shear stress in BBB function is important for developing better in vitro models and strategies to treat diseases that affect the central nervous system.

Physiological functions of the BBB

The blood-brain barrier (BBB) is a complex system that regulates the exchange of substances between the bloodstream and the brain through a combination of permeability control and active transport mechanisms. Therefore, it plays a crucial role in maintaining normal brain function and protecting the central nervous system. Being the most significant physiological functions as follows: [2]

➢ Protection. The BBB helps to protect the brain from harmful substances in the bloodstream, such as toxins, pathogens, and drugs that may have adverse effects on the central nervous system. By preventing the entry of harmful substances into the brain, the BBB helps to maintain a stable environment for normal brain function and to prevent damage to the delicate neural tissue.

➢ Nutrient transport. The BBB regulates the transport of essential nutrients, such as glucose and amino acids, into the brain to support its high metabolic needs. The BBB selectively controls the transport of those nutrients into the brain.

➢ Hormone regulation. The BBB helps to regulate the transport of hormones, such as insulin and growth hormone, into the brain to maintain normal physiological function. The control of the hormones into the brain is necessary for regulating a variety of physiological processes of the brain.

➢ Waste removal. The BBB contributes to remove waste products from the brain, such as metabolic by products and neurotransmitters, to maintain a healthy brain environment. The removal of waste from the brain helps to maintain a stable environment for normal brain function and to prevent damage to neural tissue.

➢ Homeostasis. The BBB maintains homeostasis in the brain by regulating the exchange of substances and a variety of ions to maintain a stable environment for normal brain function. This is accomplished by controlling the transport of substances into and out of the brain through different transport mechanisms.

➢ Drug delivery. The BBB presents a barrier to drug delivery to the brain, which can limit the effectiveness of some treatments for brain disorders. However, this barrier also helps to protect the brain from potentially harmful drugs and to prevent unwanted side effects in the brain. This is mainly due to the tight junctions that forms a seal between the endothelial cells as explained above.

It should be noted that BBB is not a static structure, and it can be dynamically regulated in response to various physiological and pathological conditions. For example, in response to injury, the BBB can temporarily become permeable to allow for the entry of immune cells into the brain. Similarly, in the case of neurodegenerative diseases, the BBB can become leaky, leading to the accumulation of harmful substances in the brain. [9]

Summarising, the BBB has several important physiological functions that are critical for maintaining brain health and normal brain function, and disruptions to its function can have serious consequences for brain health, as explained below.

Transport mechanisms of the BBB

The blood-brain barrier regulates and controls the exchange of substances between the bloodstream and the brain through several transport mechanisms [Fig 4], being the main ones: [10]

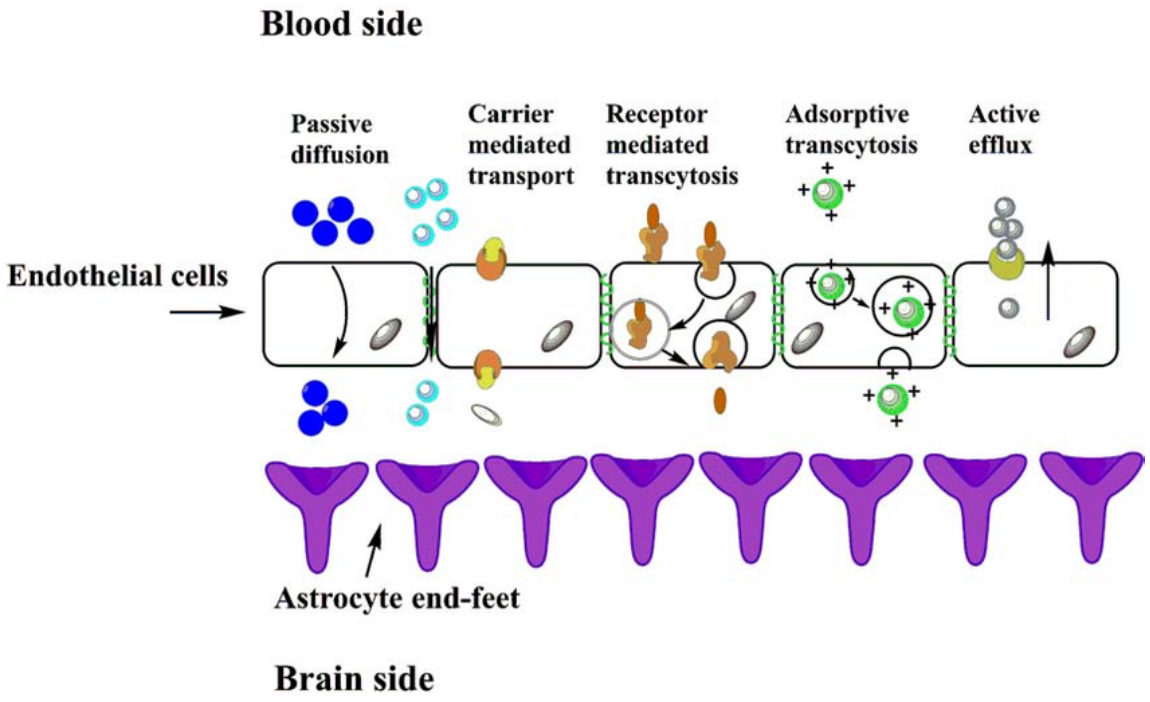


Figure 4: Transport mechanisms of the BBB

➢ Passive Diffusion. Passive diffusion is the simplest and most common mechanism for the transport of small, lipid-soluble substances, such as oxygen, carbon dioxide, and ethanol, through the BBB. These substances can diffuse directly through the lipid bilayer of the endothelial cells forming the BBB.

➢ Carrier Mediated Transport. Carrier mediated transport, also referred to as facilitated diffusion, is a mechanism for the transport of substances, such as glucose and amino acids, into the brain through specific transporters. These transporters are proteins that are embedded in the BBB cells and allow the selective transport of specific substances.

➢ Active Transport. Active transport is a mechanism for the transport of substances, such as amino acids and ions, against a concentration gradient into the brain through specific transporters that use energy from ATP hydrolysis. Sodium-potassium pumps are a great example.

➢ Active efflux. Active efflux transport, on the other hand, refers to the process of actively pumping substances against a concentration gradient. This mechanism is mediated by a family of membrane transporters called ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs) among others, which are expressed on the BBB.This mechanism is especially relevant for toxins, and thus for pharmacological drugs, which makes it a critical component in several pathologies.

➢ Transcytosis. Transcytosis is a mechanism for the transport of substances, such as antibodies and hormones, through the BBB. Transcytosis involves the formation of vesicles in the barrier endothelial cells, which transport the substances from the bloodstream to the brain. In this case, the formation of the vesicles occurs through the process of pinocytosis, which means that the vesicles are formed by invagination of the BBB cell membrane.

In few words, the BBB regulates the transport of substances into the brain through several different mechanisms, and it is the great variety and selectivity of these that allows the complex functions of the BBB to be maintained.

Disruption of the BBB by different diseases

Disruption of the blood-brain barrier can occur in a number of diseases, leading to a variety of negative outcomes for brain health. The BBB is a complex system and its disruption can have serious consequences for the function and survival of neurons.
The following are some examples of the most common diseases that can cause disruption of the BBB: [11] [2]

➢ Brain tumours. Disruption of the BBB is a common feature of brain tumours, allowing harmful substances, commonly tumour cells and toxic molecules, to enter the brain from the bloodstream. This can lead to inflammation, oxidative stress, and damage to neurons.

➢ Multiple sclerosis. Multiple sclerosis is an autoimmune disease that affects the central nervous system, causing inflammation and damage to the myelin sheaths that insulate neurons. Disruption of the BBB is a key feature of multiple sclerosis, allowing harmful immune cells and cytokines to enter the brain, causing further inflammation and damage.

➢ Alzheimer's disease. Alzheimer is a progressive neurodegenerative disease characterised by the accumulation of amyloid plaques and neurofibrillary tangles in the brain. Disruption of the BBB is a common feature of Alzheimer's disease, allowing harmful substances, such as amyloid beta, to enter the brain from the bloodstream, causing oxidative stress and inflammation.

➢ Stroke. Stroke is a sudden loss of blood flow to the brain, leading to cell death and brain damage. Disruption of the BBB is a common feature of stroke, enabling harmful substances to enter the brain causing further damage to neurons.

1.2 In vitro models of BBB

The efficacy of biomacromolecules for the treatment of diseases and lesions of the central nervous system is currently a challenge and a field with great potential. Therefore, new in vitro models need to be developed to test these new drugs and treatments.

Importance of reliable in vitro models of BBB

The development of accurate and reliable in vitro models of the blood-brain barrier (BBB) is of utmost importance for advancing our understanding of the underlying mechanisms of this complex system. In vitro models of the BBB can provide important insights into the molecular, cellular, and physiological mechanisms that regulate BBB function. These models can be used to study the effects of different drugs, disease states, and environmental factors on BBB permeability and function. [9]

These in vitro models can be used in the search for new specific treatments and drugs for diseases affecting the central nervous system. This can help to identify potential therapies and to determine the safety and efficacy of these treatments before they are tested in animal models or human clinical trials.

They also can be applied to study disease-specific mechanisms, to study the mechanisms of BBB disruption in specific diseases, such as brain tumours, stroke, multiple sclerosis, and Alzheimer's disease. This can help to identify new targets for therapy and to develop new strategies for the treatment of these conditions. [13]

From another point of view, one of the main advantages of in vitro models of the BBB is their ability to provide a rapid, cost-effective, and ethical alternative to animal models. In vitro models can offer valuable insights into the molecular, cellular, and physiological mechanisms of BBB regulation, allowing researchers to study the BBB in a controlled environment and to manipulate specific components of the system to better understand their function.

