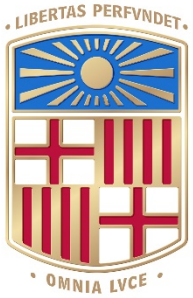

UNIVERSITAT DE BARCELONA

Final Degree Project

Biomedical Engineering Degree

“ Development of an *in vitro* microfluidic platform to mimic the blood-brain barrier ”

Barcelona, 23 January, 2023

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Abstract

There are very few models able to simulate with precision the complex structure of the blood-brain barrier (BBB): our body's most restrictive barrier which, while protecting our brain from pathogens, toxins, inflammation, injury and disease, is also the main obstacle for the delivery of drugs to the brain.

This paper examines all the aspects that have to be taken into account to properly develop, from scratch, an *in vitro* microfluidic platform to mimic the human BBB.

The thesis details every step of the fabrication of the device, provides an economic and technical analysis of its feasibility, and considers the ethical and legal aspects of its implementation to the market.

Following a thorough analysis of the different existing models of the BBB and their benefits, this work develops a design incorporating a tri-culture of human astrocytes, pericytes and endothelial cells ensembled in a 3D environment of hydrogel within a structure of PDMS .

The resulting BBB-on-a-chip (BBB-oC) is an accurate, reproducible, animal-free and cheaper alternative to in vivo models for mimicking the function and structure of the BBB. Evaluation techniques carried out in this project showed a suitable environment for the cells inside the chip, confirming their correct morphology and viability up to the 7th day. Permeability assays revealed that the barrier is size restrictive, thus allowing smaller molecules to pass through faster than bigger molecules.

To provide a clinical application to the model, permeability performance tests were conducted on two different nanotherapeutic systems which target the inhibition of Aβ fibrillation as a possible treatment for Alzheimer's disease.

Keywords: Blood-brain barrier, organ-on-a-chip, nanoparticles, microfluidics

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GLOSSARY OF ABREVIATIONS

BBB	Blood-Brain Barrier
BBB-oC	Blood-Brain Barrier-on-a-Chip
OoC	Organ-on-a-Chip
CNS	Central Nervous System
BMVEC	Brain Microvascular Endothelial Cells (also BMEC)
TJ	Tight Junctions
BM	Basal Membrane
IBEC	Institut de Bioenginyeria de Catalunya
PERT-CPM	Program Evaluation Review Technique - Critical Path Method
PET	Positron Emission Tomography
EC	Endothelial Cell
AC	Astrocyte Cell
PC	Pericyte Cell
HUVEC	Human Umbilical Vein Endothelial Cell
hCMEC	Human Cerebral Microvascular Endothelial Cell
TEER	Trans Endothelial Electrical Resistance
PDMS	Polydimethylsiloxane
PMMA	Poly (methyl methacrylate)
ECM	Extracellular Matrix
hPSC	Human Pluripotent Stem Cells
iPSC	Induced Pluripotent Stem Cells
SWOT	Strengths, Weaknesses, Opportunities and Threats
PDMS	Poly dimethyl siloxane
ZO-1	Zonula occludens-1
VE	Vascular endothelial

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1. Introduction

1.1 Motivation and aim of the Project

As arguably the most important organ in the human body, the brain requires an utterly controlled protection. The human blood-brain barrier (BBB) is a highly selective, semipermeable barrier which ensures the separation of circulating blood from the brain and the central nervous system (CNS) [1].
Composed of brain microvascular endothelial cells (BMVEC), pericytes, astrocytes, neurons, and in close contact with the basement membrane, its function is to protect the brain against pathogens, regulate molecular trafficking and maintain the homeostasis of the brain microenvironment.
These functions are so important that, in fact, most CNS diseases, such as stroke, Alzheimer's disease, depressive disorder, cognitive decline, and central infectious diseases, are associated with BBB barrier dysfunction [2].

The fact that the BBB has such a strong barrier function is actually a major obstacle for the delivery of drugs into the CNS. The tight junctions (TJ) within the brain capillary endothelium are responsible for limiting the permeability to very few molecules of very specific chemical properties: a molecular weight smaller than 400 Da and forms of less than 8 hydrogen bonds are just some of the requirements needed for drugs to be able to penetrate into the brain [3]. This high restriction makes the development of new treatments of brain diseases very difficult.

It is for this reason that *in vitro* BBB models that simulate the barrier's physiological and anatomical structure are of crucial importance. By being able to model, on a chip, the functioning of the barrier, and monitor, in real time, its response to drug delivery we will gain knowledge on how to synthetize small molecule drugs and how to bioengineer the delivery of the drug so that it enters the BBB without losing efficacy or functionality.

1.2 Objectives

The main objective of this project is to develop an in vitro micro-physiological platform that mimics the blood-brain barrier. To achieve this, the following objectives have to be fulfilled:

i. Manufacture the outer structure of the microfluidic chip.
ii. Choose the appropriate type of cells to incorporate into the model, and culture them in a sterile environment.
iii. Assemble the cultured cells into the microfluidic chip. Optimize this process by finding the accurate temperature of the reagents and sizes of the equipment.
iv. Develop precise protocols of each process to follow in the laboratory.
v. Evaluate the structure and performance of the resulting chip with microscopy techniques.

vi. Perform permeability tests to nanotherapeutic systems and asses the clinical significance of the results.

As a result, the final BBB-oC must:

i. Mimic with precision the structure of the Blood brain barrier
ii. Fully integrate living cells without damaging them
iii. Enable the study of its permeability coefficient

1.3 Structure and methodology

This project will be developed in three main parts. Firstly, the elaboration of the in vitro BBB-oC, followed by the culture of the cells and the seeding of these cells into the fabricated chip, and finally the characterization and assessment of the final assembled device.

In order to proceed with these three main focuses of the project, a previous theoretical study on the topic has been performed. Section 2 of the following paper details the main concepts surrounding the BBB and its functionality, as well as the state of the technology of organ-on-a-chip (OoC) and the research and evolution involving this technology.

A market analysis and a study of the possible solutions to be developed are also performed in sections 3 and 4 respectively. To have a knowledge on the economic cost of the project and organize the budget, a GANTT diagram has been designed in section 10.3. This just mentioned section along with the technical viability of the project will determine its feasibility.

A detailed explanation of the protocols to be followed to develop the microfluidic device has been added in the Annexes, although the main information is also detailed in the detail engineering (section 5).

Finally, an analysis of the legislation and regulation surrounding the effects of in vitro chips has also been carried out in section 11.

The whole project has been developed from March 2022 to January 2023, with the lab work being done intensively through the months of June, July and the beginning of August; and its theoretical literature research having been elaborated during the last semester of the 2021-2022 academic year and the following months from June to December 2022.

Its elaboration has taken place at the Institute of Bioengineering of Catalunya (IBEC) in the Nanobioengineering group directed by Dr. Josep Samitier. Along the whole research development of the project, guidance from senior researcher Dr. Anna Lagunas and PhD student Sujey Palma has been given, as well as tutoring from Prof. Manel Puig. .

2. Background

Before the analysis of the market sector surrounding the developed product, it is convenient to present the main theoretical concepts involving the scope of this project, as well as the state of current and previous BBB-oC model technologies.

2.1 General concepts

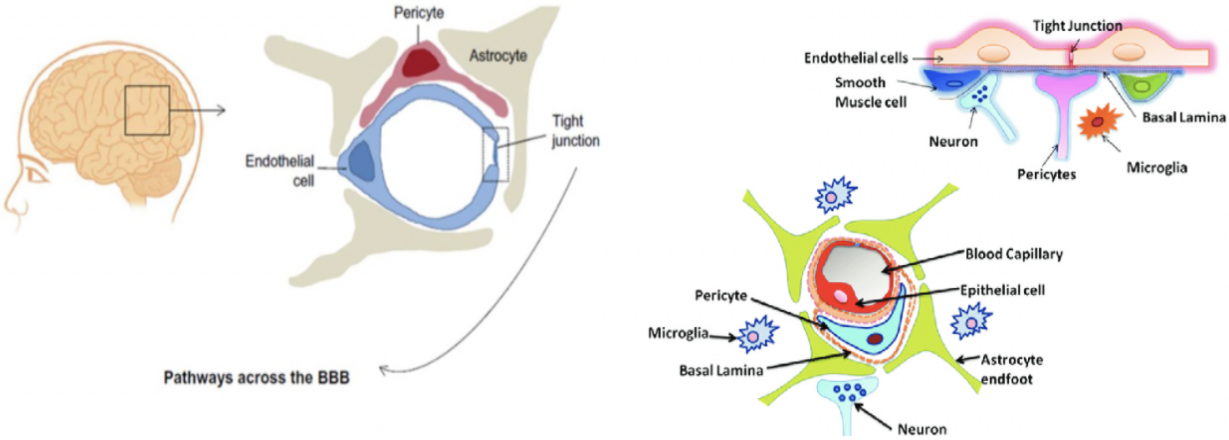
A brief look into the physiology of the Blood-Brain Barrier and the principles of microfluidic device design are exposed in this section for a better comprehension of the topic.

2.1.1 Physiology and Pathology of the Blood-Brain Barrier

The Blood-Brain Barrier is the physical and metabolic barrier that separates the CNS from the peripheral circulation [4]. Its dynamic physiological structure was first discovered in the 19th century by the German physician Paul Ehrlich after injecting a dye into the bloodstream of a mouse, when he realized, to his surprise, that it had infiltrated every tissue except the brain and the spinal cord. It wasn't until the 1960s when, due to powerful enough microscopes, that the physical layer of the BBB was shown. It was then that professionals came to the realization of the great level of protection that evolution has put into our brain; aside from the most obvious (the 7mm thick skull) the brain is also surrounded by protective fluid (cerebrospinal fluid) and the meninges. Whereas these latter provide defense against physical injury, the BBB provides a defense against disease causing pathogens and toxins that may be present in our blood.

The main anatomical constituents of the BBB are endothelial cells and the blood vessels of the vascular system of the CNS. Pericytes, astrocytes, microglia, basement membranes and the extracellular membrane also play crucial roles in maintaining the integrity of the BBB. The key structures that enable the barrier function are the TJ. These unite the endothelial cells in such close contact that only very small molecules are able to pass freely: small and fat-soluble molecules like nutrients, ions, hormones and some gases, are some examples. This selectivity, although strict, allows the brain to maintain relatively constant levels of hormones, nutrients and water, in order to ensure the fulfilment of its physiological function.

Figure 1. Schematic of the components of the Blood Brain Barrier



Schematic of the components of the Blood-Brain Barrier. The endothelial cells form the capillary, and are in contact with Astrocytes and Pericytes. The Tight Junction unites the various endothelial cells and leaves very little space in between.
Source: Panche, Archana & Chandra, Sheela & Diwan, Arvind & Harke, Sanjay. (2015). 'ALZHEIMER'S AND CURRENT THERAPEUTICS: A REVIEW.'

The BBB is dynamic and capable of rapid response to stressors including hypoxia, inflammation, trauma and pain [5].

An increased permeability of the BBB leads to an immediate malfunction and dysregulated influx and efflux due to TJ disruption, resulting in the infiltration of toxins and immune cells to the CNS. This can be caused by bacterial infection, like in meningococcal disease, where the bacteria binds to the endothelial wall and causes the TJ to slightly open. Additionally, many neurodegenerative diseases (Alzheimer's, multiple sclerosis, stroke, depressive disorder, cognitive decline, and central infectious diseases) are also thought to be originated, or at least exacerbated, by BBB dysfunction.

2.1.2 Principles of Microfluidic Device Design

Microfluidic chips require micro-scale engineering technologies to form the channels, chambers, and valves that constitute their structure, using materials such as silicon, glass, quartz or polymers. With the use of micropumps and microvalves, these microfluidic devices allow us to perform precise and complex operations with fluids at the scale of millimeter.
Microfluidic chips that make it possible to perform a series of experiments and analysis on a single chip are called lab-on-a-chip devices.

Each of the channels in a microfluidic chip needs to have a similar width scale to the biological system channel it is trying to mimic (cells, macromolecules, etc.), and through this channel design it is possible to accurately control the flow through the channel.

3D cell co-culture devices and OoC systems have also been developed to mimic entire organs or organ systems. These 3D-devices use perfusion-based media to supply nutrients to the cells and remove metabolic wastes.
The control of the physical and chemical microenvironment around the cells allows a more precise representation of the in vivo conditions of cells and makes the devices well suited for research investigations.

Since microfluidic platforms offer precise control of fluid transport at a microscale, it is a good model to simulate the BBB microenvironment. Microfluidic models provide a promising platform for the study of the functioning of BBB and the evaluation of CNS drugs.
The following section exposes the different designs, strategies and materials that have been explored in order to achieve the best BBB model.

2.2 State of the art

Due to the previously mentioned renowned permeability limitation for drugs entering the brain and the significantly big part of the population that suffer from CNS diseases, there is great interest in developing experimental models that accurately model the BBB.

The interest in modelling the BBB started years ago with the first models being developed around the late 1990s. Since then, many different types of models have been developed, each one focusing on a specific application, and giving insight into a certain property or structure of the BBB. These In Vivo models, In vitro models, and Computer models, will be studied in detail in this part of the project to have an overview on everything that has been developed and its current state.
Additionally, they have been part of the evolution of BBB models and have helped arrive to the development of the microfluidic in vitro BBB microfluidic chips, which are the solution of this project.

2.2.1 *In Vivo* Models

Normally used to perform experiments in living organisms by using intravenous injection, brain perfusion, or Positron Emission Tomography (PET), *in vivo* models have the advantage that the entirety of the experiment occurs under natural conditions and generate very reliable data, because they mimic with precision the complexities of the living physiology: the structure and geometry where the studies are performed are in fact the microenvironment where cells live, this being one of the reasons why these models are called in vivo. Experiments are to be done in animals and this

implies having to translate the obtained results to the human context. Therefore, these types of models must be interpreted as approximations, also because animal to animal variability is a reality, and the experiments have to be done in different animals in order to observe the whole process of disease development, and this implies an expensive labor and animal cost. Although there exist humanized animal models, these are not completely representative for diseases in which genetic transmission is not a majority, like for example Alzheimer's disease. [30]

2.2.2 Computer Models

Computer models are typically used for predictive purposes. Quantitative structure-activity relationships for the BBB activity are built and according to the physical and chemical properties of compounds, the prediction of drug permeability can be done. Normally, results obtained using computer simulations must be verified by *in vivo* experiments and cannot provide information about the effectiveness of a drug to the BBB as their predictions are solely based on drug structure.

2.2.3 *In Vitro* models

In vitro cell culture models have been used for decades to study various mechanisms that support the BBB physiology [6]. Traditional in vitro models involving little more than cells cultured in a petri dish have the advantage of being easy to manipulate and reproducible but cannot replicate the behavior of a tissue nor the transport of a molecule, such as a drug, through its membrane. This is why other, more complex, *in vitro* models have been thought of. One of the most common ones being the transwell models.

2.2.3.1 Transwell models

This type of model uses semi-permeable microporous inserts to culture one or more cell types: its chip design cultures a confluent monolayer of endothelial cells and can include pericytes or astrocytes [15]; creating a compartmentalized-like design that has the capability of resembling more the in vivo conditions of the tissue. For this reason, it is used to simulate the membrane of the BBB; making it easy to distinguish between the blood side and the brain enabling the testing of drug delivery efficiency and permeability.