From 2D transwell models to 3D models

Transwell cell cultures and 3D cell cultures are two different methods for growing and studying cells and cell models in vitro. The transwell model is more classical and has been used since the early 1970s, while the 3D models have been developed relatively recently. The main differences between these two methods are the way cells are cultured and the way they are exposed to their environment.

Transwell cell cultures consist of a cell culture insert with a semi-permeable membrane that separates the apical (brain-facing) and basolateral (blood-facing) compartments. The BBB cells are grown on the basolateral side of the membrane and the medium on the basolateral side represents the bloodstream. This model is easy to use and allows for the study of the BBB transport properties and drug permeability. More complex models with various cell lines can also be done. [12]

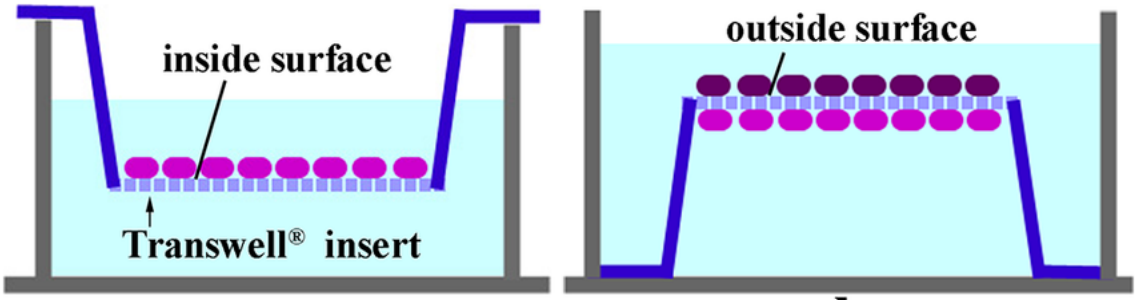


Figure 5: Transwell insert culture configurations

3D cell cultures, on the other hand, are more complex and sophisticated models that are carried out in devices called chips made of different polymers, with the aim to better represent the in vivo BBB. These cultures are typically created using hydrogels, porous membranes, spheroids and organoids to create a sandwich-like structure, and are designed to mimic the 3D structure and functionality of the BBB. 3D cell cultures allow for the study of the BBB cellular and molecular properties, including tight junction formation, cell-cell interactions, and the effect of disease or drug exposure on the BBB. It is the ability to be dynamically modulated by devices using microfluidics that gives them these superior characteristics in terms of reproducibility of the BBB. [13]

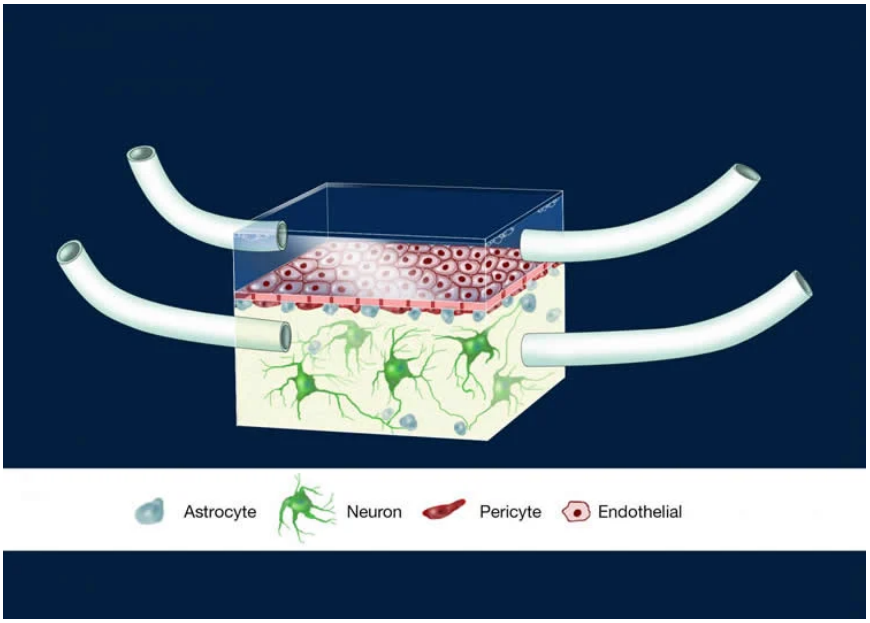


Figure 6: Illustration of a 3D culture of the neurovascular unit

Summarising, transwell cell cultures are simple, convenient and easy to use models that are well suited for the study of BBB transport properties and drug permeability, while 3D cell cultures are more complex and hard to handle models that better represent the in vivo BBB and are well suited for the study of the BBB cellular and molecular properties. More detailed information about both model types and their differences can be found in the publication [13].

In recent years, there has been a growing trend towards the use of chip device cultures in various fields of research, including the study of the blood-brain barrier. Notably, several recent publications have highlighted the potential of chip devices to provide a more physiologically relevant and functional in vitro model of the blood-brain barrier, some of the most interesting approaches being the following.

A model where they use pluripotent cells that specialise under hypoxic conditions to create their model on a microfluidic chip. They also dynamically modulate the flow, thus obtaining a highly functional model. [14]

A critical part in many models is the hydrogel matrix, as it is essential to maintain the structure of the model. So it is important to focus on this aspect and compare different strategies as it's done in [15].

In another interesting study they use a non-conventional approach in which they do not use any on-chip pump system, and the modulation of the flow is done by a gravity-based system. [16]

2. MATERIALS

In this section, the most relevant materials used to perform the experiments will be described. These are the two cell lines that were worked with, the hydrogel used as matrix and the chip devices employed for the 3D cultures.

2.1 bEnd 3 Cell Line

The bEnd.3 [BEND3] (ATCC CRL-2299) cell line is a commonly used in vitro model of the blood-brain barrier. It is a cell line derived from mouse brain microvascular endothelial cells. The cell line is characterised by the formation of tight junctions and expression of BBB-specific markers, which are crucial for the permeability of the barrier. [17]

This makes the bEnd3 cell line a useful tool for studying BBB function and transport mechanisms, as well as for testing the effects of drugs and other agents on BBB permeability and transport. The bEnd.3 cell line has also been used in other areas of research, including angiogenesis and tumour biology. Detailed and technical information about the bEnd3 cell line can be found in [17].

2.2 C8-D1A Astrocyte Cell Line

The C8-D1A [Astrocyte type I clone] (ATCC CRL-2541) cell line is a type of immortalised cell line derived from the cerebellum of a mouse. These cells are commonly used as a model system to study the properties and functions of astrocytes, which are star-shaped glial cells found in the brain and spinal cord as explained above. [18]

These cells exhibit many properties of primary astrocytes, such as the expression of astrocyte-specific markers and the ability to form a glial network. Moreover, they retain many of the physiological and biochemical properties of astrocytes and respond to various stimuli, such as neurotransmitters, cytokines, and growth factors, in a similar way to primary astrocytes. Detailed and technical information about the C8-D1A cell line can be found in [18].

The C8-D1A cell line is widely used as a tool to investigate the molecular mechanisms underlying various aspects of astrocytes. It is also used to study the

role of astrocytes in neurological disorders and to test the efficacy of potential therapeutics.

2.3 Hydrogels

During this work, two different types of hydrogel have been used which are described below. The first is a commercial hydrogel while the second is a hydrogel that is being developed by a researcher from the ZIBIO group.

The first hydrogel used was VitroGel® Hydrogel Matrix from TheWell Bioscience. This hydrogel is a formulation of multi-functional ligands that transforms into a hydrogel matrix when it is mixed with the cell culture medium, due to the calcium ions contained in it. VitroGel Hydrogel Matrix mimics the natural extracellular matrix environment, supports a wide range of cell types, has a neutral pH, is transparent, permeable and compatible with imaging systems. [19]

It was chosen for the properties explained above, the feasibility of using it with the astrocyte cell line and for its easy handling to be used as a matrix for astrocytes in cell proliferation assays on the chips. Detailed and technical product information about the VitroGel® Hydrogel Matrix can be found in [19].

The second was an alginate-based hydrogel that has been developed in the research group. These hydrogels are made from alginate, a natural polysaccharide derived from brown seaweed, and are formed through a gelation process that involves the interaction of alginate with divalent cations, such as calcium ions.

Alginate-based hydrogels are a type of hydrogel that are commonly used in the development of in vitro models, often used to create 3D cultures or co-cultures of cells. This hydrogel was used as a barrier for the endothelial cells grown in the chip devices, being its function to maintain the structure necessary to recreate the BBB. [15]

The components of an alginate-based hydrogel typically include alginate, a crosslinking agent like calcium ions, and an aqueous phase. The alginate provides the polymer network of the hydrogel, while the crosslinking agent and aqueous phase provide the structure and hydration to the gel, respectively. [20]

To form the alginate-based hydrogel that was used [Fig 7], alginate was first dissolved in phosphate buffered saline (PBS) to create an alginate solution. Calcium carbonate ($CaCO_3$)was then added which acts as a source of calcium ions that are necessary for crosslinking with the alginate polymer. When dissolved in an acidic environment, calcium carbonate releases calcium ions that react with the alginate chains to form a stable hydrogel network. This acidic environment occurs when the alginate and calcium carbonate solution is mixed with another solution of Glucono-delta-lactone (GDL) in PBS. In the presence of PBS, the GDL undergoes hydrolysis to produce gluconic acid, which acidifies the environment causing the $CaCO_3$ to act as a crosslinker for hydrogel formation. GDL functions as a time- and pH-dependent acidifier that gradually lowers the pH of the alginate solution, leading to the solubilization of calcium ions from calcium carbonate. The combination of both provides a robust method for the formation of the hydrogel.

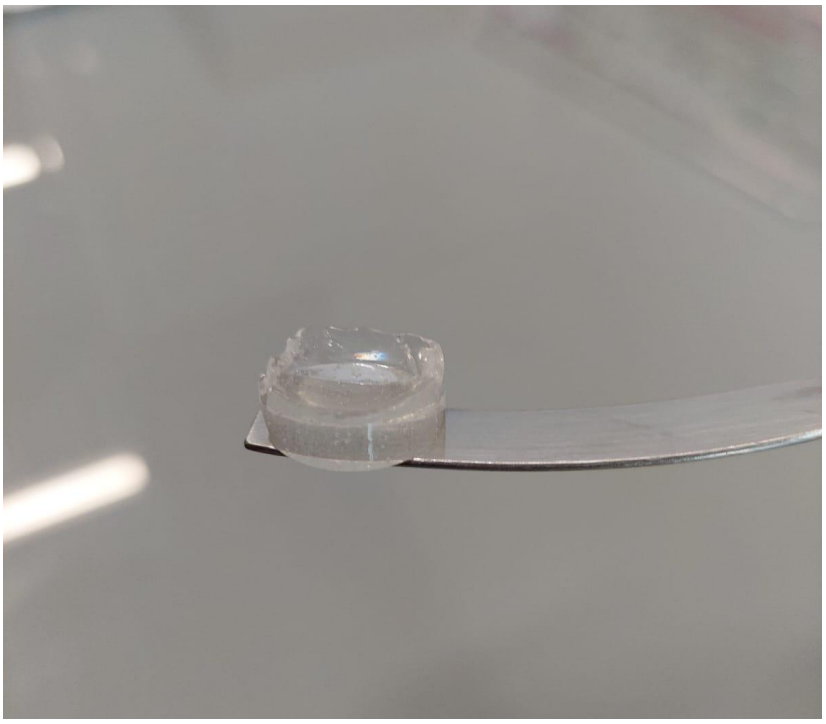


Figure 7: alginate-based hydrogel developed in the research group

2.4 Microfluidic Chip Devices

Microfluidic chip devices are a type of in vitro cell culture system that use microfluidic technology to create a controlled environment for cell cultures. They are based on the concept of microfluidics, which involves manipulating and controlling fluids at the microscale.

A microfluidic chip device typically consists of a chip made from a polymeric material, which contains microscale channels and chambers. The chip is designed to allow for the flow of fluids, like cell culture media for example, into and out of the microscale chambers where cells are grown. This allows for the precise control of the physical and chemical parameters that influence cell behaviour, such as flow rate, shear stress, and nutrient delivery.

In the context of blood-brain barrier (BBB) research, microfluidic chip devices are used to create more realistic in vitro models of the BBB. The microscale channels and chambers can be designed to mimic the BBB structure and environment, achieving a 3D cell co-culture and physical conditions like the shear stresses similar to in vivo models. This method allows for the study of BBB function, transport mechanisms and drug permeability in a controlled and reproducible manner. [13]

Two different devices were used, both from the company BEOnChip, one being the BE-Gradient Standard model and the other a customised version of it. Both devices are made of the same lipophobic thermoplastic material and have the same characteristics such as compatibility with optical microscopy techniques and no unspecific substance absorption by the chip. The difference between them is that the standard model uses a physical separation between the channels and the central chamber, while the customised version relies on a hydrophobic treatment to keep each fluid in its corresponding compartment, allowing closer contact. Both devices can be seen in [Fig 8], and the dimensions of the chip in [Tab 1]. [21]

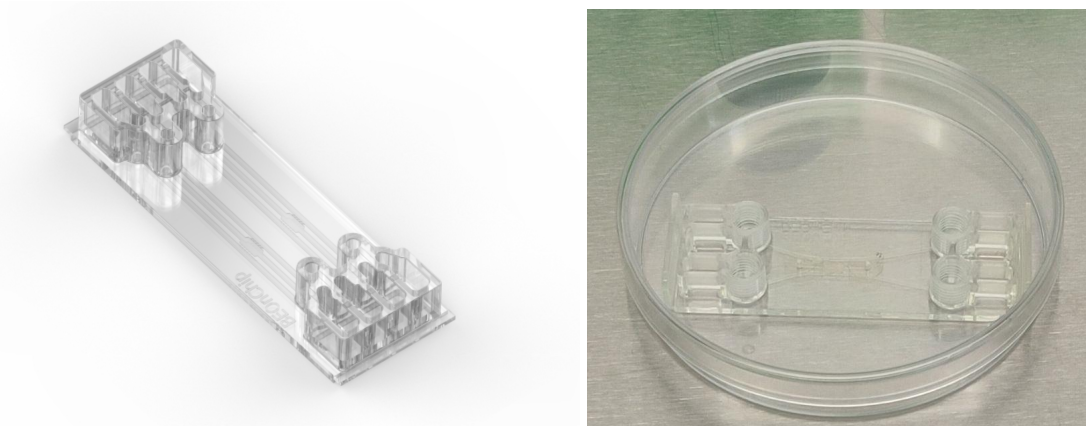


Figure 8: Chip devices from BEOnChip

Table 1: Chip device dimensions

	Height	Width	Lenght	Total volume
Central channel	300 µm	1 mm	39 mm	12,6 µL
Lateral Channels	300 µm	1 mm	50 mm	14,5 µL
Chamber	300 µm	2 mm	4,6 mm	3 µL
Inlet/Outlet	8 mm	Ø = 2,3 mm		18,4 µL
Reservoir	6 mm	3,6 mm	7 mm	151,2 µL

3. METHODS

This section will describe the experiments performed and the procedures followed to do them, starting with the assays carried out on transwell cell cultures and following with those carried out on chip devices.

3.1 2D Cell Cultures

In this first trial, the two cell lines mentioned above in the materials section were cultured, in order to better understand their handling and to see their proliferation. Both were cultured in a humid incubator with a CO_2 level of 5% using T75 flasks, as described below.

The cell line bEnd.3 [BEND3] (ATCC CRL-2299) was cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% Fetal Bovine Serum (FBS), 50 U/mL penicillin, and 10 mg/mL streptomycin. Cells were passaged with 0.25% trypsin-EDTA when the culture reached 80% confluence, and ratios of 1:6 to 1:10 were used. The culture medium was changed every 2-3 days.

The cell line C8-D1A [Astrocyte type I clone] (ATCC CRL-2541) was also cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% FBS, 50 U/mL penicillin, and 10 mg/mL streptomycin. Cells were passaged with trypLE-EDTA when the culture reached 80% confluence, and ratios of 1:4 to 1:6 were used. The culture medium was changed every 2-3 days.

3.2 Monoculture BBB model on transwell

This assay consists of culturing bEnd3 cells in a 24 well plate using transwell inserts with a pore size of 0.4 μm, thus obtaining a membrane of endothelial cells on the insert. First, the insert was moistened by adding 1 ml of culture medium on it and leaving it in the incubator for two hours. Afterwards, the transwell was placed in a 24 well plate and endothelial cells were added in the basolateral side with a density of 3×10^4 cells per insert. Finally, DMEM culture medium was added to both the apical chamber and the basolateral chamber and placed in the incubator. Culture medium was changed periodically until the cells were confluent and form a monolayer. An illustration of the set-up can be seen in [Fig 9].

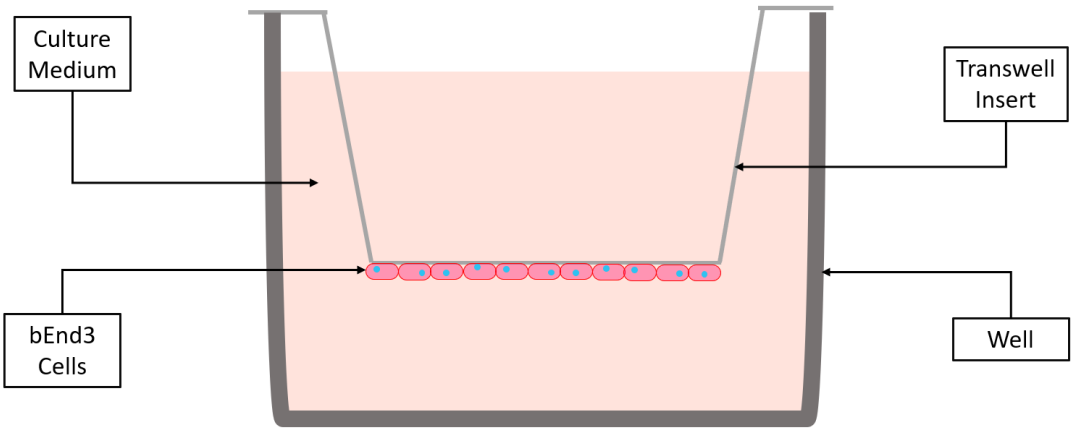


Figure 9: Set-up of the monoculture BBB model on transwell insert

3.3 Trans-Endothelial Electrical Resistance of the 2D BBB model

Trans-endothelial electrical resistance (TEER) is a viable measurement to determine the formation of tight junctions and the integrity of the barrier. In this experiment, the *EVOM3* voltmeter was used to measure the TEER of the monolayer described in the previous section 3.2. First, the *EVOM3* electrode was left in a 0.15 M NaCl solution overnight. Then, and prior to measurements, the probe was immersed in 70% isopropanol for 10 minutes followed by rinsing with water. The inserts with the cell monolayer and the empty control inserts were then transferred to a new well plate, where culture medium was added again to both the apical and basolateral sections. For TEER measurements, one end of the probe was carefully immersed in the apical space and the other end in the basolateral space as shown in [Fig10]. Finally, the inserts are placed back in their original well plate and new medium is added. This measurement was done in triplicates up to 8 days.