It is a user-friendly and cost-effective model, making it possible to easily manipulate experimental conditions such as temperature and concentration. This model, however, is unable to replicate some key (complex) characteristics of the BBB: the endothelial cell

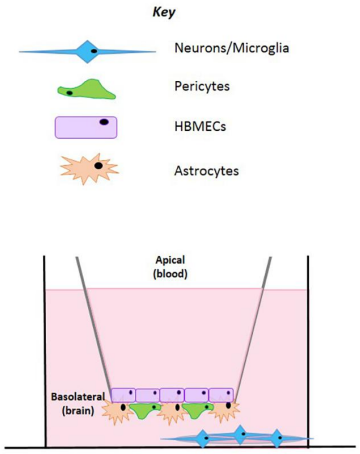


Figure 2. Scheme of a transwell model

monolayer lacks the direct interaction with other cell types and the representation of shear stress, as it is a **static model**[1]. So, it does not capture the complex architecture of the BBB.

2.2.3.2 Humanised dynamic fibre based in vitro models

Humanised dynamic fibre based (DIV-BBB) in vitro models were designed in 2006 [21] to improve the lack of shear stress presented by the Transwell models, and introduced the system of culturing the ECs in the capillaries. This was done because the capillaries allow the inducement of the physiologic levels of shear stress (generated by intraluminal flow) as well as the ability to be surrounded by other chambers that can simulate the different brain regions. This 3D dynamic in vitro model proved to be more reliable and realistic than the previous Transwell model. However, it required a long culture time to reach the transepithelial/transendothelial electrical resistance (TEER), which is a very sensitive and reliable method to confirm the integrity and permeability of the layer by the measurement of the electrical resistance [22]. Moreover, it also made it difficult to observe different cellular behaviours because it integrated all the BBB areas in one chamber, and the large thickness of the wall of the capillaries reduced the contact of the ECs with the other cell components (pericytes, astrocytes, neurons) which reduced the realisticness of the model.

2.2.3.3 Brain slice models

Brain slice models have also been used for the studies of BBB. These models use slices of organotypic hippocampus and culture them on a membrane to study the functions of the BBB under different pathological and physiological conditions. Although they present all cell types and cell interactions, it is not convenient to work with fluorescent immunostaining of biomarkers, which limits the research investigations.

2.2.3.4 BBB spheroids

BBB spheroids are another recent development that consist of a 3D BBB in vitro model with a mixed culture of human primary brain ECs, human primary pericytes, and human primary astrocytes [16]. These three cell types are in direct contact with each other, ensuring a spontaneous self-assembling process. This spheroid model, previously used for the study of tumour cells to analyse the mechanisms of organogenesis [17], liver physiology [18] and cellular viral infectivity [19], is cost-effective and is limited in its ability to recreate a realistic and relevant BBB morphology [15]. However, reproducibility of the experiments is limited, and shear stress would not be represented in all cells.

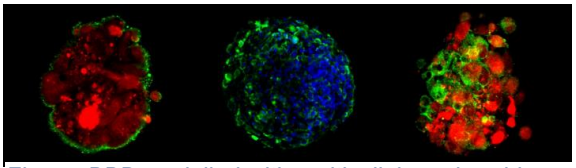


Figure. BBB modelled with multicellular spheroids

[1] See section 4.1 Study of in vitro BBB designs

2.2.3.5 Microfluidic in vitro models

Advances in microfluidic technology and nanofabrication have resulted in the generation of in vitro organ-on-chip (OoC) BBB models capable of mimicking the basic function of the BBB in vivo. They overcome the disadvantages of other in vitro models like transwell or DIV providing a highly controlled cellular microenvironment and being able to assess physiological and pathological responses of the BBB to stimuli.

Astonishingly, in vitro microfluidic BBB chips are capable of incorporating shear stress on endothelial cells, mimicking the in vivo conditions present in the human brain endothelium. This is why microfluidic BBB-model chips have great potential for CNS disease models, drug screening, permeability and neurotoxicity testing, and many other applications. They can also take into account the effects of blood flow in the neural tissue.

In order to properly design a microfluidic in vitro model of the Blood-Brain Barrier, it is essential that it resembles as much as possible to the main features of the in vivo Blood-Brain Barrier. These features are [1]:

- 3D vessel-like structure of the endothelial cells
- Cell-cell interactions
- Flow-induced shear stress on endothelial cells
- Thin basal membrane (BM)

The ideal microfluidic device should accomplish the mimicking of these structures and features in order to provide a reliable model of the BBB. To date, shear stress and cell-cell interactions have been successfully integrated in various in vitro models. However, a faithful replication of the BM is more difficult to achieve: it should have a thickness of 100 nm and be made of biocompatible materials. The BBB models developed to date tend to lack on the reality of the dimensions of the vasculature: the normal in vivo human capillary has a diameter of 7-10µm, while the modelled systems replicate them with a size ~600-800µm, altering realistic transport exchange mechanisms [20].

Different types of designs have been developed to try to find the best possible solution:

Sandwich Design (also called stack configuration)

As one of the first microfluidic in vitro BBB models, it resembles an evolution of the classic transwell design mentioned in the in vitro segment before[2]. It is composed of an upper and a lower PDMS channel separated by a porous membrane. Endothelial cells are located in the upper channel and pericytes, astrocytes and other brain cells are in the lower channel. This method of culturing the different types of cells in the two sides of the porous membrane provides a microenvironment that is similar to the one in vivo: a neural chamber next to a vascular chamber. [31]

[2] See section 2.2.3 "*In vitro* models"

Parallel Design (also called in flank configuration)

In this planar parallel configuration, two aligned channels are horizontally separated by an array of PDMS microchannels. This PDMS barrier simplifies a lot the binding and assembly of different BBB model devices together, and allows a good cell-cell interaction. However, this design still doesn't achieve the desired thickness of the native BM. [32]

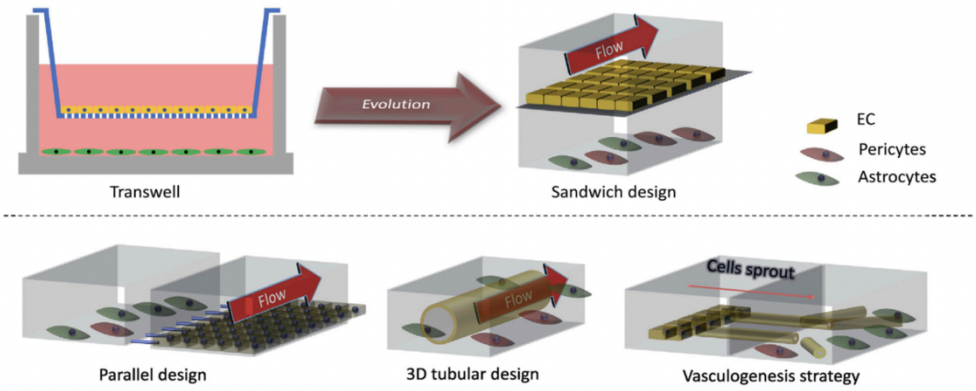
3D Tubular Structure Design [1]

This construction focuses on cylindrical microchannels, which improves the constant shear stress along the inner walls.

Vasculogenesis Design [1]

This less common approach aims to reconstruct the microvessels de novo, as opposed to the majority (and the previously mentioned) models where the microvessels are predetermined scaffolds like microfluidic channels.

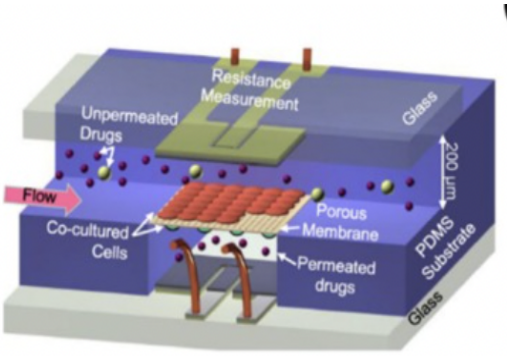
Figure 2. Design models for the BBB



Source: A. Oddo, B. Peng, Z. Tong, et al., Advances in Microfluidic Blood-Brain Barrier (BBB) Models, CellPress, 2019.

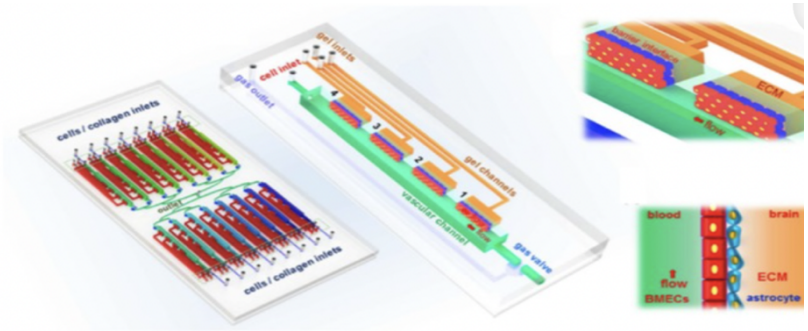
Other typical designs include chips composed of 16 independent functional units where each unit consists of four BBB regions that mimic the whole BBB, shown in Figure 3 and multi-layered channel structures that add two sets of electrodes and obtain dynamic flows with a PDMS substrate, as represented in Figure 4.

Figure 3. Chip with 16 independent functional unit



Source: Xu H., Li Z., Yu Y. et al. A dynamic in vivo-like organotypic blood-brain barrier model to probe metastatic brain tumors. Sci Rep 6, 36670 (2016).

Figure 4. Multi-layered channel with PDMS substrate and electrodes



Source: Jiang, Lili & Li, Shu & Zheng, Junsong & Li, Yan & Huang, Hui. (2019). Recent Progress in Microfluidic Models of the Blood-Brain Barrier. Micromachines.

Figure 5. Comparison of the size of a microfluidic device to a coin

Source: M. Campisi, Y. Shin. T. Osaki, V. Chiono, R.D. Kamm, 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, perycites and astrocytes. Biomaterials Elsevier, 2018.

As it has been shown, many microfluidic models have been designed with different structures and for different purposes, most with a porous membrane segmentation and a separation of the culture of endothelial cells and the rest of the cells (astrocytes, neurons), but in general each design varies from the others.

Also, we have seen that while in vivo models mainly shed light into the behavioral, structural, and systemic effects of BBB disruption, *in vitro* models achieve to identify key cellular, molecular

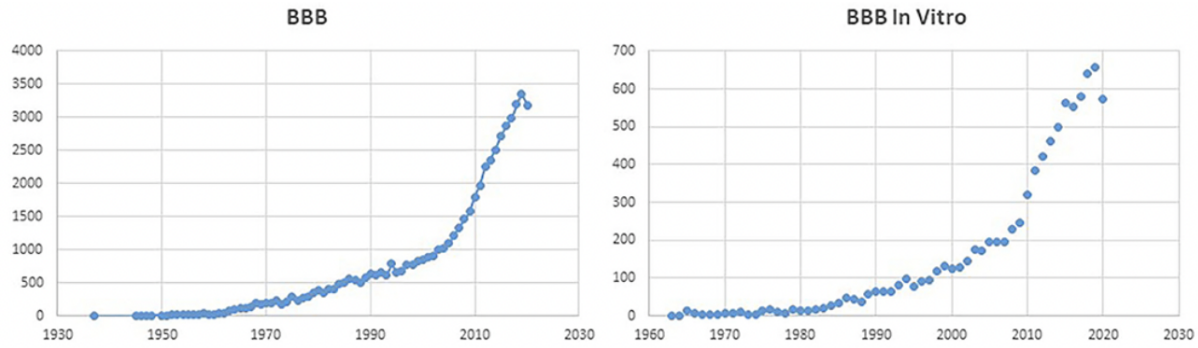
players, targets and regulators that facilitate the comprehension of the fluidity, regulation and integrity of the BBB and its effect on tested drugs but can also lack the ability to represent complex structures. As opposed to the traditional in vitro systems in which cells are simply cultured in a Petri dish and no accurate representation of the tissue microenvironment is achieved, microfluidic in vitro systems such as OoC successfully solve this problem: designs engineered to incorporate mechanical, geometrical and biochemical factors that simulate the *in vivo* environment of the tissue whilst it all being captured in an in vitro platform. These devices get much closer to the characteristics that were envied from animal models by also adding them to their structure, making the benefits of working with them in vitro many more than the problems that in vivo animal modelling proposed.

2.3 State of the situation

As we have seen, the situation regarding the development of new possible models to simulate the BBB have been on the rise for several years now.

Medical progress has historically depended on scientific discoveries, and in the case of diseases involving the brain and CNS, this progress is in utter need, as many of these diseases have no cure and are little known. This has boosted the investigations and the popularity of new approaches to model the BBB. The following figure illustrates this mentioned exponential increase for BBB related topics.

Figure 6. Number of PubMed searches for the terms BBB and BBB In Vitro



Source: Williams-Medina, Alberto & Deblock, Michael & Janigro, Damir. (2021). In vitro Models of the Blood–Brain Barrier: Tools in Translational Medicine. Frontiers in Medical Technology.

The complexity of the BBB and the major obstacles it creates for brain drug delivery, considering that the BBB is the primary cause of treatment failure leading to disease progression [8], the development of an ideal platform which can predict the behavior of a drug once delivered is extremely crucial. It is for this reason that the situation surrounding the development of BBB models is constantly evolving.

The first reported use of a Transwell apparatus was in the early 1990s. Since then, the model has changed significantly by modifying the type of materials employed, adding filters to allow porosity and allowing cell-cell interactions. Dynamic models[3] of the BBB have gained importance as they can better address the complexity of tissues and organs. recently, a new study [7] has proven that the use of ultrasounds can temporarily open the BBB and facilitate the transport of drugs, giving rise to a new non-invasive and localized method of drug administration.

Next-generation in vitro BBB models involve spatial organization of different cell types in 3D microenvironments. These models are a consequence of the advances in stem cell technology and tissue engineering. Truly promisingly, next-generation BBB models improve accuracy of gene expression of cell sources and microenvironments so that the models can achieve high human-specificity (age, sex, ethnicity), as well as disease state-specificity and brain region-specificity [9].

With the increasing interest in BBB models and the rapidly evolving technology, the situation surrounding models to mimic the BBB is promising. Great amount of research has been done and various developments have been found, so there is plenty to work with and improve so that we can finally obtain the ideal and accurate model for the BBB.

[3] See section 4.1 “Study of in vitro BBB designs”. In this section, dynamic models are more thoroughly explained and discussed.

3. Market Analysis

Now focusing only on the BBB type of model developed in this project, we will look into the influence of the *in vitro* microfluidic BBB-oC models in the market and analyze their evolution through time as well as their possible future perspectives.

3.1 BBB-oC Market Sector

The market sector contemplated for our *in vitro* microfluidic BBB-oC model is the biomedical research industry, as well as the clinical research industry for hospitals. The device will be used for (i) research studies involving the testing of drugs to deliver to the brain for those people with incurable neurodegenerative diseases, such as Alzheimer's Disease, (ii) the study of the physiological mechanisms and pathophysiology of many diseases, (iii) the analysis of methods to improve certain aspects of already existing drug treatments, for instance the reduction of secondary effects .

By being able to perform permeability tests and drug delivery assessments on our BBB-oC, we will be avoiding having to use animals as subjects to study, therefore we will not only be creating a cruelty free environment but also a more accurate obtention of results, as our model will be focused on human studies and human cells, and we will be eliminating the lack of specificity caused by the translation of results from animal to human. The BBB-oC is also fit to mimic the complex system that is the BBB, providing with more reliable data whilst being a much more accessible, reproducible and easy to analyse method than in vivo experimentation on animals.

In vitro microfluidic BBB-oC models can be applied to the testing of drug delivery, as mentioned before, but also to drug discovery, personalised medicine, toxicology, and brain research in general [1]. The chosen model enables cell co-culture and is suitable for high resolution imaging; it can integrate sensors and electrodes to improve its performance and provide opportunities for single cell manipulation.

Different studies have been developed using *in vitro* microfluidic BBB-oCs and have proven significant remarks:

- An establishment of a human BBB co-culture model mimicking the neurovascular unit using induced pluri and multipotent stem cells research done in 2017 by Appelt-Menzel et al. [10] demonstrated the effectiveness of penetration of diazepam, ibuprofen, caffeine, celecoxib and other brain disease related drugs on the BBB model.
- Another study performed in 2018 by Montaux et al. [11] showed how a PDMS chip combined with microelectrodes captured the synaptic activities and dynamic process of rat primary cortical neurons.