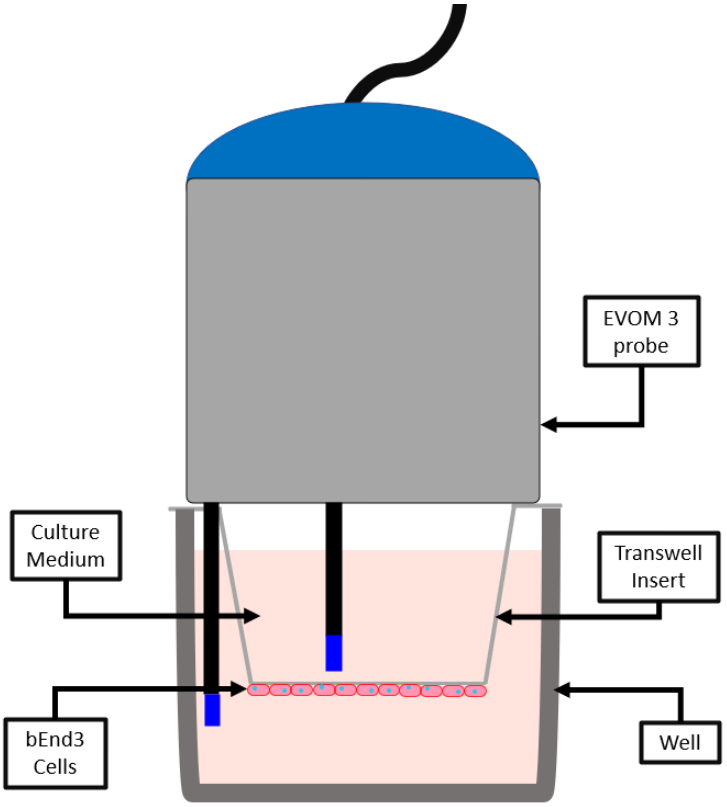


Figure 10: Set-up of the trans-endothelial electrical resistance measurement of the 2D

Then the TEER value was calculated with the following equation:

TEER= (Rt – Rc) * At

Rt is the measured resistance of the inserts with cells. Rc is the resistance of the inserts without cells (control). A is the area of the transwell insert membrane (0.33 cm2).

3.4 Permeability assay of the 2D BBB model

In order to analyse barrier permeability and barrier integrity, two fluorescently labelled dextran compounds (Sigma-Aldrich) were used, one of 4 kDa and the other of 20 kDa. First, endothelial cells were cultured for 5 days on a transwell insert until a monolayer was formed, as explained in section 3.2. Once the monolayer has been formed, the inserts with cells and also empty ones without cells (as a control) are transferred to a new well plate, where 800 µl of culture medium was added to the basolateral chamber. Then the medium from the apical chamber of the inserts was aspirated and 250 µl of the solution containing the fluorescently-labelled dextran was added at a concentration of 1 mg/mL, an illustration of the set-up can be seen in [Fig 11]. Then, the plate was placed in the incubator for 90 minutes.

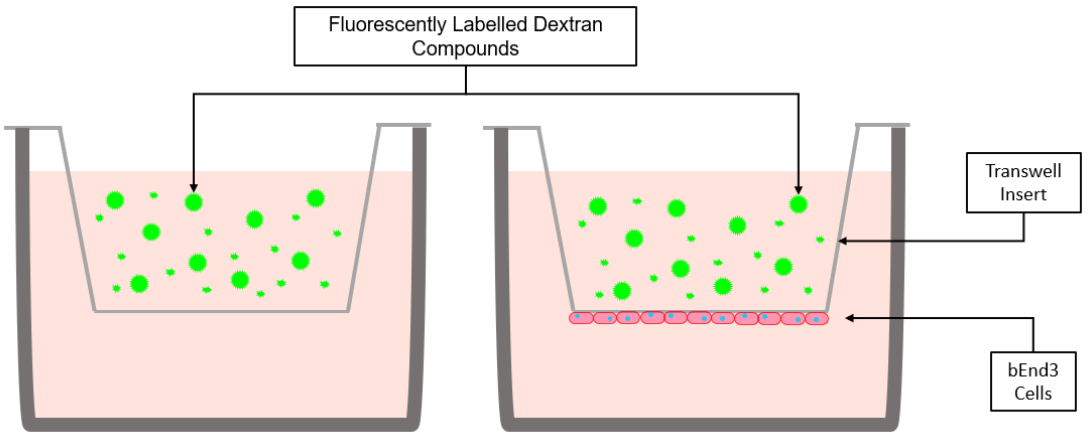


Figure 11: Set-up of the permeability assay on the 2D BBB model. Left, insert without cells. Right, insert with cells

Afterwards, the medium from each basolateral compartment was collected in Eppendorfs and mixed appropriately, then 100 µL aliquots from each Eppendorf tube were transferred to a 96 well plate. Finally, the fluorescence intensities were measured in triplicates using a microplate reader from BioTek (model Synergy H1), with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

3.5 Tight junction immunostaining

As the objective in this case is to stain the tight junctions, the first step is to culture the cells until they form a monolayer as in the section 3.2, to achieve close contact between the cells and thus develop tight junctions. This was done on transwell inserts from which the porous membrane where the cell monolayer was formed was removed for immunostaining. As explained in section 1.1. tight junctions are formed by different transmembrane proteins. The target proteins to be stained in this case were Zonula occludens 1 (ZO1), with a Rabbit polyclonal to ZO1 tight junction protein staining.

Once the monolayer was formed, the immunostaining process was started: First, the culture medium was removed and the cells washed with PBS; then, to fix the cells, 350 µl of a 4% solution of formaldehyde in PBS was added for 10 minutes. Once the cells were fixed, the porous membrane of the insert containing the cell monolayer was cut in order to work with this sheet in the following steps. The next step was to place this membrane in a 24 well plate and add 300 µl of a permeabilization buffer (0.5% Triton-X100 in PBS), leaving it for 7 minutes and then washing it 3 times with PBS. The primary antibody (Rabbit anti-ZO1 tight junction protein antibody (ab216880) from ABCAM) solution was then prepared, consisting of a 1:100 dilution of the antibody in PBS with 1% bovine serum albumin (BSA) and 0.1% Tween 20. A wet chamber was used to apply the solution by placing a piece of parafilm with the paper side facing upwards on the base of the wet chamber, then a drop (10 µl) of the primary antibody solution was added onto the parafilm and the membrane with the cell monolayer was placed in such a way that the cells were in direct contact with the solution. The chamber was closed and incubated overnight.

The following day, the membrane was removed from the wet chamber and placed in a 24 well plate with the cells side facing up, and washed 3 times with a solution of 0.5% Tween 20 in PBS. The secondary antibody (Alexa Fluor® 488 Donkey anti-Rabbit IgG Antibody (Product #R37118) solution, which was a 1:200 dilution of the antibody in PBS with 1% BSA and 0.1% Tween 20, was then prepared and applied in the same way as above. A piece of parafilm was placed at the base of the wet chamber and a drop of the secondary antibody solution was added, followed by placing the membrane with the cells in direct contact with the solution, closing the chamber and incubating it for 2 h at 4 ºC.

After completion of staining of both antibodies, the membrane was removed from the wet chamber and placed on a 24 well plate with the cell monolayer facing upwards. Finally, it was washed 2 times with 0.5% Tween 20 in PBS solution and 2 times with PBS. The membrane was preserved in PBS until its observation under the microscope (Nikon Eclipse TS 2).

3.6 Cell proliferation on chip devices

In this first trial using the chip devices, a simple cell proliferation assay was performed to see how the cells grew in the chip environment. This was done with both astrocytes and endothelial cells.

In the case of astrocytes, these were seeded in the chip embedded in hydrogel. For this, first the astrocytes were detached from their T75 flask using trypLE-EDTA and a solution with 1×10^{6} cells per millilitre was obtained, then this solution was mixed with the VitroGel® Hydrogel Matrix with ratios of 1:1, 1.5:1 and 2:1 respectively. Finally, the solution was seeded into the central channel of the chip, as shown in [Fig12]. Then, culture medium and PBS were added to the corresponding chambers of the chip to maintain humidity levels. The chip was incubated in a petri dish for 5 days.

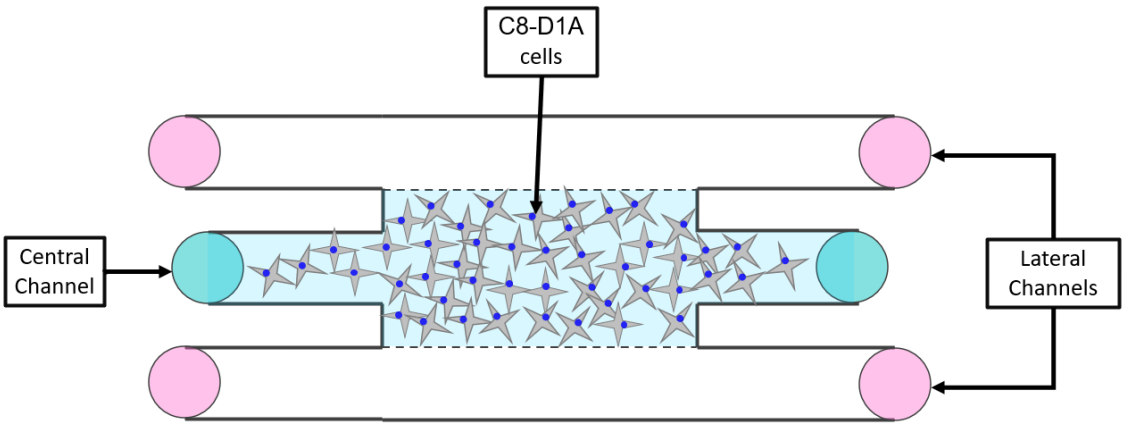


Figure 12: Set-up of the C8-D1A astrocyte cells proliferation on chip devices

In the case of endothelial cells, no hydrogel was used. Accordingly, they were detached from the T75 flask using 0.25% trypsin-EDTA and the solution was adjusted to 1×10^{6}. This solution was directly seeded into the side channel of the chip, as illustrated [Fig 12].. Then, culture medium and PBS were added in the corresponding chambers and the chip was incubated in a petri dish for 5 days.

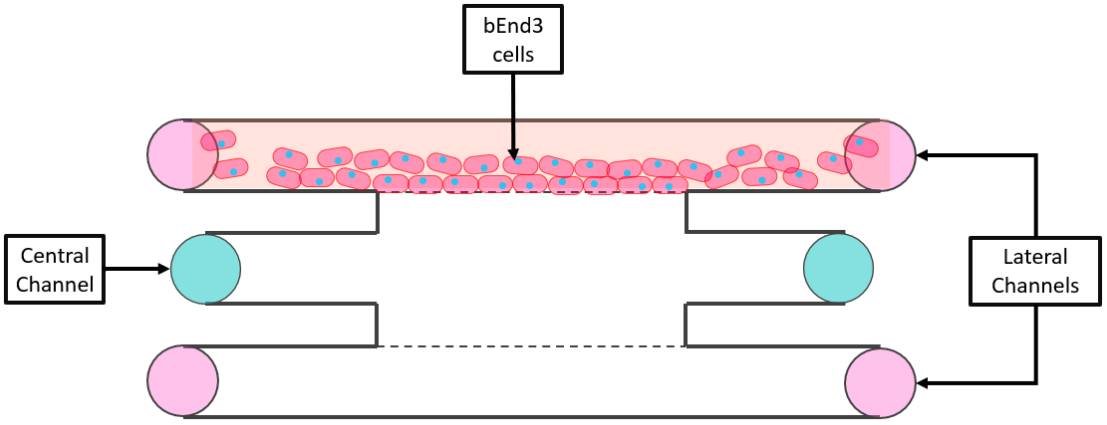


Figure 13: Set-up of the bEnd3 endothelial cells proliferation on chip devices

3.7 Monoculture BBB 3D model

The aim of this experiment was to try to reproduce the structure of the BBB by having a space filled with endothelial cells which is differentiated from the contiguous space, thus forming a monolayer of cells at the interface between the two. For this purpose, endothelial cells were seeded in one lateral channel of the chip while the central channel (the adjacent one) was filled with hydrogel to act as a barrier for the cells.

The chip device was first placed in a petri dish and incubated overnight to pre-warm it. Once the time had elapsed, the two solutions needed to form the hydrogel were made, the first being PBS with 1% mass/volume of alginate and 0.25% mass/volume of calcium carbonate ($CaCO_3$), and the second PBS with glucono delta lactone (GDL) in a ratio of 2:1 moles in relation to the $CaCO_3$ of the first solution (2 mol GDL / 1 mol $CaCO_3$). To form the hydrogel, both solutions must be mixed at a ratio of 1:1, so the two solutions were brought inside the clean bench along with the chip device, due to the need to work quickly with it to prevent it from polymerising before seeding. The solutions were mixed in an eppendorf and 15 µl of hydrogel was seeded in the central channel of the chip, leaving it for 2 h inside the biosafety cabinet at room temperature for the first stages of polymerisation, then it was placed in the incubator and left for 2 days until the hydrogel polymerised completely.

After this time, endothelial cells were seeded by detaching them from the T75 flask using 0.25% trypsin-EDTA and a solution of 1×10^6 cells per millilitre was made. 20 µl of this solution was seeded into the side channel of the chip. The configuration being as shown in the illustration [Fig 14]. Finally, culture medium and PBS were added to the appropriate reservoir chambers to maintain humidity and the chip was cultured for up to 5 days.

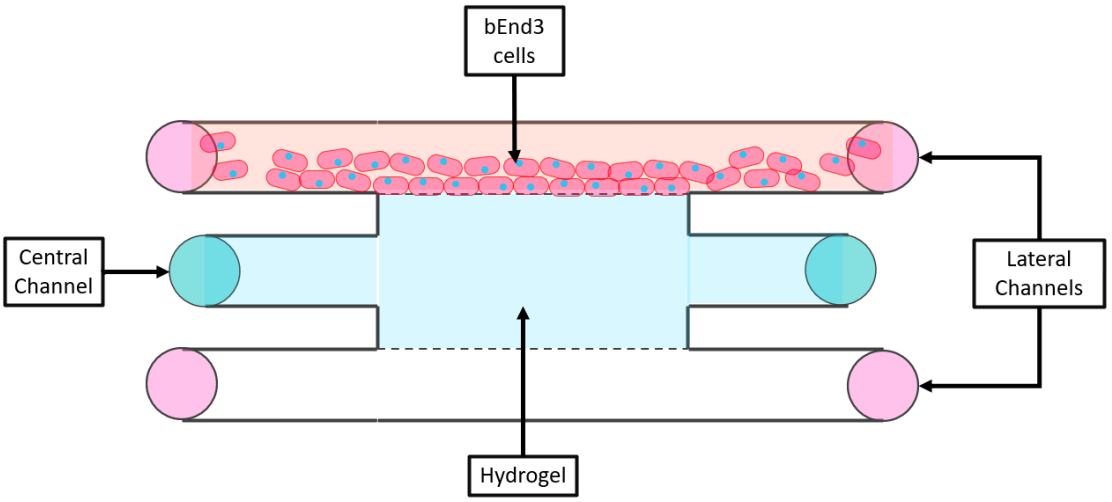


Figure 14: Set-up of the Monoculture BBB 3D model

4. RESULTS & DISCUSSION

In this section, the results of the experiments will be presented and discussed. Starting with the assays carried out on transwell cell cultures and following with those carried out on chip devices.

4.1 2D Cell Cultures

This assay was a simple cell culture of the two cell lines that have been worked with, the goal was to get both cell lines to proliferate normally with respect to what is indicated by the distributor. This was done to become familiar with the handling of the cell lines and to know their proliferation speed, thereby finding out how fast they reach confluence. The culture of both cell types can be seen in [Fig 15].

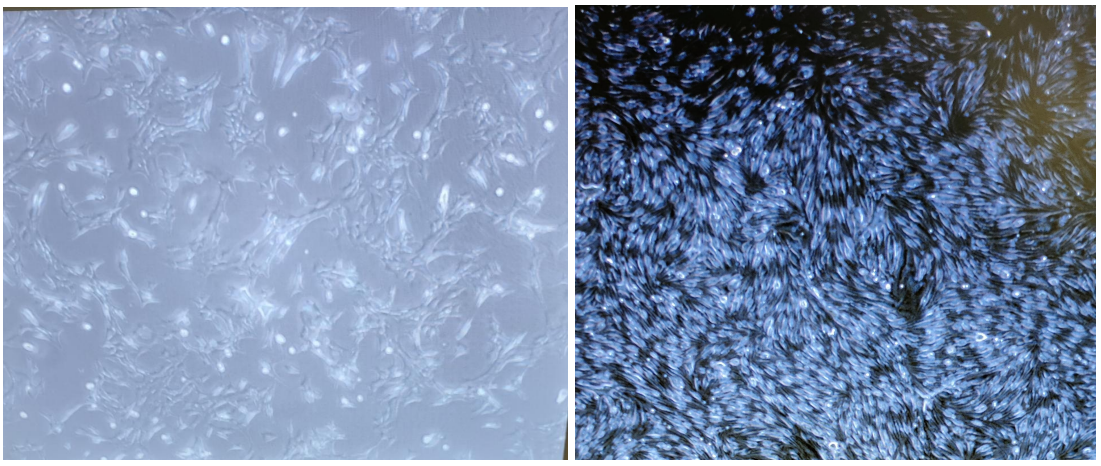


Figure 15: Left, culture of C8-D1A astrocyte cells. Right, culture of bEnd3 endothelial cells

This provides a better understanding of both cell lines in order to be able to work better in the following experiments.

4.2 Monoculture BBB model on transwell & TEER measurements

In this experiment, a monolayer of endothelial cells was first cultured on the porous membrane of the transwell insert and then tested for proper formation and integrity by measuring the transendothelial electrical resistance TEER.

Transendothelial electrical resistance is an indicator of barrier formation and tight junction formed on it, therefore, is a recommended measurement prior to further experiments. This has been performed as described in methods section 3.3 and the results are as follows [Tab 2], [Fig 16].

Table 2: TEER measurement values. Highest value (green)

Replicates	Day 1		Day 2		Day 3		Day 4	
No cells	201 ohm	204.3 ohm	216 ohm	211.3 ohm	201 ohm	203.1 ohm	199,6 ohm	203.5 ohm
	202 ohm		217 ohm		202 ohm		199,6 ohm	
	203 ohm		218 ohm		203 ohm		199,6 ohm	
Cells	204 ohm	204.3 ohm	219 ohm	**285.3 ohm**	204 ohm	203.1 ohm	199,6 ohm	203.5 ohm
	205 ohm		220 ohm		205 ohm		199,6 ohm	
	206 ohm		221 ohm		206 ohm		199,6 ohm	

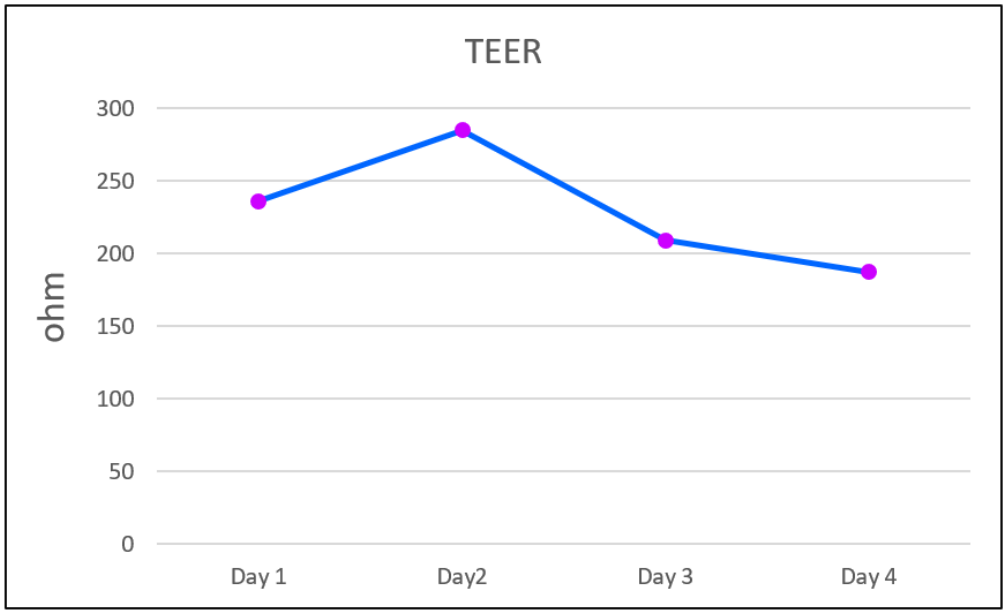


Figure 16: TEER graph from the inserts with cells

As shown in [Fig 16], the TEER measurement values started to increase until it reached the maximum value on day 2, and then decreased during days 3 and 4. This behaviour is logical, since at the beginning the cells proliferate forming the barrier and tight junctions are also formed, making the TEER increase. Then, after the second day, the TEER starts to decrease, indicating a deterioration of the barrier, probably due to the lack of stimuli. Regarding the maximum TEER value, it has a mean of 285 ohm [Tab 2], a value that is in line with what is reported in the literature. [14]

It is also worth noting the variations in the values of the replicates [Tab 2], which together with the experience of use where slight changes in the positioning of the probe were seen to alter the values of the measurements, affect the reliability of the test. Even so, the results were positive.