- A free-filtering BBB chip constructed by human Brain Microvascular Endothelial Cells (BMECs) showed efficient filtration effects of different drugs through the BBB. These drugs penetrated with sufficient concentration to the diseased part of the brain, being able to have the expected therapeutic effect [12].

There have been several patents granted for different *in vitro* BBB designs:

- "Blood-Brain barrier model" patented by Eric V. Shusta et al. in Wisconsin Alumni Research Foundation, Application No.: 13/218,123. The model comprises culturing primary brain microvascular endothelial cells upon a permeable support in the presence of neural progenitor cells. [13]
- "Blood-Brain barrier model" patented by Athena Neurosciences Inc., Application No.: 91/05038. *In vitro* model of a BBB comprising a porous solid support upon which is disposed of a confluent monolayer of brain microvascular endothelial cells. [14]

Moreover, research of OoC in similar fields have been also developed and have provided insights into possible solutions for other diseases, exaggerating the potential of the OoC technology, not only on BBB modelling. Some of the examples that show this potential are:

- The detection of thrombotic risk in vessels-on-chip [24]
- The discovery of targets for metastases in cancer-on-chip [25]
- A test for kidney toxicity in kidney-on-chip [26]
- Drug effects on neurons and glia cells-on-chip [27]
- Drug discovery in disease model for ALS [28]

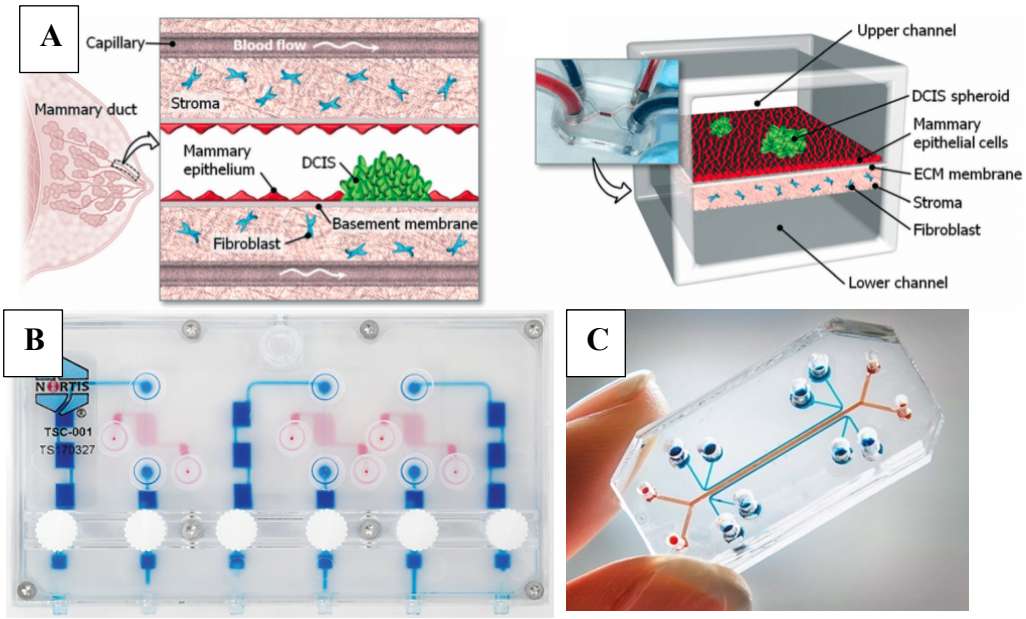


Figure 7. A) Breast tumor-on chip, B) kindey-on-chip, C) Blood vessel-on-chip

These OoC studies have provided evidence that this type of technology is promising and can approach studies in a much more cost-efficient manner, as well as an accurate and precise methodology. BBB-oC have been already designed and tested, proving to be an affordable solution for drug-targeting and human organ modeling.

Despite the market already having various models for *in vitro* BBB microfluidic chips, no ideal model capturing with precise accuracy the physiological, anatomical and pathological microenvironment of the human BBB has been developed. In fact, having so many BBB-oC designs generates a huge variability in terms of comparing test results between them, as each design incorporates different cell types, coatings, device material, membrane thickness, and size of the channels and chambers of the chip. These differences generate variation on the results of the permeability tests for the same drug, and therefore a consensus has to be reached among researchers for future high-throughput screening applications of the BBB-oC for more industrial processes of drug testing; for instance, the establishment of a universal value for the permeability coefficient that the modelled BBB should have in order to be proven effective, and for the value of the human BBB permeability. Other aspects like the usability of the devices (if they are ready to use or their cells need a prior differenciation), the incorporation of sensors, and the compatibility of the devices to be observed through microscopy are key aspects that prove that the development of new BBB-oC is in utter need.

3.2 Historical evolution of *in vitro* BBB models

As it has been shown in previous sections, the efforts in generating *in vitro* BBB models started several decades ago with the increasing interest in finding treatments for the wide part of the population affected by neurodegenerative diseases.

The years of model development have resulted in a range of well-established and characterized models created in different laboratories. The first models were based on pig, bovine, rat, and mouse endothelial cells and allowed a significant amount of information on the physiology and pathophysiology of the BBB. However, as stated before, the animal-to-animal variance and the conversion to human data became a problem. For this reason, human tissue started to be used. Although this latter form of tissue is more difficult to obtain and leads to more ethical precautions, human brain endothelial cells provide more accurate results.
The different models generated during the past 40 years behave in non-identical ways, leading to small differences in the way small laboratories treat the results and come to conclusions, although all validated models continue to be used and have obtained valuable research data.

For instance, the different microfluidic design models for BBB exposed in section 2.2.4 have all suffered changes through the evolution of the market: the sandwich design has gone from being a 2D design in 2012 to a 3D design able to mimic metabolically critical physiological functions of the neurovascular unit in 2018 [1]. Similarly, the parallel design has gone from using PDMS as a substrate and being a 2D model in 2012 to a 3D design with a basis of Extracellular Matrix (ECM) in 2018 [1].

The progress of the development of microfluidic devices has enabled the knowledge of the field to grow, getting research much closer to finding important breakthroughs on the delivery of drugs for the CNS.

3.3 Future perspectives

Microfluidic chip models exhibit unique advantages in the BBB research. Generating *in vitro* models reflecting the properties of the BBB can, and will, revolutionise the treatment of many neurodegenerative diseases, as well as the discovery of new drugs available for treatment, and the possibility to create a much more personalised medicine for each patient, by using patient-derived cells in the BBB-oC and therefore having direct insight on how the drug is targeting that patient's body.

New developments will make possible the creation of the ideal *in vitro* model of the BBB, which will allow mechanistic studies of the BBB TJ, transporters, enzymes, cell trafficking and signaling to be suitable for rapid and effective screening of BBB permeability for new CNS and brain drug candidates.
An improvement of the evaluation system for drug toxicity and safety will also be given by the future *in vitro* models, leading as well to the successful launch of new drugs on the market.

Another future application of *in vitro* BBB microfluidic models can be an integrated deep learning system to the microfluidic chip, in order to avoid the errors of system and human factors [1]. Therefore, the combination of multiple technologies may further optimise the applications of BBB microfluidic chips and can bring even more technological innovation to human brain research. Similarly, the combination of *in vitro* BBB modelling with known genetic markers of diseases may will give ground-breaking insight into BBB pathobiology [9].

Moreover, a very promising future for the design of microfluidic BBB models is related to the type of materials used as substrates for the cellular growth: graphene's properties have been recently studied to increase transmural pore size and electrical conductivity, which may be beneficial to improve the passage of nutrients and drugs across the barrier [9].

Also, as mentioned, one of the most attractive applications of BBB-oCs is the use of patient-derived cells which can improve the predictive value of the models for drug delivery.

4. Conception Engineering

As it has been shown, the importance of modelling the human BBB has led to the development of various different types of models and designs. However, this project aims to implement only one of them. In order to choose which model is best for this project, a further study of the possible solutions has been done. The proposed solution will not only be based on the positive aspects of the model itself but also on the realistic possibility of a fourth-year degree student being able to develop it.

The previous study of the state of the art technology in section 2.2 exposes the many different types of models that have been created to simulate the BBB. These models not only differ in the design but also in their applications. It is for this reason that an evaluation of their advantages and disadvantages is needed in order to properly choose the type of BBB-model that will be implemented in this project.

The following table demonstrates the main characteristics (positive and negative) of the three big groups of models available to simulate the function, structure and physiology of the BBB.

Type of model	Advantages	Disadvantages
Computer	Can be used to predict BBB permeability	Results obtained need to be verified by in vivo experiments Does not provide information about the effectiveness of drugs
In vivo	Closely mimics the physiology and microenvironment of the BBB Can be used to predict the outcomes of new drugs and therapies Generates reliable data	Experiments have to be performed in animals Results need to be translated into human conditions, so accuracy is lost (discrepancy from the human BBB) Animal to animal variability High cost and labour Low efficiency for high-throughput screenings
In vitro	Highly efficient Easy to construct and manipulate experimental conditions such as temperature User friendly and cost-effective Some of the models mimic with precision the main characteristics of the human BBB Very technologically advanced	Not all *in vitro* models replicate the cell-cell interaction of the BBB Some models lack the representation of the shear-stress

Table 1. Schematic representation of the three main modelling options *for the BBB*

Having analysed these three big types of models, and evaluating the options for each one, this project will develop an *in vitro* model for the BBB, as it is the more advanced technologically and it allows the best simulation of the human barrier compared to the computer and in vivo models, being a cheap, accessible (performed in the lab) and accurate representation of the tissue. Moreover, it offers a great number of applications and the future holds many new opportunities for its development.

4.1 Study of *in vitro* BBB designs

Now that we have established that the model implemented will be *in vitro*, we have to perform a further selection of the different types and characteristics of *in vitro* models, as there are many and each one provides different properties. It is important that in the choosing of the type of *in vitro* model we evaluate the characteristics and properties we think fit best to the application we want to give to our BBB model, because, as we have seen, the structure and design of the model not only depends on the level of technology but also on the application it serves.

4.1.1 Static and dynamic models

One of the main features that defines *in vitro* models is being static or dynamic.

Static *in vitro* BBB models have been used for many decades and have allowed us to understand the basic activities of the functioning of the human BBB. The models are easy to build and have been widely used for simulating the biomolecule transport through the BBB *in vitro*. However, these static models need to be integrated with microfluidic devices to reveal the mechanism that the BBB uses to function in the presence of shear stress. This shear stress representation is very important to have in models, as it is a key feature of the human in vivo BBB. The lack of this feature results in the model to be less realistic.

The most typical static *in vitro* BBB model is the Transwell Model, also mentioned previously[4].

Dynamic *in vitro* BBB models, on the other hand, include the feature of shear stress. They generate more reliable data and allow the coculture of the different types of cells of the BBB (ECs, astrocytes, neurons, pericytes) and do not inhibit the crucial function of TJs. Dynamic models better simulate the complexity of the BBB.

DIV-BBB and Microfluidic BBB models are two of the most common dynamic models.

4.1.2 2D and 3D configurations

Another important aspect that determines the type of *in vitro* model is the fact that the microenvironment can be 2D or 3D.

[4] See section 2 “State of the art”.

In vivo, cells are organised in a 3D type of structure. This type of organisation is required for the correct development of cellular processes like differentiation; moreover, positioning effects and polarisation have an impact on how cells interact with each other [23]. This is one of the reasons why 2D models fail to recapitulate, with accuracy, the architecture of the BBB, because, in 2D *in vitro* BBB models, ECs differentiate spontaneously and acquire a cell phenotype which is much more generic, losing the key BBB-like properties.
3D *in vitro* BBB models are a step closer to the ideal BBB model, as they allow all cell types to be in direct contact with each other. This feature is one of the keys to properly mimic the in vivo human BBB, as we have seen in previous sections.
Moreover, endothelial cells and pericytes form 3D regular structures on their own, as they are cells prone to adhesion. This is an extra opportunity that 3D models can simulate and 2D cannot.
2D systems also ignore the 3D structure of blood vessels, and this consequently produces a lack of interaction between cells and their environment.

4.1.3 Cell type

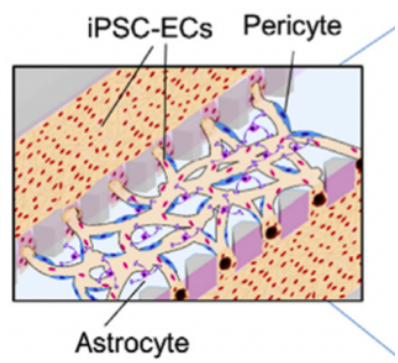
The type of cells that are used in the cultures have to be defined. These cells will determine the behaviour of the model and its similarity to the in vivo BBB. First of all, an important decision to make is if the introduced cells will be of human or animal origin. As we have mentioned before, while co-cultures with cells from different species are advantageous in terms of accessibility and ease of genetic manipulations, cross-species compatibility remains a problem. Having human cells is a much better option as these compatibility problems disappear, but the availability of human cell sources is in this case one of the problems to consider. To solve this, immortalised cell lines are used, although it has been seen that they make TJs to be less stable [22].
Human cells offer a better BBB phenotype [33]. Primary ECs are the main cell type that maintains the physical barrier between the blood and the brain.

Models can also choose to have a co-culture of cells or a uni cell culture: having cells from different types coexist with each other in the model offers a much more realistic microenvironment, as this is what happens in our brain.
Human Umbilical Vein Endothelial Cells (HUVEC) are the most common EC sources for research, as they can form barriers with the desired permeability. However, they offer a poor model for cerebral vasculature [15], as they are umbilical endothelial cells. It is for this reason that the equivalent source of EC to implement in brain models are Human Cerebral Microvascular Endothelial Cells (hCMECs) These are human cerebral microvascular endothelial cells. They are

easy to obtain and culture in the lab, and they provide good properties to imitate their function *in vivo*. However, some studies [34] have shown they are lack some properties to reproduce the BBB. Another option is to use hPSCs: we can derive astrocytes, pericytes and neuronal cells from them, and they can be used in co-culture systems, in which they show presence of many TJs, and with this composition, the barrier has similar characteristics to the BBB in vivo.

The use of Induced Pluripotent Stem Cell Derived ECs (iPSC-ECs)[5] has the advantage of studying endothelial physiology in different conditions and states of cell differentiation, and they can be co-cultured with brain PCs and ACs.

Figure 8. Example of a co-culture of iPSC-ECs with PCs and ACs



The image shows the microvascular 3D network that these cells adopt when together, mimicking the BBB structure in vivo.

Source: M. Campisi, Y. Shin. T. Osaki, V. Chiono, R.D. Kamm, 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, perycites and astrocytes. Biomaterials Elsevier, 2018.

4.1.4 Scaffold materials

For the model to be realistic, and therefore recapitulate all the features of the *in vivo* BBB, there has to be a concrete choice of the materials used, especially for the case of the basal membrane, which has to have a thickness of ~100 nm and be made of biocompatible materials.

Some of the options for the materials are:

[5] "Induced pluripotent stem (iPS) cells are a type of pluripotent stem cell derived from adult somatic cells that have been genetically reprogrammed to an embryonic stem (ES) cell-like state through the forced expression of genes and factors important for maintaining the defining properties of ES cells." - Lei Ye, Cory Swingen and Jianyi Zhang. Induced Pluripotent Stem Cells and Their Potential for Basic and Clinical Sciences. Current Cardiology Review. Feb 2013

- PDMS: it is cost effective, can be easily shaped, it is biocompatible and optically transparent. This last property facilitates the visualisation with imaging techniques. It is also non-toxic nor flammable, it is gas and water permeable and has a fair amount of flexibility.