This data was of great importance to determine at what time the barrier model is in its best conditions, knowledge that was necessary for further experiments. Summarising, using 24 well plates and seeding 3×10^4 cells per well, the best barrier conditions are obtained two days after seeding.

4.3 Permeability assay of the 2D BBB model

This assay aims to analyse the permeability of the cell monolayer that was developed on the transwell inserts as a model of the BBB. For this, two fluorescently-labelled dextran compounds with molecular weights of 4 KDa and 20 KDa respectively were used, to better determine the level of permeability.

As explained in section 3.4., the fluorescence intensity was measured with a Synergy H1 microplate reader from BioTek, the values of the measurements being as follows [Tab 3]. The fluorescence part of the culture medium was then subtracted, and the mean values of the replicates were calculated. These values were then normalised using the measurements of the control for each compound. In the control, these compounds were added on empty inserts (no cells), so their values represent the maximum fluorescence intensity. The normalised results are highlighted in [Tab 3], and have been plotted into a graph in [Fig 17].

Table 3: fluorescence intensity values (arbitrary units) from the permeability assay. Green, average values. Purple, normalized values

	C1 (4kDa)	Insert 1	C2 (20kDa)	Insert 2
Replicates	36095	9095	22391	4437
	37368	6887	22846	3191
	35926	6486	24319	3235
Average	**36463**	**7489**	**23185**	**3621**
Normalized	**100%**	**21%**	**100%**	**16%**

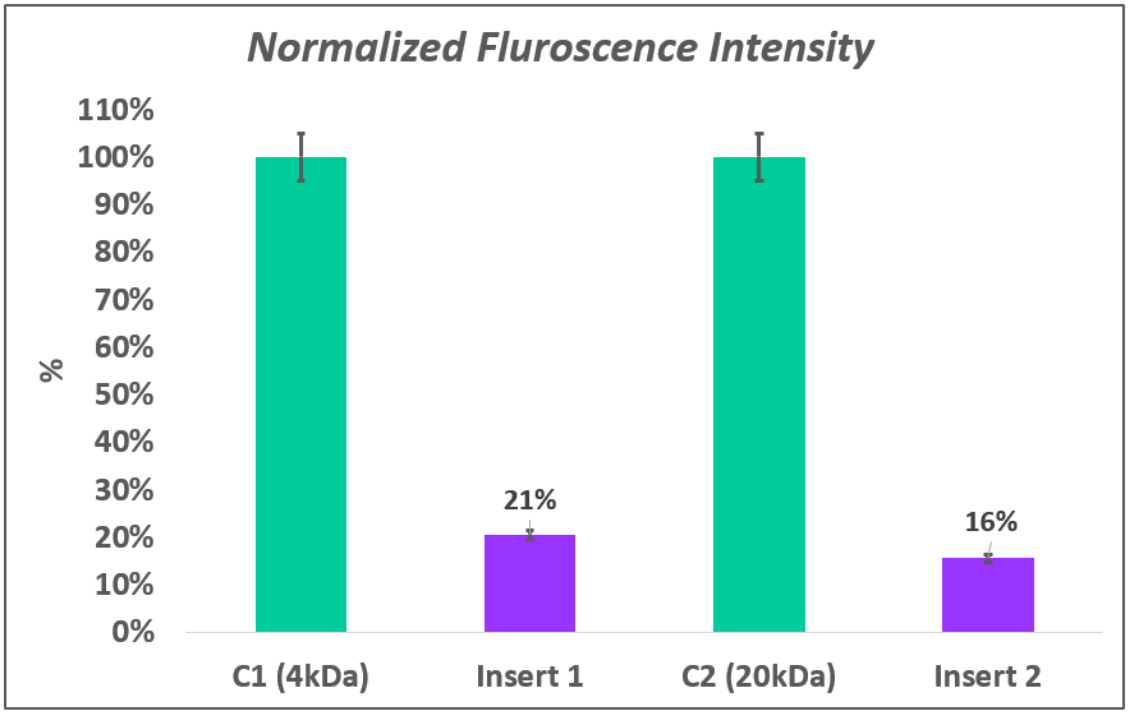


Figure 17: Permeability assay normalised values results graph

Looking at the fluorescence intensity values of 20.5% and 15.6% for the 4 KDa and 20 KDa compounds respectively, it can be seen that the cell monolayer was able to block the passage of the studied compounds. This is a positive result, as one of the main characteristics of the BBB is its low permeability. It is also worth noting that although the intensity value for the 4 KDa compound is higher, as it is a smaller molecule and crosses the barrier more easily, it does not differ much from the value for the 20 KDa compound. This is also a positive result, as it indicates that the cell monolayer is able to block compounds of different molecular weights, just like the BBB in vivo.

These results were relevant to check the permeability of the cell monolayer developed, a property that is really necessary to test the passage of drugs through the barrier, which is what this in vitro model is intended for. This, together with the TEER results, ensures the correct formation of the cell monolayer.

4.4 Tight junction immunostaining

To complement the tests already carried out, immunostaining of the tight junctions was performed to visualise them under the microscope. Performing the process as described in section 3.4, the following images were obtained [Fig 18] [Fig 19].

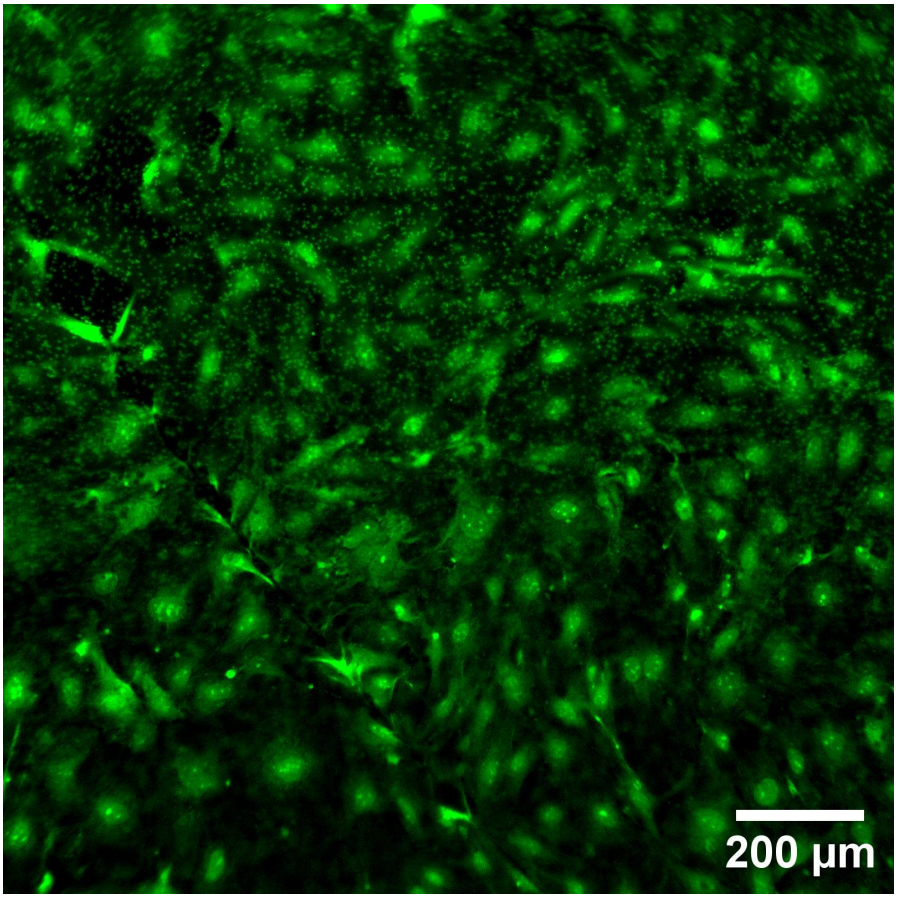


Figure 18: Green staining image

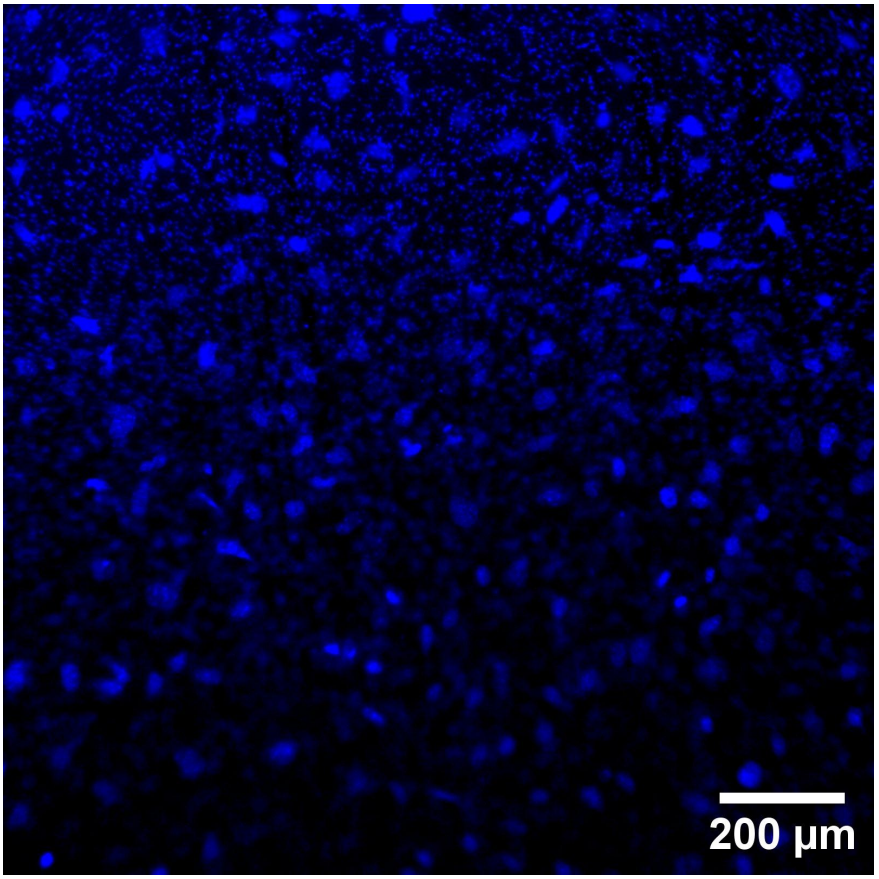


Figure 19: Blue staining image

In these images the dye is too intense and the structures of the tight junctions cannot be clearly differentiated, and there is also a lot of background noise, which makes visualisation even more difficult. On the other hand, the blue dye image also helps to differentiate the cells which are attached to the porous membrane, and how densely they form the barrier.

These images were the result of the first attempt to stain the ZO1 proteins, so the lack of experience in this procedure led to suboptimal results. As these images can be improved, better results will be obtained when this experiment is repeated and the protocol is optimised.

4.5 3D Cell Cultures on chip devices

In this first assay using the chip devices, the proliferation of bEnd3 endothelial cells and C8-D1A astrocytes was tested. The aim was to achieve normal proliferation of both cell lines in the chip device. This served to get familiarised with the handling of the microfluidic devices and to see how the cells behaved in them. The culture of both cell types can be seen in [Fig 20].

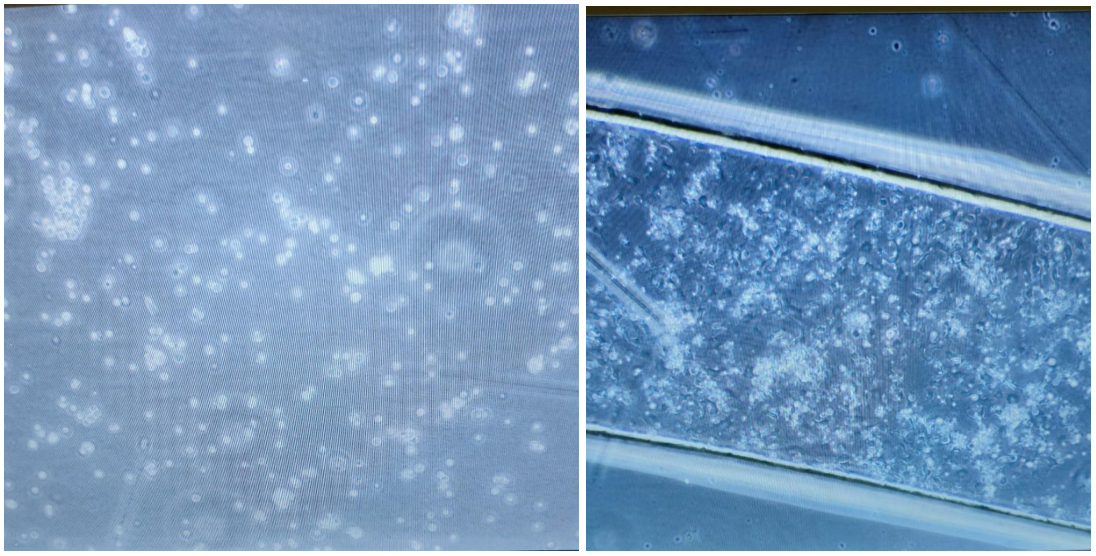
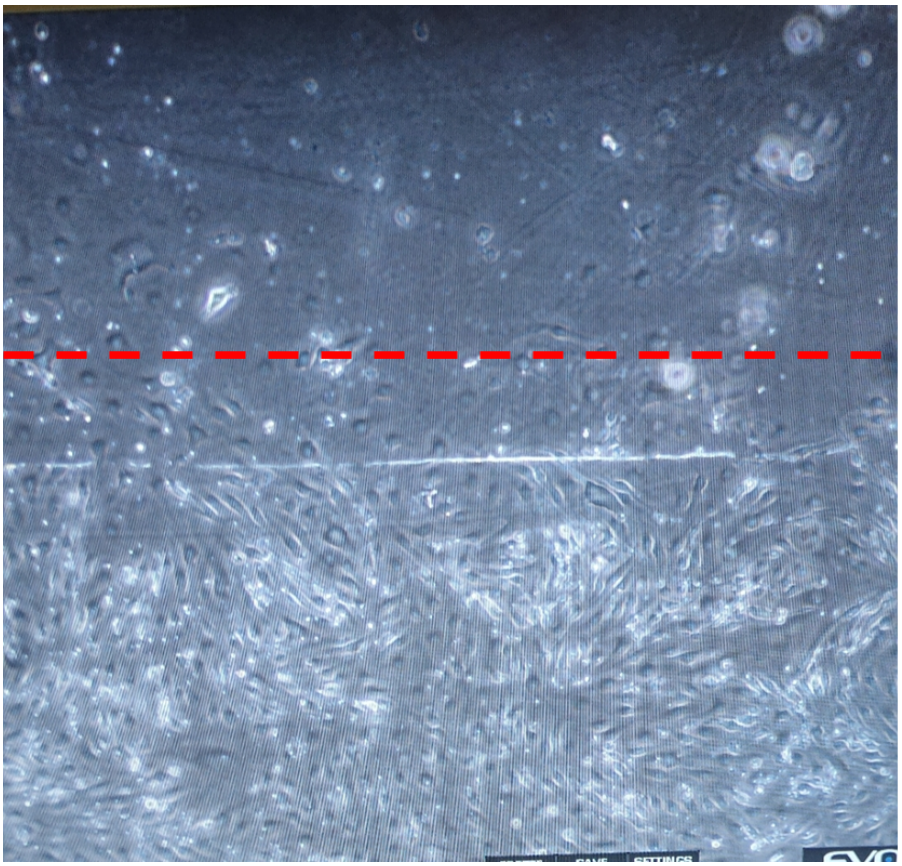


Figure 20: Left, culture of C8-D1A astrocyte cells on chip device. Right, culture of bEnd3 endothelial cells on chip device

Both cell types were able to proliferate in the device. They lasted until day 4, where they look a bit more shredded as it can be seen in the image [Fig 20], when it was necessary to renew the culture medium using microfluidic pumps, which could not be done. In the case of astrocytes, these were seeded embedded in hydrogel, the hydrogel was able to contain the cells but it lacked integrity and could not stay in its channel, it leaked. Although these results are not completely positive, they provide experience with the use of the chip devices in combination with cells, which is necessary for the following experiments.

4.6 Monoculture BBB 3D model

In continuation from the previous experiment and making it more complex, endothelial cells were cultured in one channel and hydrogel in the other. The aim was to simulate the BBB structure at the interface between the channel with cells and the channel with hydrogel. This experiment provided a lot of information and knowledge about the behaviour of the hydrogel with the cells, the hydrogel inside the chip and the handling in general with this type of microfluidic devices. The images of the results are [Fig 21], [Fig 22].



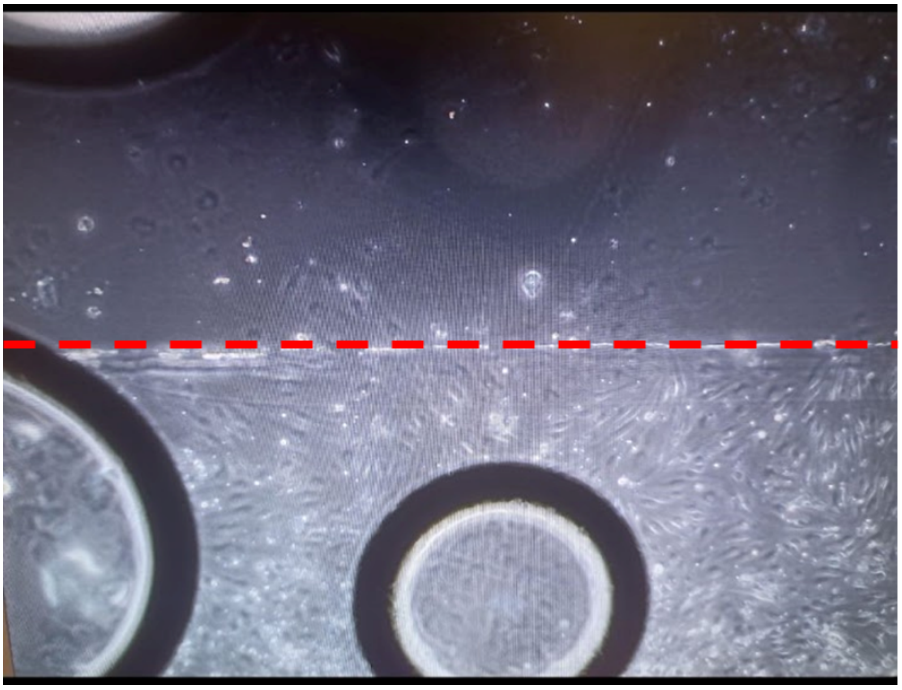


Figure 21: Monoculture BBB 3D model. The red lines show the differentiation between the hydrogel space and the cells. Top, replicate 1. Bottom, replicate 2.