  However, PDMS presents a hydrophobic surface, which implies that it needs to be modified in order to be used for the BBB models, and this modification can lead to the leakage of non-cross linked monomers into the culture medium.
- PMMA: It is transparent and thermoplastic, it has better light transmission and a higher chemical stability than PDMS. It is also more compatible with organic solvents. One drawback of this material is that it is difficult to process into complex micro or nano structures.
- ECM gels: using Collagen is another very plausible material option. These gels have similar properties to the basal membrane of the *in vivo* BBB, and the characteristics of hydrogels can be easily modified by changing the materials: biocompatibility, porosity and stiffness can be changed by the degree of crosslinking, the composition of polymers and the type of treatment they are submitted to.

  Hydrogels are fitted for co-culture systems, as they can be loaded with astrocytes, pericytes and neurons.

Combining materials such as using PDMS + collagen gel, or PDMS + 3D printed plastic is also a possibility. Additionally, Polyethylene terephthalate (PET) and polytetrafluorethylene (PTFE) are very good in terms of transparency, they offer a good observation.

The following table shows a brief summary of the mentioned options to consider and the choices to make in order to start the design of an *in vitro* BBB.

FEATURES	POSSIBLE OPTIONS			
TECHNOLOGY OF THE MODEL	Static		Dynamic	
STRUCTURE OF THE MICROENVIRONMENT	2D		3D	
TYPE OF CELLS	Human Animal	Co-culture Uni-culture	hCMEC, HUVECs, hPSCs or iPSC-ECs	
TYPE OF MATERIALS	PDMS	PMMA	ECM gels	Combinations

Table 2. Schematic of the recompilation of choices to be made to develop an in vitro BBB model

4.2 Selected *in vitro* BBB design

Once all the possible solutions have been thoroughly analysed taking into account their characteristics and their inconveniences, a final decision has been made considering the application of this project and the fitting of each of the options to this application.

Considering the importance of designing a BBB model capable of portraying the most physiologically relevant structures, with the most accuracy in the realisticness of the features presented *in vivo*, the model that fits best is clearly a **dynamic** model. From the different types of dynamic models, a **microfluidic** system has been chosen, as it can incorporate a platform where different cell types are in contact with each other, as opposed to the one chamber mechanism of the DIV-BBB. Additionally, the thickness of the wall in most microfluidic models resembles more the actual *in vivo* size, allowing the mentioned contact between the different BBB cell types.

The structure of the microenvironment will be **3D**, as it is the type of organisation required for the correct development of crucial cellular processes that determine the behaviour of the cells in the model, and it will better capture the architecture of the BBB.

The model will incorporate **human endothelial cells (hCMEC/D3)** that will be in **co-culture** with **human brain PCs (HP) and ACs (HA)**, specifically: human hippocampal astrocytes (HA-h) and human brain-vascular pericytes (HBVP) (all of them are commercialy available), to mimic the organisation and structure of the brain microcirculation observed *in vivo*. Moreover, hCMEC/D3 have been selected as they are easy to obtain and culture in the lab, as opposed to iPSC-ECs, which require a very strict process to control the differentiation of fibroblasts into brain endothelial cells. Moreover, for the research application that our model aims to have, it is not necessary to work with a specific patient's cell; if that were the case (if the model was aimed to be used for therapeutic reasons), iPSC-ECs would be of more interest as we could directly isolate the patient's fibroblasts and differentiate them into ECs, being able to obtain information about how that patient responds to certain drug treatments. If this were needed in the future, this cellular model could be incorporated into our chosen configuration due to its versality.

The 3D microfluidic system will be composed of **PDMS** using standard photolithography techniques, and the structure design of the system will be a single layer central chamber with two fluid side channels. The channels, representing the blood vessel wall, will be connected to the central chamber, simulating the brain, with small triangular structures that leave very little space between them. These triangular structures will perform the barrier function once the cells and **hydrogel** are added. This design offers holes of different sizes as ways of entry to the 3D structure. This structure corresponds to a **parallel type** of design, in an in flank configuration.

The HA-h and HBVP will be seeded in the central chamber (simulating the brain) and the hCMEC will be in the side channel. The HA-h and HBVP will be introduced with a fibrin hydrogel (simulating the ECM) so that the central chamber will have a certain depth and create a 3D structure. Within this hydrogel, the hCMEC/D3 will be able to adhere to it, creating direct contact between the three types of cells. The small triangular structures will inhibit the HA-h and HBVP to filter to the side channel, enabling a clear separation between the brain and the blood spaces.

Considering it all, our proposed solution consists of a 3D BBB microfluidic model developed from human endothelial cells co-cultured with human brain PCs and ACs, with a design that ensures dynamic and direct cell contact with each other.

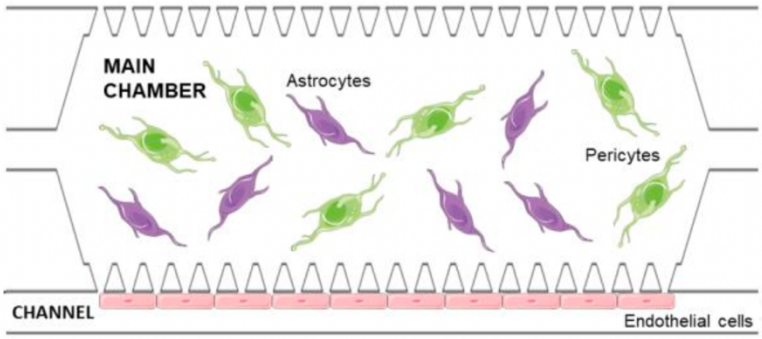
The following table shows a schematic of this proposed model, with the solution highlighted with respect to the other options previously considered.

FEATURES	POSSIBLE OPTIONS			
TECHNOLOGY OF THE MODEL	Static	**Dynamic**		
STRUCTURE OF THE MICROENVIRONMENT	2D	**3D**		
TYPE OF CELLS	**Human**	Uni-culture	**hCMEC**	
	Animal	**Co-culture**	HUVECs or hPSCs or iPSC-ECs	
TYPE OF MATERIALS	PDMS	PMMA	ECM gels	**Combinations**

Table 3. Comparative schematic of the proposed solution. All considered options are shown, while the selected options are highlighted.

Additionally, figure 8 shows the visual schematic of what the design is expected to have.

Figure 9. Schematic of the design of the chosen microfluidic OoC



This is a detailed schematic of the microfluidic BBB-oC that will be developed in the lab. On the lower side channel, endothelial cells are seeded and adhered to the wall of hydrogel that limits, along with the triangular structures, the brain chamber seeded with astrocytes and pericytes. Culture medium will be introduced and changed daily through small holes, not depicted in the figure, that are in contact with the channels.
Source: bioRxiv 2022 Sujey Palma et al.

5. Detail Engineering

In order to carry out the project and actually put the theory into practice, a series of steps have to be taken. This section will explain in detail all these necessary processes and protocols that have to be followed in order to obtain a functional model of the BBB in a microfluidic device.

As it has been mentioned throughout this paper, the *in vitro* model of the BBB consists of the implementation of cells into a microfluidic device that simulates the properties, structure, and functions of the *in vivo* BBB. Therefore, the first step of the process is the design of the device. This design mainly takes into account the morphology of the barrier: we want to simulate the BBB, therefore, we need the brain chamber to be connected, through a restrictive membrane (the barrier), to the blood vessel. Once this design is complete, we need to fabricate the device. This fabrication includes the addition of a semi-solid substance into the mould containing the blueprints of the design so that it can become a 3D chip with the functional chambers and channels. It will be in these chambers and channels where we will need to add the cells, as the device without the cells is just a scaffold. The introduction of the cells into the device will truly transform it into *the in vitro* model we are aiming for. These cells, carefully selected and cultured, will be introduced through the holes that the design presents and will adopt a functional role in the chip, giving biological sense to the fabricated *in vitro* BBB model.

Once the device is fully simulating the BBB with its cells and ECM, we will need to characterize it to evaluate its performance. Different techniques such as fluorescent microscopy, bright field microscopy and permeability assays will be carried out in order to visualize and quantify the goodness of the model, as well as its similarity to the *in vivo* BBB.

Finally, to assess the possible clinical application of the model, a drug delivery nanosystem will be tested through the fabricated BBB-oC. The permeability of the barrier will be computed with and without the drug in the nanosystem, so that a conclusion can be reached on the capacity that the

drug has to cross the barrier. Additionally, a previous evaluation of the state of the chip will be performed through imaging techniques.

As a result, these three aforementioned processes:

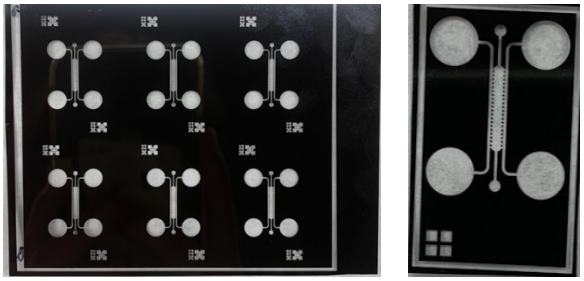
1. fabrication of the microfluidic device,
2. culture of cells, and
3. seeding of the cells into the microfluidic device,

Will allow the full development of the BBB-oC as a viable product that can be submitted to tests and assays that will evaluate the goodness of the model. These evaluation experiments will be discussed in the following section. Now, the three main steps of the BBB-oC production will be thoroughly described.

5.1 Fabrication of the microfluidic device

Firstly, the device is designed with AutoCAD[6] and printed as an acetate mask. This mask is treated with photolithography techniques so that a master (mould) is obtained. This master (Figure 9) consists of a very thin silicon wafer with the design engraved in it.

Figure 10. Master with 6 designs engraved with photolitography



The main chamber (simulating the brain) can be distinguished, connected through little openings to the blood vessel chambers. The circles correspond to the holes from where the cells will be introduced into the device. Source: myself, in IBEC lab

In order to create the 3D chip into which the cells will be introduced, it is necessary to transfer this design into a PDMS structure with soft lithography: first, we prepare the PDMS mix and then we pour it into the master/mould. This structure (Figure 11) is then cured in the oven for 2 h at 65 °C.

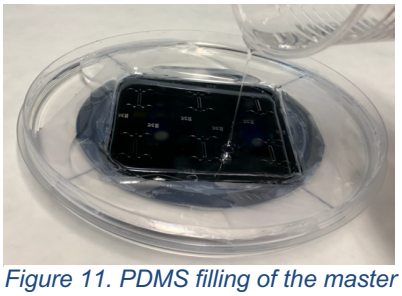


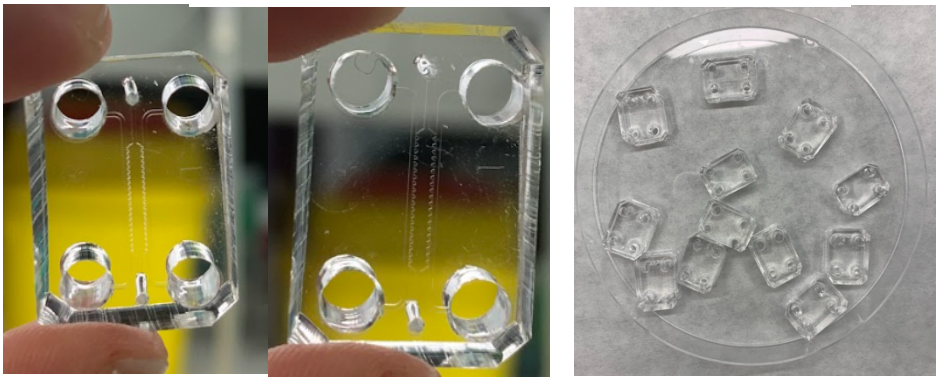
Figure 11. PDMS filling of the master

[6] ***AutoCAD*** *is a commercial computer-aided design (CAD) and drafting software application. Developed and marketed by Autodesk. -* https://en.wikipedia.org/wiki/AutoCAD

The preparation of PDMS is done in a 10:1 ratio (elastomer: curing agent (CA)). The role of the curing agent is to create a much more dense and solid-like substance. Each mould should have approximately 40 g of PDMS (36 g elastomer + 4 g CA). Each mould has 6 individual device designs engraved.

Once the PDMS has cured, the design engraved on the mould will have been successfully transferred into the PDMS structure, and we will have obtained the scaffold for our device. The following step is to carefully extract the solidified PDMS from the mould (this mould is reusable if not damaged and can be used to create more scaffolds) and cut the individual designs. On each scaffold, with the use of an awl, we pierced the holes. These holes will be the passage of entry of the cells into the simulated BBB.

Figure 12. Solidified PDMS scaffolds



The design is engraved and the channels connect to the open holes. Dust particles and dirt can be clearly observed, these pictures are taken before the cleaning process in clean room conditions. Source: myself, in IBEC lab

Before adding the cells, it is crucial that these device scaffolds are bound to a surface: a glass coverslip, so that when the cells are introduced there will be no leakage. The bonding process is done in a clean room and through an irreversible oxygen plasma treatment[7] for 30 s at high mode (10.5 W).

Finally, the microdevices are left in the oven overnight at 85 ºC.

The exact protocol to follow for the fabrication process of the microfluidic device is shown in Annex 1.

5.2 Culture of cells

The culture of cells is necessary so that when we introduce the cells into the microfluidic device, they are confluent and functional.

[7] Low pressure plasma activated bonding consists of a process that bonds two surfaces together with the use of vacuum, high frequency electrical field between electrodes and process gases. These components, combined with the manual change of oxygen and pressure levels inside the machine allows the added oxygen to bond the two inert surfaces (PDMS and glass, normally made of Si-O components) together, liberating radicals of the surface and hydrogen bonds, so that when the process is over, the surfaces can bond together.

Cells need certain conditions to stay alive, and *in vitro* these conditions need to be strictly kept so that the cells are tricked into thinking they are still inside the human body.
The chosen cells to culture are:

- HA-h, passage 2, freezed on 8/03/2022
- HBVP, passage 3, freezed on 22/10/2021
- hCMEC/D3, passage 3, freezed on 19/02/2018

These cells are obtained from commercial sources and all treatments and cultures are performed in a sterile culture hood (class II) with strict disinfection.

The preparation and culture of each type of cell starts with supplementing the medium with specific substances that enrich it and give it properties more to the liking of the cells, to ensure their survival and functioning.
The HCMEC/D3 medium was supplemented with endoGRO™, a prepared culture media kit with basal medium and supplements that prevent masking effects, cell stress and any possible damage that can influence the experimental results. Apart from endoGRO™, the medium was additionally supplemented with antibiotics and FGF-2.
Similarly, HA-h and HBVP mediums were supplemented with Fetal Bovine Serum (FBS)[8], antibiotics and astrocyte / pericyte growth supplement respectively.
Antibiotics are frequently added to the mediums to prevent contamination. Although the working conditions are very strict in terms of disinfection, bacteria could still appear and modify the growth of the cell population, causing an alteration in the results obtained.

Following the preparation of the mediums comes the coating of flasks. The flasks are the recipients where the cells will be introduced to, and they require a specific coating on their surface so that when the cells are poured, they can properly adhere. This coating mimics the ECM of cells. As it has been mentioned previously, apart from the importance of the ECM on being 2D or 3D, it also plays a crucial role[9] on the development of cells as it is in constant close contact with them, and it is composed of various proteins. One of them being collagen.
In this case, the flasks were coated with collagen Type 1, extracted from rat tail at room temperature. After the flasks are coated and the collagen has adhered to the surface, the remaining liquid has to be aspirated. In the case of Ha-h and HBVP cells, the coating is with poly-L-lysine.

[8] FBS provides hormonal factors, attachment and spreading factors, as well as transport proteins.