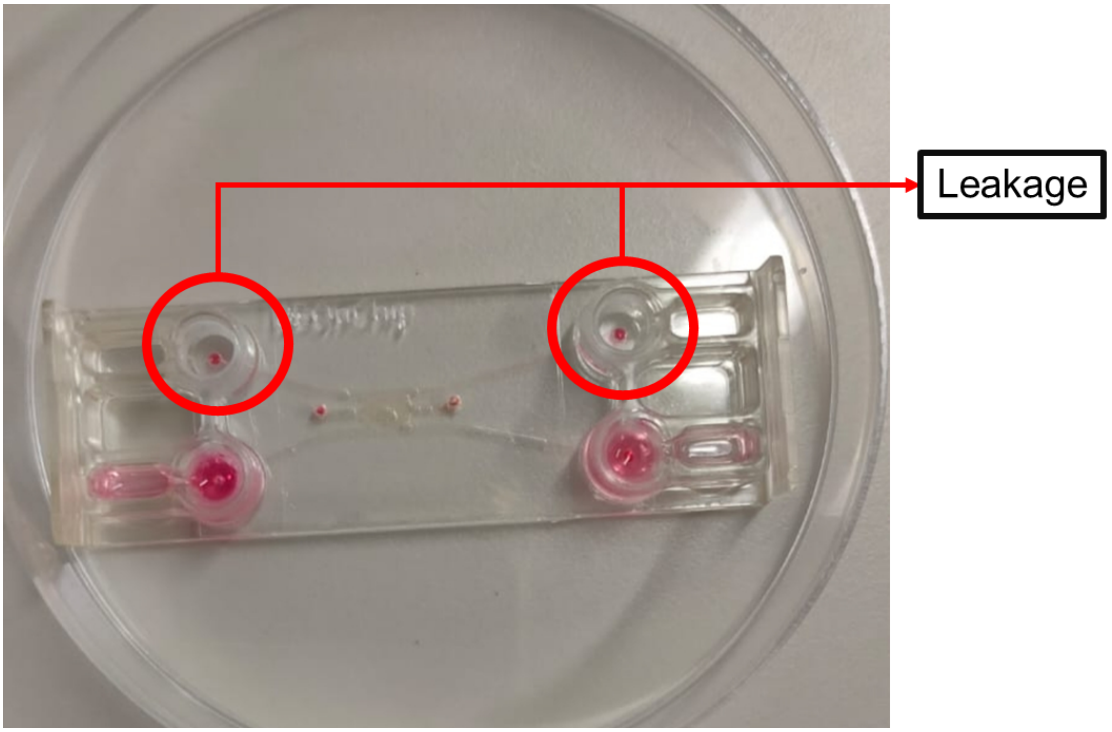


Figure 22: Monoculture BBB 3D model on chip device. Leakages highlighted in red

At the best of the attempts the hydrogel was able to contain the cells to a considerable extent [Fig 21], but in many other cases it was not able to and the cells together with the medium migrated into other channels [Fig 22]. To check that this was not directly due to the properties of the hydrogel, the interaction of the hydrogel with the cells was tested in a petri dish [Fig 23].

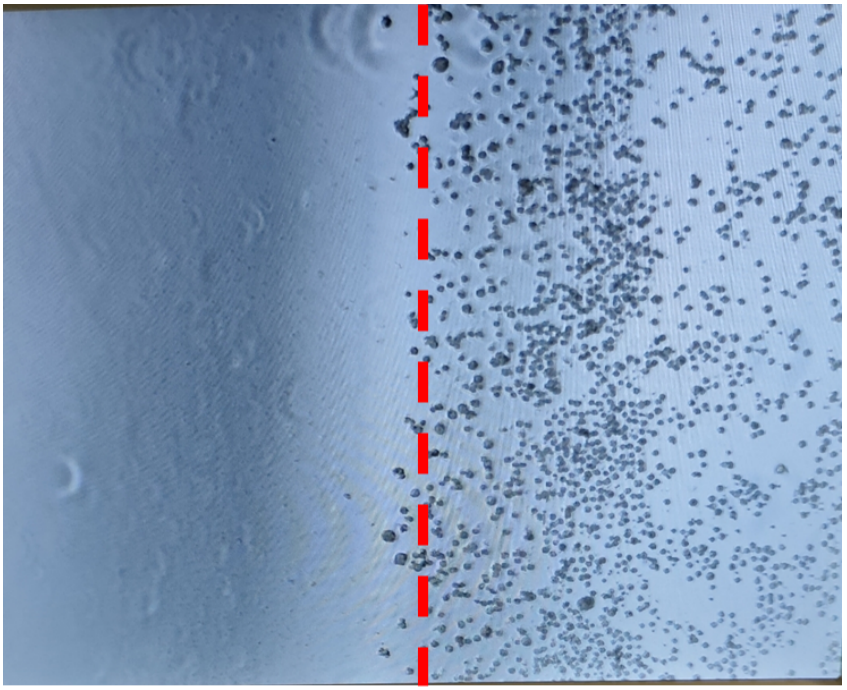


Figure 23: Hydrogel surrounded by endothelial cells in a petri dish. The red line shows the differentiation between the hydrogel space and the cells

As can be seen in the image above [Fig 23], there is a clear differentiation between the space with cells, although these did not proliferate properly, and the space without. Making clear the capacity of the hydrogel to contain the cells. So, the problem described above is due to factors of a different nature. On one hand, despite seeding the hydrogel correctly and creating a moist environment using PBS and a petri dish, bubbles formed in the hydrogel and it dried out, causing the hydrogel chamber to lose its integrity and not be able to contain the cells. On the other hand, the type of hydrogel used, despite polymerising to a large extent during the first day when cells were not yet seeded, it continues to polymerise during the following days, a process which is negatively affected by the temperature of the culture conditions, causing the hydrogel not to solidify as well as it should, compromising its integrity. It is the sum of these two problems that makes it difficult to achieve good and reproducible attempts.

Moreover, the lack of experience in handling microfluidic devices makes more complex models that use more than one channel difficult to perform correctly. This is mainly due to the difficulty of seeding the different fluids in the device accurately, and keeping each fluid confined in its space.

Summarising, despite the problems described, the results indicate that the model approach has potential, as most of these problems could be solved with greater experience working with microfluidic devices. This experiment is also an important prelude to more complex experiments where different cell lines are co-cultured.

5. CONCLUSIONS

Regarding the main objective of the project to develop a three-dimensional in vitro model of the BBB, this is still far from being achieved. But the first steps to do so have been taken, and as it is part of a long-term European project it will be further developed.

Both endothelial cells and astrocytes were successfully cultured within the device, and a more complex model was made using endothelial cells and hydrogel in contiguous channels to generate a structure. The biggest obstacles were the lack of experience in handling microfluidic devices, as the seeding of the fluids was problematic, making good attempts difficult to reproduce. The hydrogel would also need to be improved to withstand the culture conditions while maintaining its integrity.

Concerning the model carried out on transwell inserts, significant progress was made. Positive results were obtained in the TEER and permeability measurement tests. Although the immunostaining process of the tight junctions can be improved to obtain better images than those achieved.

The model using the chip devices and the one using the transwell inserts should be developed in parallel, thus, improving the processes and protocols of both to achieve more complex models.

With regard to microfluidic chips as a technology for creating three-dimensional cellular models in vitro, they are clearly the present and the future for simulating complex cellular structures, such as in this case the blood-brain barrier. As discussed in previous sections, these have a number of advantages over traditional models, and are and will be used to develop new therapies and test new drugs. Technology that combined with other advancements such as drugs modelled by algorithms and artificial intelligence, improved fluid control systems and the capabilities of chip manufacturing technologies, will lead to the development of more complex and efficient therapies.

6. FUTURE LINES

As this work is part of a bigger project which is under development, it should be continued. Further experiments will be carried out in the future to achieve the project's objective, some of which are discussed in this section.

The first major milestone would be to co-culture both cell lines on the chip device, being in one channel the astrocytes on a hydrogel matrix and in the other channel the endothelial cells in the corresponding wall, thus achieving a three-dimensional cell structure. The hydrogel has a critical role in this, so the development of the research group's hydrogel will be necessary, or as another option, finding a commercial hydrogel that suits the needs of the experiments.

Continuing, the next step could be the implementation of shear stresses on the endothelial cells channel. Using a microfluidic pump system to generate a flow that mimics natural physiological conditions to create shear stresses, these shear stresses will cause the cell structure to develop tight junctions and other structures, which are essential to reproduce the characteristics and properties of the blood-brain barrier.

It is also important to achieve immunostaining on the chip, in order to evaluate barrier formation and expression of tight junctions. It is also important to improve the immunostaining protocol on transwell inserts to obtain better images.

Additionally, during the development of all experiments and tests, the chip device should also be improved. Working on aspects such as the seeding of the liquids to make handling easier, improving the design to have a better cell structure, or improvements such as the implementation of electrodes to be able to measure the TEER.

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Fig 7: Author´s graph. Provided by Sara from the research group.

Fig 8 Left: Be-gradient standard BEOnChip. Available at: https://beonchip.com/product/be-gradient-standard/

Fig 9: Author´s graph.

Fig 10: Author´s graph.

Fig 11: Author´s graph.

Fig 12: Author´s graph.

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Fig 22: Author´s graph.

Fig 23: Author´s graph.