[9] It has been seen that the ECM interferes in the survival, the integration, adhesion, and mechanical forces the cell is submitted to, as well as the stimulation of different signaling patwhays.

Once the flasks are ready to host the cells, these have to be prepared. Cells are normally freezed in liquid nitrogen once they arrive from the provider so that they are cryopreserved. Therefore, to use them the first step is to unfreeze them. Once they are completely thawed, they are transferred to a conical tube and centrifuged. The pellet is removed and resuspended with the previously prepared medium (each type of cell with its respective medium). Afterwards, the cell suspension is placed onto the pre-coated flask and incubated at 37 ºC in a 5% pCO_2 humidified incubator.
The medium needs to be changed the next day, and every two or three days thereafter.

Once the cells are approximately 80% confluent, which normally happens around the fourth day, they need to be trypsinized to detach them from the surface of the flask. The trypsinization process includes disaggregating the cells by resuspending them and putting them in a new medium so that they are ready to be introduced into the microfluidic device.

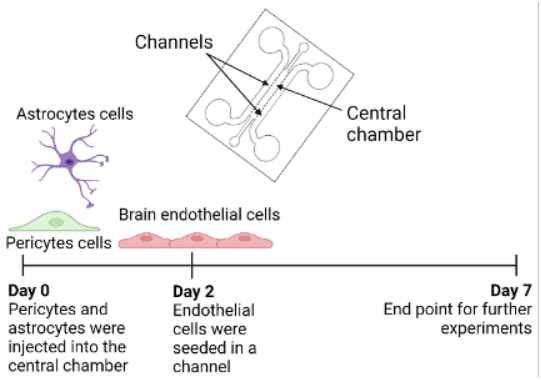
The exact protocol to follow for the culture of each type of cell is shown in Annex 2, as well as the trypsinization protocol, added in Annex 3.

5.3 Seeding of cells into the microfluidic device

Up until this point, the two main parts of the functional BBB-oC have been prepared independently: the physical microfluidic device has been casted and attached to a glass coverslip, and the three different types of cells have been cultured in rich mediums. However, in order to assemble the final BBB-oC, these two independent parts have to join.
The cells will be added to the microfluidic chip with constant sterile conditions, under a class II cell culture hood.
As it has been mentioned before, the microfluidic device is composed of two main channels and a central chamber. During this seeding process the HA-h and HBVP are introduced within a fibrin hydrogel, acting as their ECM, into the central chamber, as depicted in Figure 12. The endothelial cells are added to the side channel 2 days after the seeding of HA-h and HBVP. Due to the fact that this microfluidic device (OoC) is 3D, these endothelial cells are able to adhere to the wall of hydrogel and consequently be in direct contact with the other cell types through the gaps between the microposts that separate the two chambers in the chip (triangular figures). As mentioned, this provides the model to simulate much better the *in vivo* conditions of the distribution of cells. As Figure 12 also shows, the timeline for the seeding of the three different types of cells is fixed: the HA-h and HBVP have to be seeded first and cultured during 2 days before the seeding of the hCMEC/D3 into the side channel, this is because it seems that the contact with astrocytes and pericytes favors the formation of a better BBB. Then, the medium has to be changed every day and it is not

until the seventh day after the assembling of all three cell types that the permeability assays and experiments can be performed.

Figure 13. Timeline of the BBB-oC cell seeding

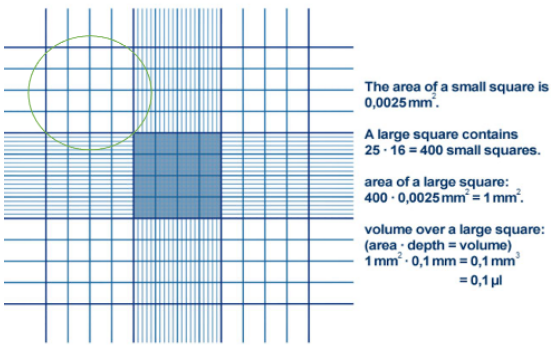


Source: Integrated micro-TEER system into BBB-on-a-chip for permeability evaluation of multi-functionalized gold nanorods against Alzheimer's Disease. Sujey Palma-Florez, Adrián López-Canosa, Francisco Morales-Zavala, Oscar Castaño, M.J. Kogan, Josep Samitier, Anna Lagunas, Mónica Mir.

Preparation of the hydrogel and seeding of HA-h and HBVP

The hydrogel is composed of fibrinogen and thrombin. Its preparation is based on the mixing of 50 µL filtered Fibrinogen 3 mg/mL and 1 µL of thrombin with PBS. Each chip requires 15 µL of this prepared hydrogel resuspended with 40000 cells of each type (HA-h and HBVP at day 0). The counting of the cells is done with a Neubauer Chamber where with the deposition of only 10 µL of cells and with an optical microscope we can count the number of cells within the 4 quadrants of the chamber (as shown in Figure 13). The resulting number of cells is multiplied by a 10^4 factor to obtain the number of cells per mL. Therefore, if the average number of cells in one quadrant of the Neubauer Chamber is 25 cells, this will mean that our solution has 250000 cells/mL, and considering we want 40000 cells in each chip, we need to take 160 µL of such cell type and mix it with the hydrogel.

Figure 14.Scheme of the quadrants in a Neubauer Chamber.



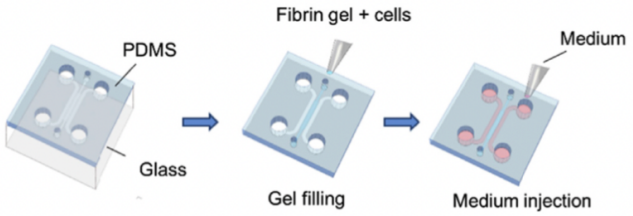
Source: Protocol obtained from the IBEC oneDrive shared folder of Nanobioengineering group

The 40000 cells of each cell type are mixed in an eppendorf tube and centrifuged at 1000 rpm during 5 min. The supernatant is discarded, and the pellet is resuspended with the 50 µL fibrinogen + 1 µL thrombin (hydrogel). 1 5µL of this mix are taken and introduced through one of the holes of the central chamber of the chip.

Before incubating the assembled chip for 15 min at 37°C and 5% pCO_2 to polymerize the hydrogel, it is optimal to check with the optical microscope if the introduced cells have reached the central chamber only and have not filtered into the side channels as well. If that were to happen, it would mean the PDMS structure is not well attached to the coverglass slip and therefore all cells would spread and mix, meaning the barrier function does not work. These faulted chips would need to be thrown away.

Otherwise, the chips removed from the incubator are completely polymerized and have to be supplied with medium, so that the cells inside the chip continue to be viable. The medium introduced is a 1:1 mixture of endothelial and astrocyte medium (EM:AM medium), and it is introduced through the larger holes of the chip, enabling the supply of medium to all cells.

Figure 15. Scheme of the process of seeding the cells and hydrogel (fibrin gel) into the microfluidic device



See how the process is finished by adding the medium into the larger holes of the structure. Source: M. Campisi, Y. Shin. T. Osaki, V. Chiono, R.D. Kamm, 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes. Biomaterials Elsevier, 2018.

Seeding of the hCMEC/D3

After 2 days of HA-h and HBVP having been added to the chip, and their medium having been changed every day since then, we proceed to add the Endothelial cells. These cells are added to one of the side channels of the chip, and as opposed to the seeding of the HA-h and HBVP, in this case no preparation of hydrogel is needed as it is already there. The hCMEC/D3 will adhere to the present hydrogel and therefore will be in contact with the other two types of cells. 100000 hCMEC/D3 will be seeded on each chip.

For the seeding of the hCMEC/D3, we need to previously coat the side channel with collagen so that when the cells are added they can adhere properly and they find their environment comfortable. It is for this reason that once the collagen has been added to the channel and has been incubated, the hCMEC/D3 are injected

with a pipet and the chip is turned sideways for 1 h 30 min. This is done so that, by the force of gravity, the hCMEC/D3 fall into the hydrogel and are forced to be in direct contact and adhere to it.

The exact protocol to follow for the seeding of the cells into the microfluidic device is specified in Annex 4. A more precise explanation on the functioning of the Neubauer Chamber is also added in Annex 5.

The resulting chips with all three types of cells: 40.000 HA-h, 40.000 HBVP, 100.000 hCMEC/D3 are the final product of the experiment. Each one models the Blood Brain Barrier as the HA-h and HBVP are alive in the brain channel (central chamber) in a comfortable environment of fibrin hydrogel, and the hCMEC/D3 are seeded in the side channel although also in contact with the hydrogel, and therefore, the HA-h and HBVP. Consequently, the BBB-oC is completely fabricated and ready to be submitted to assays evaluate its performance.

6. Experimental validation of the assembled BBB-oC

At day 7 of the BBB-oC cell seeding, the evaluation of its performance can begin. This evaluation consists in a series of microscopy images that show the correct functioning of the assembled *in vitro* BBB, as well as permeability assays to quantify and monitor the entrance of certain nanosystems into the barrier and through the brain.

Firstly, the viability of the cells in the neurovascular cell arrangement with its endothelial and neuronal zone needs to be assessed, so that other assays can be performed on the chips. Once the images prove that the seeded cells in the BBB-oC are viable and alive, permeability assays that compute the barrier function of the chips test the activity of different nanodrugs through its pathway can be performed.

This section will show the different evaluation experiments that have been carried out to prove the functioning of the designed chips and its application on drug screening, as well as the discussion on the results obtained from each assay.

6.1 Characterisation of the neurovascular network into the microfluidic device

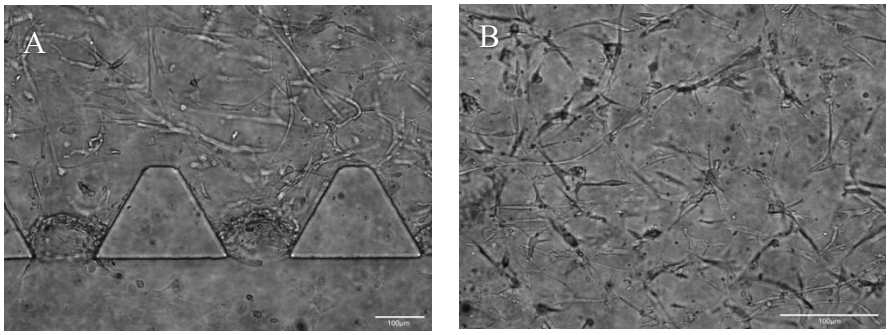
This first evaluation of the designed BBB-oC focuses on proving that the cells introduced into the chips are viable and have the morphology of healthy cells capable of being submitted to further assays. To do this, optical images of the microdevices have been taken using an inverted optical microscope (Olympus IX71) with an integrated CCD Hamamatsu camera. Moreover, brightfield (BF) images have also been taken to show the arrangement of the three different cell types within the chip, and fluorescence images have been taken with the Leica SP5 confocal microscope.

6.1.1 Cell morphology and disposition inside the device

The following figure is the first glance of the created BBB-oC seen from the microscale. It allows us to see how the cells have self-organised themselves within the chip and how well they have adhered to the specific coatings we deliberately added.

Figure 16A shows how the ECs are gathered in a compact form at the interface between the channel and the hydrogel. This disposition of the ECs adhered to the hydrogel just as in the *in vivo* BBB has been achieved by the technique in which the ECs were seeded, explained in section 5.3, where the chips were turned sideways after adding the ECs so that by the force of gravity they would come into contact with the coated collagen wall and form this disposition. Figure 16B, on the other hand, shows the hydrogel part of the chip (the brain chamber), where astrocytes and pericytes are displayed in the 3D scaffold.

Figure 16. Optical images at day 7 of seeded BBB-oC. A) Endothelial zone Eat 10x. B) Hydrogel zone at 20x. Scale bar 100 µm.



Moreover, the cell morphology was observed through the staining of F-actin from the cytoskeleton. Figure 17portrays the characteristic star-spread shape of ACs and PCs displayed in the matrix, which confirms the correct position and morphology of these cells in the seeded BBB-oC. The figure also shows the location of the ECs in between the endothelial channel and the central chamber, reaffirming what was previously seen in Figure 16A.

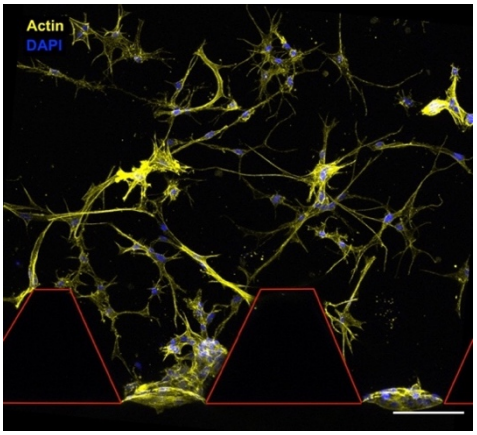


Figure 17. Microscopic confocal images of the neurovascular cells seeded in the microdevice. Microposts' structures have been manually drawn for clarity. Scale bar 100 µm

6.1.2 Cell viability assay

To follow the studies concerning the correct positioning and morphology of the cells in the microdevice, cell viability assays were also performed to check if and how cells survived during time. For this reason, alive cells were stained in green and dead cells in red. Images were taken every day from day 3 to day 7 of the endothelial channel and the hydrogel zone. As shown in Figure 18, the live/dead assay clearly shows fully viable cells (powerful green signal) and puts the designed BBB-oC in position to perform any further assays.

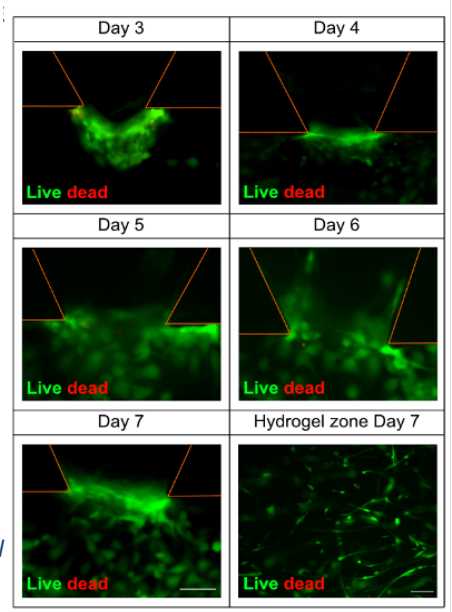


Figure 19. Live/dead assay of endothelial cells at 20x and hydrogel zone at day 7 at 10x.

6.1.3 Detection of TJs

Finally, to complete this first evaluation of the seeded cells in the microdevice, an immunostaining of the TJ-associated protein ZO-1 and the adherens junction VE-Cadherin was carried out to see the TJ linking the ECs in the chip.

Demonstrated in Figure 18, VE-cadherins clearly form the connection in between endothelial cells, being supported by ZO-1. The fact that the endothelial channel presents TJs is important as they are the key to the impermeability of the BBB.

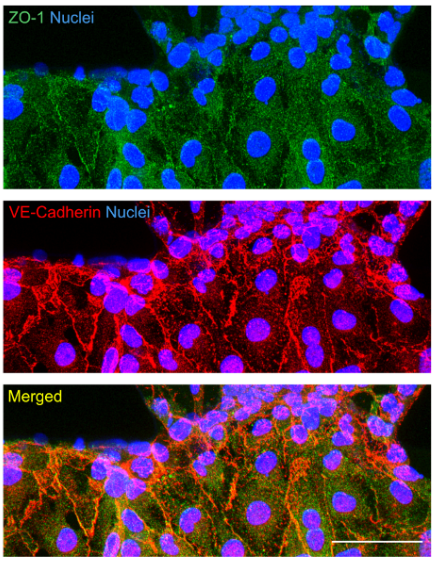


Figure 20. Fluorescence confocal images of TJs formation between ECs in BBB-oC. ZO-1 is stained in green, VE-cadherin in red, and nuclei stained in blue.

The completion of this first evaluation of the neurovascular network formed in the fabricated BBB-oC confirms that the seeded cells are positioned as expected (AC and PC in central chamber, and EC in the side channel), that they are viable and present the correct morphology, and that the ECs are joined by TJs which increase the resistance of the barrier and therefore allows it to perform its main function.

However, to properly assess the BBB-oC and define its similarity to the *in vivo* BBB, there needs to be quantitative data that proves that the cells are indeed behaving as a physical barrier. It is for this reason that the following subsection analyzes the permeability coefficient of the barrier when two well-known molecular tracers cross it.

6.2 Permeability assay

Sodium Fluorescein (NaFl) and Dextran70-FITC (D70) are two fluorescent tracers of different molecular weight. NaFl and D70 have an atomic mass of 376 Da and 70000 Da respectively. They have been previously used as standard tracers to validate the correct permeability performance of the BBB according to size-exclusion [35]. For this reason, computing the permeability coefficient of these well-known tracers in our fabricated BBB-oC is a correct way to evaluate if the chip presents the expected barrier resistance and restriction to size or not.

The two tracers are prepared as a solution with EM:AM medium and introduced to the endothelial channels of the chip. Once introduced, fluorescence images are to be taken through an inverted fluorescence microscope every 3 min for 1 h. The resulting images need then to be analysed by a software (ImageJ) so that the amount of fluorescence is quantified, and the permeability coefficient can be calculated. This calculation is done following the Permeability Coefficient formula detailed by Campisi et al. [15]:

$$P = \left(\frac{1}{ICht1 - ITt1}\right) * \left(\frac{ITt2 - ITt1}{(\Delta t)}\right) * \frac{VT}{A} \text{ cm/s}$$

Where ICht1is the fluorescence intensity in the channel at the initial time, ITt1 is the intensity in the tissue (brain chamber) at the initial time, and ITt2 is this same intensity at the end time. Δt is the difference between the initial and end time in seconds, VT is the volume of the central chamber in cubic centimeters and A refers to the surface of the endothelial wall (hydrogel) in square centimeters.

As the fluorescent tracers are introduced through the endothelial channel, the intensity in the brain chamber at the initial time is expected to be near 0 (the fluorescent molecules won't have crossed the barrier yet and therefore no fluorescence should be detected initially in the central chamber). As time passes, the tracers go through the barrier and fluorescence increases in the central chamber (ITt2 increases).

Due to the size difference between the two fluorescent tracers, the permeability coefficients are expected to differ as well: NaFl should have a higher permeability value than D70.

The results obtained by the group at IBEC (prior to my stay) are presented in the following figure.

As the figure shows, the assay confirms the theoretic hypothesis and therefore demonstrates the correct barrier-function of the designed BBB-oC.

NaFl introduced through the endothelial channel of the BBB-oC crossed the barrier with a permeability coefficient of P = 4.76 10^{-6} cm/s, much higher (faster) than the larger molecule, D70, with P = 1.11 10^{-6} cm/s. This significant difference between permeabilities suggests that the fabricated BBB-oC presents a size-dependent exclusion, letting through smaller molecules easier than larger ones.

Moreover, a control where the fluorescent tracers were introduced in a chip without ECs was also performed. The results, showing that both tracers entered the brain zone with similar permeabilities between each other (NaFl 4.30 10^{-6} cm/s and D70 4.61 10^{-6} cm/s) reaffirms that the resistance of the barrier is due to the presence of ECs.

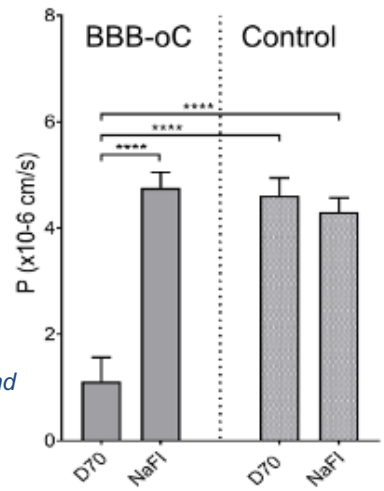


Figure 21. Permeability assay of standard fluorescent tracers D70 and NaFl.

6.3 Discussion

The completion of the evaluation of the neurovascular network formed in the fabricated BBB-oC confirms that the seeded cells are positioned as expected (ACs and PCs in central chamber, and ECs in the side channel), that they are viable and present the correct morphology, and that the ECs are joined by TJs which increase the resistance of the barrier and therefore allows it to perform its main function.

Moreover, the permeability assay suggests that the BBB-oC has a barrier function that is restrictive to size, where smaller molecules go through faster than bigger ones. Additionally, all P values obtained are of the order of 10^{-6} cm/s, which is closer to the *in vivo* BBB P value of 10^{-8} cm/s than other microfabricated *in vitro* models, like the Transwell, which reaches a P of 10^{-4} cm/s.

With this, the chip is now suitable to obtain reliable results in the testing of therapeutic and clinical particles that need information on their crossing through the BBB.

7. BBB-oC application for clinical research

This section focuses on the analysis of the permeability performance of two nanosystems that can be applied for clinical purposes.

Nanosystems, also referred to as nanostructures, are one of the results of the great development of nanotechnology in recent years. The research for the methods in which these nanosystems can be designed to improve the delivery of drugs has proven to be very effective, showing that the way in which the different molecules self-assemble increases the solubility of poorly soluble drugs, reduces the cytotoxicity and improves the therapeutic efficacy [6]. Knowing these advantages, many different nanosystems have been developed for different disease targeting drugs, including drugs for the CNS diseases.
It is for this reason that being able to test how well these nanosystems penetrate into the BBB is crucial for the development of the drug. Consequently, permeability tests of the different elaborated nanosystems in our fabricated BBB-oC are key.
Sometimes the manufacturer of the nanosystem is not only interested in knowing the permeability of their nanosystem but also on other aspects such as the mechanism of entry to the BBB.

The study of two different nanosystems: AuNP-PEG-POM and GNR-PEG-Ang2/D1, have been analysed. Although I have carried out only the tests concerning the AuNP-PEG-POM nanoparticle, the GNR-PEG-Ang2/D1 will also be thoroughly explained as it is of importance for comparing the obtained results.

The name given to the nanosystems is due to the different molecules/particles that compose them. This way, it can be seen that both of the studied nanosystems in this project share the -PEG structure.
PEG refers to polyethylene glycol, a polymer which is very hydrophilic and suitable for the delivery of drugs as it is biocompatible. In both of the studied nanosystems, PEG covers the GNR/AuNP as a type of coating to improve water solubility.
The scaffold of both nanosystems is a gold nanoparticle, with the difference of one being specifically a gold nanorod (GNR). Gold is a metal that has been widely used as a scaffold for nanostructures in drug delivery due to its favourable optical and chemical properties [3].
Gold nanoparticles have a highly adjustable morphology (especially rods) [2] and their surface can be easily modified with different functional groups. This makes them suitable for forming nanosystems as they are prone to being conjugated with peptides to target the cell nucleus, or with polymers to improve the stability of the drug delivery [36]. In the GNR-PEG-Ang2/D1, GNR's photothermal properties proved to be a key on the inhibition of the growth of a studied molecule.

7.1 GNR-PEG-Ang2/D1

The purpose of the analysis of this nanosystem is the research of a drug for a possible Alzehimer's Disease therapy.

TheGNR covered with PEG is conjugated with a lipophilic peptide Ang2 (Angiopep-2) that is thought to allow the entrance of the nanoparticle to the brain through its binding to the low density lipoprotein receptor-related protein 1 (LRP1) present in the brain endothelium. The whole nanosystem is functionalized by the D1 peptide (QSHYRHISPAQV), with a high affinity for Aβ monomers and oligomers avoiding Aβ aggregation.

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In order to know if the Ang2 peptide is in fact the shuttling agent that allows the nanosystem to cross the barrier, a permeability test is required.

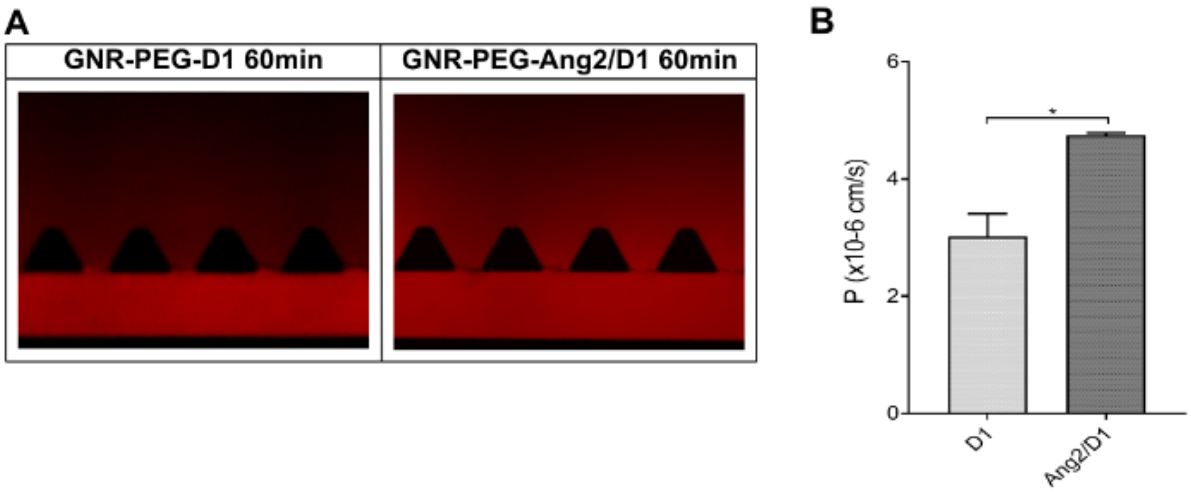
Permeability performance of Angiopep2:

To carry out this permeability test, two nanosystems have to be prepared: GNR-PEG-Ang2/D1 and GNR-PEG-D1. The latter is the control. The two solutions of nanoparticles functionalized with the fluorescent label Alexa674 are introduced, separately, into the endothelial channel of the BBB-oC,, and monitored with the fluorescent microscope. Images are taken every 3 min for 1 h. Then, the permeability coefficient is computed by extracting the fluorescence intensity values of the images with the ImageJ Software and substituting them into the Permeability Coefficient formula developed by Campisi et al. [15], as shown before.

The results of comparing the permeability coefficients of the GNR-PEG-Ang2/D1 and GNR-PEG-D1 suggest that Ang2 enables the crossing of the nanosystem through the endothelial channel into the brain. Figure 20A demonstrates the significant difference in fluorescence intensity at the end of the experiment in both the control and the Ang2 functionalyzed nanosystem, showing a much higher penetration of fluorescent nanoparticles in the case where Ang2 is present.

Moreover, Figure 20B supports this reasoning mathematically by showing the significant difference between the P values of the nanosystem shuttled by Ang2 (GNR-PEG-Ang2/D1 P = 4.74 10^{-6} cm/s) being much higher than the control GNR-PEG-D1 (P = 3.02 10^{-6} cm/s).

Figure 22. Results of the permeability test concerning the effect of Ang2 in the entrance of a nanosystem through the BBB. A) Fluoresence images at the end of the permeability test. B) Histogram with the permeability coefficient values of the nanosystem and its control



Note that to perform this experiment, a western blot was previously carried out to verify the presence of the LRP1 protein in the endothelial channel.

Aside from proving that the Ang2 peptide acts as a shuttling agent of the nanoparticle into the endothelial barrier, it is also interesting to analyse its mechanism of entry. Dynasore is a small cell-permeable molecule that inhibits the activity of dynamin, a regulator for clathrin-mediated endocytosis, which is the main mechanism for internalization of cell-surface molecules and surface-bound cargoes.

If the nanoparticles in the studied system were to enter the brain chamber by a dynamin-dependent endocytosis, by performing a permeability test where this type of endocytosis is blocked, the results would show a lower permeability coefficient in the cases where dynasore is present.

Permeability performance with the inhibition of dynamin-dependent endocytosis by Dynasore:

The permeability assay was performed adding 80 µM of Dynasore in cell medium and incubating for 30 minutes. The GNR-PEG-Ang2/D1 nanosystem stained with A674 in the 80 µM dynasore was injected through the endothelial channel of the BBB-oC and images were taken every 3 min for 1 h. Apart from a first control of GNR-PEG-Ang2/D1 where the nanosystem is injected without any treatment, another control of DMSO was carried out as the Dynasore stock was solubilized in DMSO. Therefore, to rule out the possibilities of the endocytosis-inhibition being due to DMSO, images with this control were also taken and analysed.

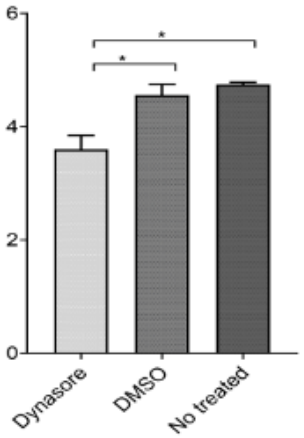


Figure 23. Permeability experiment results with Dynasore as an endocytosis inhibitor.

The permeability results of this experiment, presented in Figure 22, concluded that the entrance of nanoparticles through the barrier was reduced when dynamin-endocytosis was inhibited, passing with a P = 3.60 10^{-6} cm/s. Moreover, DMSO proved to have no implication in the entry of GNR-PEG-Ang2/D1, as its permeability coefficient showed no significant differences to thus of the nanosystem alone.

7.2 AuNP-PEG-POM

The permeability performance of this nanosystem is carried out for two different reasons, similar to the previous case:

- To study the effect that the POM has on the nanoparticle's entrance to the BBB
- To study if the mechanism of entry of the nanosystem is due to dynamin-dependent endocytosis

The conclusions of the obtained results will be compared to the GNR-PEG-Ang2/D1 case to provide more perspective into the different applications and results the BBB-oC can have.

This nanosystem, as opposed to the previous one, has POM as the functional unit. POM (polyoxometalate) is polyatomic ion that has historically been of interest in physics applications due to its ability to self-assemble in soft matter. However, the compound has now gained importance in the biological field as it has been shown that its properties can help in cancer therapy and Alzheimer's disease. In the case of cancer, it is believed that POM could help achieve the targeting of the treatment by only killing cancerogenic cells, therefore reducing most of the adverse effects of the therapy, like the falling of the patient's hair. On the other hand, POM has an application in Alzheimer's as an inhibitor of the aggregation of β-amyloid fibers.

Therefore, the addition of POM to possible drug nanoparticles could lead to the discovery of new treatments for both cancer and Alzheimer therapies. It is for this reason that its effect on the entrance to the BBB is crucial.

Permeability performance of AuNP-PEG-POM and AuNP-PEG:

An assay was conducted to compute the permeability coefficient of AuNP-PEG-POM. The result was compared to the control, which is the nanosystem without POM (AuNP-PEG), so that a conclusion could be reached on whether POM had an effect on the entry of the nanoparticle through the BBB or not. The resulting graph is shown in figure 23.

The results show no significant difference on the permeability coefficient when the nanosystem presents the POM compound, which means that its addition does not affect the entrance to the BBB.

Both nanosystems have similar permeability coefficients (AuNP-PEG-POM 3.210^{-6} cm/s and AuNP-PEG 3.310^{-6} cm/s).

This is a positive result as it means that POM, although it does not enhance the transport across the BBB, it also causes no blockage and can potentially be used as a drug in a nanosystem targeting the BBB.

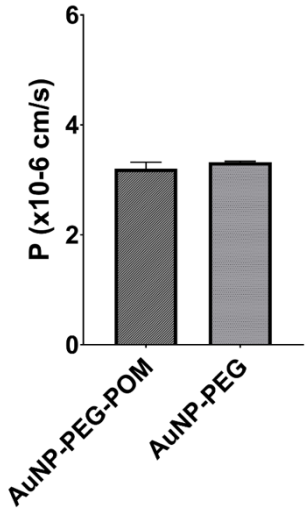


Figure 24. Permeability assay between the nanoparticle with and without POM.

In figure 24 we can clearly see how the nanoparticle goes through the barrier as the fluorescence intensity increases significantly in the central chamber.

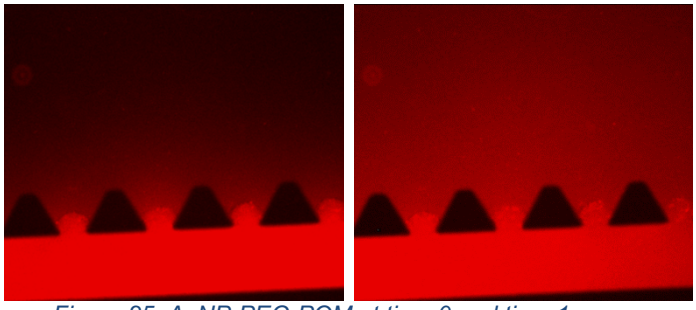


Figure 25. AuNP-PEG-POM at time 0 and time 1.

Permeability performance with the inhibition of endocytosis by Dynasore:

Similarly to the GNR-PEG-Ang2/D1 nanosystem, to see if dynamin-dependentendocytosis is the mechanism of entry of the nanoparticle into the endothelial barrier, a permeability assay with Dynasore was performed.

In this case, the results showed that the inhibition of dynamin-dependent endocytosis by Dynasore did not affect the permeability. In fact, the permeability coefficient values of the nanosystem with Dynasore (3.610^{-6} cm/s) have no significant difference from those of the nanosystem without Dynasore (3.210^{-6} cm/s). This is represented in Figure 25 Consequently, this means that the AuNP-

PEG-POM nanosystem uses an entry mechanism to the BBB different from dynamin-dependentendocytosis.

However, it can be seen that when DMSO is added to the medium the permeability increases in a significant way. The exact reason for this is unknown and would require the realization of many more assays which are not included in the scope of this assessment, as the conclusion we wanted to reach concerns whether the entry mechanism is dynamin guided or not. Therefore, the effect of DMSO is not innert and we have to bear in mind its effects.

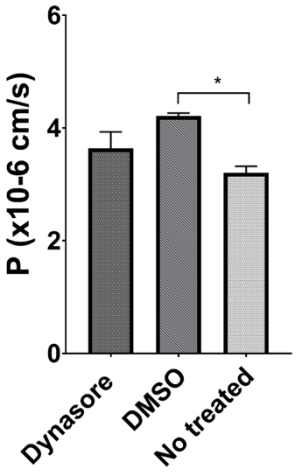


Figure 26. Permeability experiment results with Dynasore as an endocytosis inhibitor.

These results are completely opposite to those of the previously studied nanosystem, GNR-PEG-Ang2/D1, as its P values showed a reduction of the entrance of the nanosystem when dynamin-dependent endocytosis was blocked. Moreover, in this case, we observed that DMSO had no effect on the permeability and was used simply as control.

As we can see, each nanosystem has its own structural and chemical properties that determine its behaviour through the BBB, and assays are needed to show how this behaviour can affect the application of the nanosystem. Those have been successfully addressed through our BBB-oC model.

8. Technical viability

The developed BBB-oC has proven to provide consistent results. However, in the process of fabricating and testing the chip, certain technical challenges have appeared. These have to be considered to perform a fair analysis of the viability of the product.

Brittle glass coverslip

Firstly, the design of the microfluidic chip requires the assembly of a glass coverslip to the PDMS structure so that when the cells are added they remain in the device, and so that they can be observed in the microscope. This entails more risk of breakage of the chips during their manipulation, as the required glass coverslip is very thin and delicate. This proneness to breaking increases due to the fact that the chips need constant manipulation from the moment the cells are seeded to the moment of the last experiment: during this period of time, the medium of the cells has to be changed daily, and this process inevitably involves putting pressure on the glass coverslip.

Strict sterile conditions

Secondly, the culture of cells needs to be done in sterile conditions, under very strict cleaning, and any little mistake during the manipulation of the cells or their medium can lead to the contamination of the whole sample. This increases the risk of failure of the project, as it can lead to having to repeat the process various times and consequently exhaust the economic resources.

Slight inefficient workflow

Many of the processes needed for the fabrication part of the device require long periods of waiting. For example, the time during which the PDMS structures are solidifying in the oven, and the time spent bonding the structures to the glass coverslip in the clean room. These processes are very long and interrupting because the following step of fabrication cannot be done until the previous finishes, so the waiting hours are essentially wasted hours if the sole aim of the worker is the fabrication of chips.

By bearing these technical issues in mind the fabrication process of the BBB-oC can be optimized, although, essentially, they are drawbacks of the project.

STRENGTHS	WEAKNESSES
- 3D design - Direct contact between cell types - Similarity to the *in vivo* BBB - Realistic and accurate results - Easy manufacturing of the chip	- Doesn't incorporate neurons - Lacks the simulation of shear stress - Easy breaking of the chip - Strict sterile conditions - Slight inefficient workflow - Possible PDMS leakage
OPPORTUNITIES	**THREATS**
- Great market potential - Alternative to animal testing - Affordable to produce in laboratories - Possibility to revolutionize personalized medicine - Increasing research on the topic	- Lacks product standardization - New technology - Room for design improvements

Table 4. SWOT analysis of the designed product

The internal strengths and weaknesses of the proposed solution are portrayed in the first row of Table 4.

As it states, the microfluidic BBB model has the crucial advantage of simulating a very realistic model of the human BBB due to the 3 dimensionality of its design. This feature provides key similarity to the human BBB.

Moreover, the model counts with the incorporation of three different cell types that are in direct contact with each other. It is important to add that these cells are human-derived, which increases the value of the obtained results as they are applicable to human morphology.

The structure of the design produces a perfusable network with its channels being comparable to human arteriole and capillary diameters. This allows a transport very similar to the actual human vascular network.

Another important feature of the product is that its manufacturing is relatively easy and does not require machines of significant cost nor specified trainings for personnel.

Although these are extraordinary characteristics for a BBB model to have, and essentially determine its performance, the model also has some weaknesses that could be improved in order to achieve an even more accurate representation of the BBB. These weaknesses are in part based on the type of material used (PDMS), which requires a modification of its surface before treating it because it is hydrophobic, and this modification can lead to the leakage of unwanted particles into the culture medium, which could result in variations in the outcomes of the study.

Possibly the main drawback of the model, but also the one with an 'easy' solving is the lack of neurons as a cell type in the model; only three types of cells are seeded in this chip, and neurons are not included. This is because the treatment for neurons is much more specific and they are very delicate, therefore, they were not considered for the optimization of the chip. Moreover, the group lab where the chip is produced does not have many providers for neurons yet and is currently working on a partnership which can lead to the help of a specialist in culture of human neurons *in vitro* to aid with the implementation of them to the chip.

Additionally, the actual model does not represent shear stress. This can be solved by the addition of automatized medium pumps, which would increase the cost.

The fragile glass material supporting the chip causes its easy breaking. The slight inefficient workflow and strict sterile conditions of cell manipulation are two other drawbacks which have previously been explained.

The second row of the SWOT analysis exposes the external opportunities and threats. This model offers the possibility of designing a pathological model based on patient-derived cells. This is a great opportunity for developing drug tests on the cells of patients, to evaluate the performance of the treatment with much more accuracy. Moreover, this patient-derived pathological model can certainly give more information surrounding the progression of the disease and can offer solutions to a more personalized therapeutic approach. This exponentially increases the interest in the development of the product for industrial purposes and incentivizes the interest for research.

The manufacturing of the product is economically viable and much more ethically responsible than animal testing.

The main threats that surround the designed product are the lack of regulations in the European Medicines Agency (EMA). This is a consequence of the new technology it offers, which also leaves new room for improvements to be made as research progresses.

9. Economic viability

The accomplishment of this project implies a certain economic cost. In order to have an idea of what this cost is and where it comes from, a study of the economic aspects of the project has been performed.

It is important to take into account that this project has been developed under the supervision and institution of IBEC laboratories. Therefore, the materials and machines have not had to be specifically bought for this project, and consequently the cost of their use is reduced. However, most of the laboratory materials needed to perform the experiments, such as pipette tips or glass pipettes, can only be used once and need to be thrown away after their use. It is for this reason that when evaluating the cost of the project, the amount of used units of each of these materials has been taken into account and their cost has been fully considered, as opposed to the non-fungible material (such as microscopes) which price has not been taken into account in the final sum of the budget but has been portrayed in the table anyway to have an idea of what the total cost of the project would be if we were to finance it all from scratch.

Table 7 shows the list of materials used for the whole development of the project. In the table, the mentioned separation between the one-use materials and the non-fungible materials is represented. The subtotal sum considers only the cost of all the materials that have been 100% paid for (the price of the exact number of units/bottles of each of the materials). This includes the bottles of reagents for each of the experiments, which price has also been adapted to the quantity used of each reagent.

Additionally, considering the project has been carried out by me, the cost of human resources has also been taken into account. This cost is linked to the amount of time dedicated to the development of the project, which, if we consider the time spent at the laboratory and the time dedicated to the research of literature and the writing of the project, sum to a total of 670 h. Supposing that the salary is 25 €/h, this would add up to a theoretical total of 16.750€ due to human resources. I have used the word theoretical because no actual amount of money has been paid, due to this project being part of a subject of the degree, and therefore counting as compulsory.

Item	Price
Laborarory Reagents	
EndoGRO™ Basal Medium (Cat #SCME-BM)	168,30 €
ScienCell™ Astrocyte Medium (AM) (Cat #1801) *ScienCell™ Pericyte Medium (PM) (Cat #1201)*	270,00 €
Innoprot™ Dulbeccos's Phospate-Buffered Solution (DPBS)	85,00 €
Sigma-Aldrich™ Collagen Type I Solution from rat tail	205,20 €
SYLGARD™ 184 Silicone Elastomer Base + *SYLGARD™ 184 Silicone Elastomer Curing Agent*	158,60 €
Gibco™ Trypsin 100 ml	15,50 €
Gibco™ Fetal Bovine Serum (FBS), 500 ml, ref. 10270106	136,60 €
Sigma-Aldrich™ Fibrinogen 100 mg	78,31 €
Sigma-Aldrich™ Thrombin, T4648-1KU	70,34 €
One-use Material	
20-200µl pipette tips, 1000 pieces	22,80 €
50-100µl pipette tips, 1000 pieces	25,85 €
1-50µl pipette tips, 1000 pieces	25,25 €
Sigma-Aldrich™ 10 ml serological pipettes, 200 units	80,10 €
Sigma-Aldrich™ 25 ml serological pipettes, 200 units	149,00 €
Sigma-Aldrich™ 5 ml serological pipettes, 200 units	78,70 €
Sigma-Aldrich™ 2 ml aspiration pipettes, 1000 units	575,00 €
Sigma-Aldrich™ 24 mm x 60 mm cover glasses, 1000 units	364.00 €
Sigma-Aldrich™ Eppendorf Conical tubes, 500 units	161,00 €
Sigma-Aldrich™ Corning 50 ml Centrifuge tubes, 50 units	30,00 €
Sigma-Aldrich™ Cell culture flasks, 20 units	45,80 €
Sigma-Aldrich™ Petri Dishes, 50 units	14,00 €
Millex Syringe Filter	117,00 €
Nitril Santex Gloves, 100 units	9,90 €
Sigma-Aldrich™ Corning 15 ml Centrifuge tubes, 48 units	45,20 €

Other material	
Neubauer chamber	58, 90 €
Printed Masters with design (2)	364,92 €
SUBTOTAL	**3180,06 €**
MilliSentials™ Aliquotinc Pipette Controller	746,00 €
Sigma-Aldrich™ 20-200µl Nichipet Eco pipette (Z710180)	542,00 €
Sigma-Aldrich™ 100-1000µl Nichipet Eco pipette (Z710199)	542,00 €
Sigma-Aldrich™ 100-1000µl Nichipet EX-Plus II pipette (Z717355)	459,00 €
Plasma Etch PE-25 Low-Cost Plasma Cleaner	6.000,00 €
Jenco™ compound optical microscope (Z737607)	591,00 €
Olympus IX71 inverted fluorescence microscope	10.745,96 €
JP Selecta™ Drying and Sterilization Oven	1.650,80 €
Pyrex 250ml Precipitate glass	106,93 €
Sartorious Precision Balance 220g-1mg	2.000,00 €
Apple MacBook Pro	1.500,00 €
Human Resources	
670h of work	16.750, 00 €
TOTAL	**44.813,75 €**

Table 5. Economic Viability of the project

The amount of material considered in this table corresponds to the needed quantity to produce 50 microfluidic chips. Therefore, the cost of producing, from scratch, these 50 microfluidic chips with the steps explained in the Detail Engineering section, is 3180.06 €. Note that this price includes all the reagents and one-use material, as well as the price of the masters, which are printed and treated with photolithography techniques, and the Neubauer chamber. These last two materials have been considered in the final budget even though they are not one-use materials because their use is solely for the fabrication of the chips and are not needed for other experiments.

As for the rest of the materials in the list, adding their price to the total budget gives us an idea of what it would cost to develop the project for the first time without any institution behind, and therefore having to buy every single thing just for the purpose of the project. This list includes my

personal computer, with which the project has been written, and its acquirement was independent and prior to the project.

Naturally, this last list of non-fungible materials exponentially increases the cost of the project. The exact difference between the cost of the project considering the price of the whole of the equipment/reagents whereas the cost of just the used quantities/units of materials is: 41.633,69 € for 50 chips, which if we approximate it to the production of just one chip, becomes a difference of 832,67 €.

The reason behind considering the budget for more than just one single chip is that when doing the experiments, errors can involuntarily occur and damage the chips or its cells, making it impossible to use those chips for the permeability evaluation or any other possible analyses and assays. For this reason, many chips are produced and seeded but only few of them actually get to the final stage.

For the purpose of the evaluation of the economic viability of the project, the computed budget of fabricating a chip in the lab has been compared to the cost of buying a finished chip to an external company.

AimBioTech is a company that produces chips with the same design used for this project, and a box of 25 chips costs 400 $ (800 $ for 50 chips). This box includes just the chip itself, not the cells or reagents. Therefore, considering that the cost of producing 50 chips (just the structure) in the lab is less than 50 € because it involves just the mixing of elastomer + curing agent and the solidification of the chips in the oven (without counting the long term investment of 364 € for the masters, which can be reused many times), it is much more cost-effective to produce the chips in the lab as opposed to buying them directly. Emulate is another company that sells OoC devices. As opposed to AimBioTech, Emulate doesn't sell just the chips, but the complete series of materials and reagents needed. Concretely, Emulate sells packs of 12 chips including the cells and the reagents. The price of these 12 BBB-oC is 130000$, which is significantly much higher than the budget at IBEC for producing 50 of the same chips.

As a conclusion of this analysis of the economic aspects of the project, it can be stated that producing the BBB-oC in a laboratory is more rentable than buying the product to a third-party. Even more so considering the benefits obtained from becoming a client for companies that aim to test their drugs or nanoparticles with the laboratory manufactured device.

10. Execution schedule

This section focuses on the techniques that have been implemented to organise the project and to achieve productivity.

The execution of any project, this one included, requires a high level of organisation and planification of the tasks that have to be done and the time required to carry them out. Without this project management, the development of the tasks could be affected by distractions that cause the project to go off track or focus on something that is not in the established scope. Having a clear view on what is to be done, and when, is also key in working productively and effectively. It is for this reason that the management and planification stage of the project development must not be underestimated.

The following sections show the three techniques that have been used for the organisation of this project, as well as their meaning and utility. The techniques performed are: a Work Breakdown Structure (WBS), a Program Evaluation and Review Technique with its Critical Path Method (PERT-CPM), and a GANTT diagram.

10.1 Work Breakdown Structure (WBS)

The Work Breakdown Structure (WBS) is based on the breaking of the scope of the project into different, smaller, tasks. The main groups of work form a 'package' and are decomposed into the smaller tasks needed to perform to accomplish them. This hierarchical decomposition of the work in 'packages' allows a clear division of the scope of the project and offers a visual representation of the amount of work that has to be done in each 'package', so that the steps that need to be followed to proceed with the development of the project is key.

The WBS of this project is presented in Figure 26:

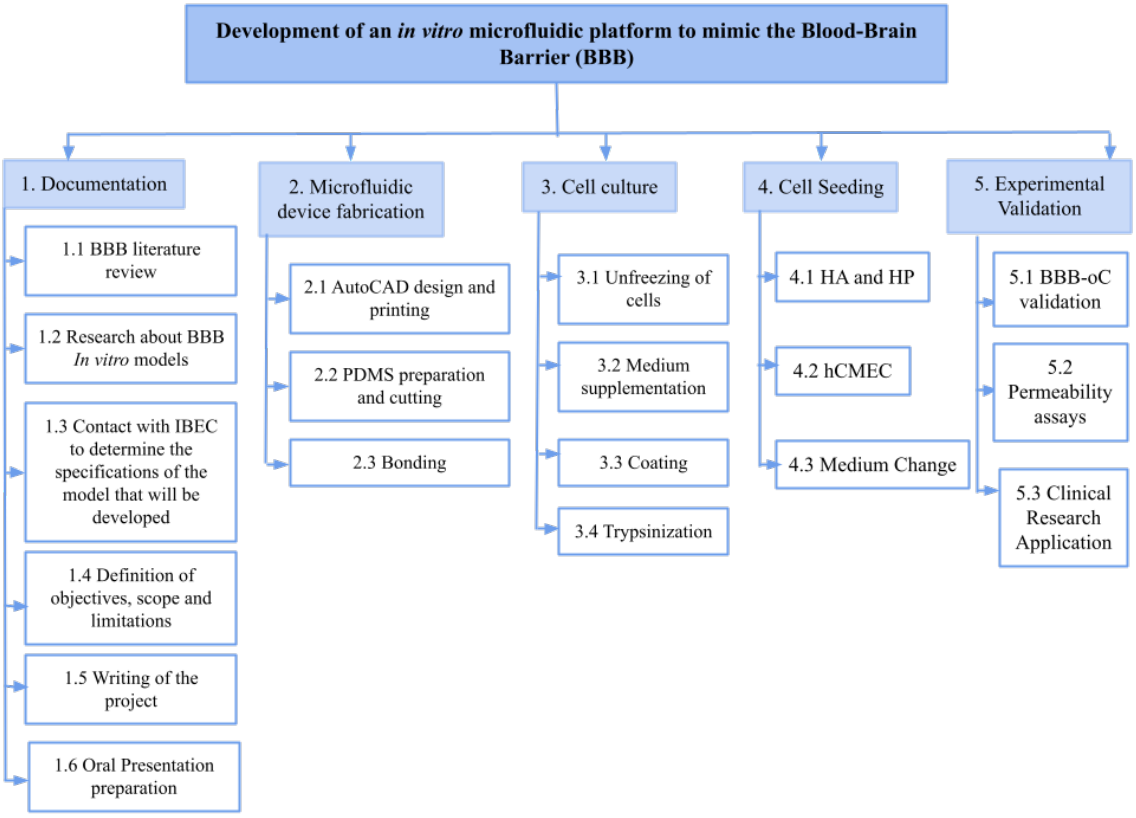


Figure 27. Work Breakdown Structure (WBS) of the project

As it is portrayed in Figure 26, the project is divided into 5 packages of work. Each of them has several tasks that need to be accomplished so that the package of work is complete. In order to understand more specifically what each task consists in, a WBS dictionary has been elaborated:

1. Documentation

Nº	NAME	DESCRIPTION
1.1	**Literature review**	Extensive research on the characteristics, functions and structure of the BBB, as well as the diseases associated with BBB dysfunction.
1.2	***In vitro* BBB model research**	In depth review on the functioning BBB models. Origins, development, and state of situation.
1.3	**Contact with IBEC**	Due to the fact that the fabrication of the model and the laboratory work is done within IBEC's facilities and under their supervision, a first contact with them to establish what will be done during the research period is necessary. The information required to obtain is the type of design model they use, the equipment available, and the limitations that the research may have considering its duration.
1.4	**Scope, objectives and limitations**	Determine the aim of the project, what it intends to solve and how. Specify the range of the topic that will be covered and be aware of its limitations.

1.5	**Writing of the project**	Development of the written project that will be submitted. This process begins in the writing of the 'Avantprojecte' in late March 2022 and ends in January 2023 with the submission of TFG.
1.6	**Oral presentation**	Preparation of the power point presentation and organization of the explanation to be done during the oral exposition of the final project

2. Microfluidic device fabrication

Nº	NAME	DESCRIPTION
2.1	**AutoCAD**	Digitalization of the design with AutoCAD software and printing of the acetate masks for the photolithography treatment.
2.2	**PDMS**	Preparation of the PDMS substance (weighing and mixing of elastomer and curing agent) and cutting the solidified result into the chip structure.
2.3	**Bonding**	Bonding of the PDMS chips into glass coverslips with plasma treatment. This step requires the previous cleaning of all the chips and coverslips. To be done in a certified clean room

3. Cell culture

Nº	NAME	DESCRIPTION
3.1	**Unfreezing of cells**	Unfreezing of the cells from the liquid nitrogen tank they are stored in.
3.2	**Medium supplementation**	Supplementation of the medium of each type of cell to enrich it with substances that allow the survival of the cells.
3.3	**Coating**	Coating of the flasks that will host the cells during their culture time before seeding them into the chip. Each flask contains a different type of cell and therefore a specific coating.
3.4	**Trypsinization**	Detachment of the cells from the coated flask in order to centrifuge them and insert them into the chips.

4. Cell seeding

Nº	NAME	DESCRIPTION
4.1	**HA and HP**	Seeding of the astrocytes and pericytes into the chips.
4.2	**EC**	Seeding of the endothelial cells into the chips.
4.3	**Medium change**	The medium has to be changed every day after the seeding of cells into the chip.

5. Experimental validation

Nº	NAME	DESCRIPTION
5.1	**BBB-oC validation**	Characterisation of the assembled BBB-oC by microscopy imaging techniques. This evaluation was performed prior to

		my stay at IBEC so I just participated in its reporting and writing.
5.2	**Permeability assays**	Assays to compute the permeability coefficient of different compounds across the assembled BBB-oC.
5.3	**Clinical research applications**	Addition of pharmaceutical nanodrugs into the BBB to assess their performance.

10.2 PERT-CPM diagram

In order to coordinate the different tasks that have to be done, and reach the deadline of the project without delay, a PERT-CPM diagram has been made. This diagram visually represents the tasks that have to be done, their timing and their relationship. It identifies which tasks come first and which are the following to be executed. With the computation of the 'early' and 'last' time, it is able to find the path which will determine the length of the project (the critical path).

These concepts will be further explained along this section.

Firstly, a table with the chronological dependence of each task and its duration has been elaborated.

ID WBS	ID PERT	Previous task	Time (in days)
1.1	A	-	10
1.2	B	A	5
1.3	C	-	2
1.4	D	C	1
1.5	E	-	9 months
1.6	F	E	3
2.1	G	D, B	5
2.2	H	G	1
2.3	I	H	2
3.1	J	D,B	1
3.2	K	J	1
3.3	L	K	1
3.4	M	L	1
4.1	N	M, I	1
4.2	O	N	1
4.3	P	O	1
5.1	Q	P	10
5.2	R	Q	5
5.3	S	F, R	5

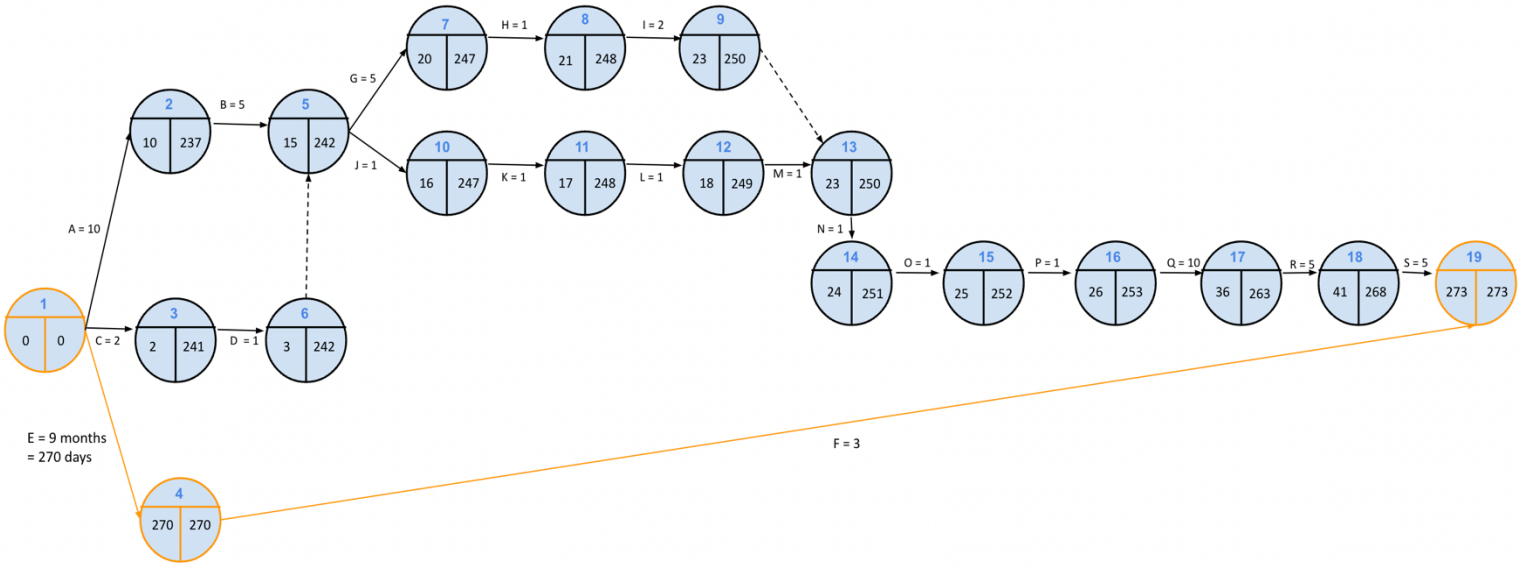
Table 6. Table of task precedencies and timings

From these precedencies, the chronological order of the tasks is set. This way, we know that one task cannot begin if its precedent has not finished, although various tasks can be done simulataneously . By computing the early time and last time we can see which is the critical path.

To understand what this means, a proper definition of each of these terms is provided:

- Early time: minimum time necessary in order to perform a task. It is the earliest moment in which a task can be carried out.
- Last time: latest time in which a task can be done. It is the maximum time that an activity can reach a node.
- Critical path: path composed of all the activities that, in case of there being a delay, would modify the final timing of the project. Therefore, it is the longest possible path that affects the total timing of the project.

Figure 28. PERT-CPM diagram



We can see that our project's critical path is composed by tasks E,F highlighted in orange. This critical path makes sense, as the project cannot be finished until the writing part is over, and this is the process that takes the longest.

10.3 GANTT diagram

Finally, the series of tasks have been displayed against time in a GANTT diagram to visualize the temporal evolution of the project's course, so that we have a clear view on when each activity

begins and ends, how long each task is scheduled to last, which activities overlap with other, and most importantly, to set the start and finish date for the project.

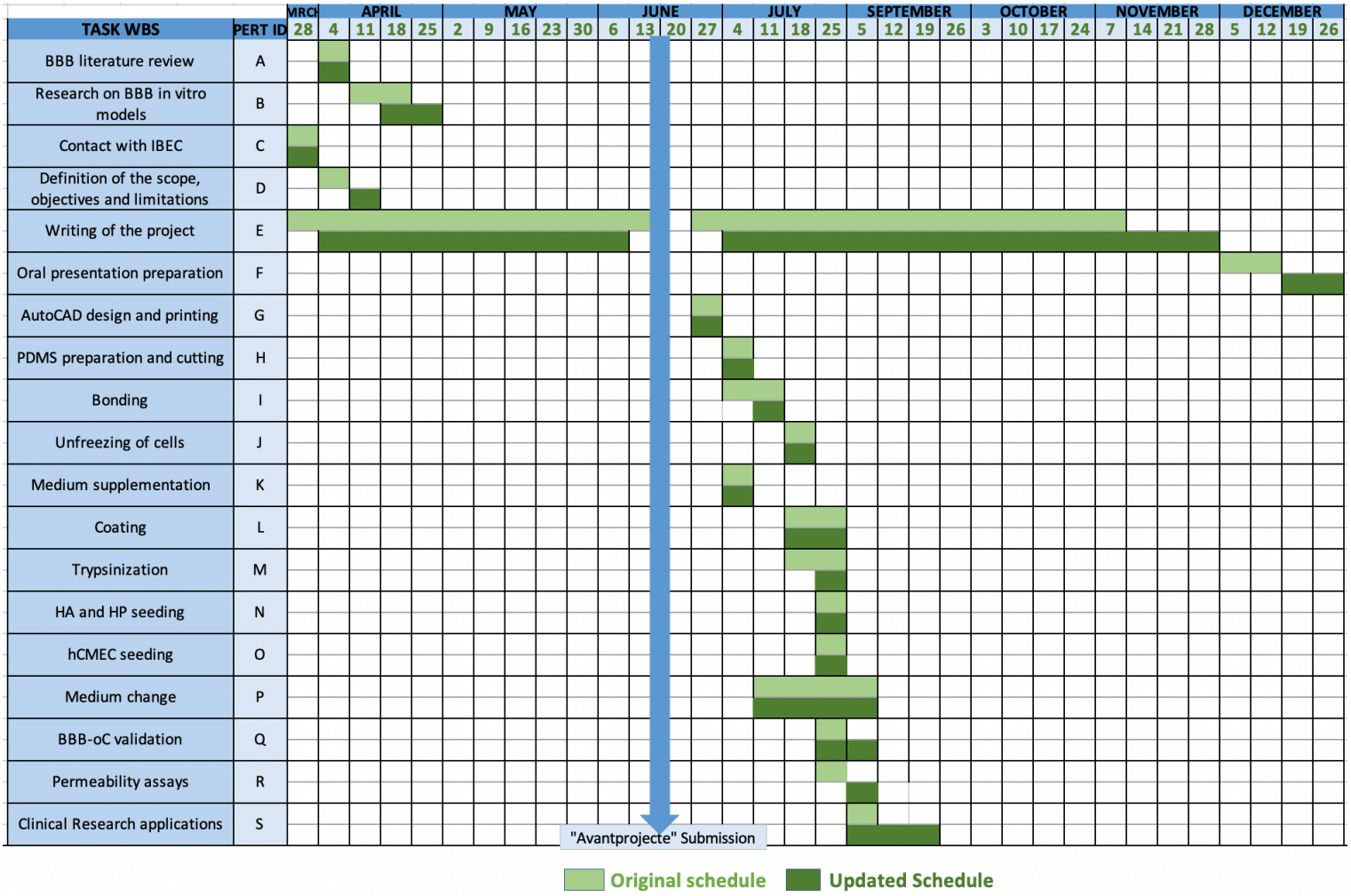


Table 7. GANTT Diagram

The display of the diagram is as follows: on the left are the list of tasks to be done, and each column represents the week of the month when the task will be carried out.

The diagram also shows two different schedules, as the original planned schedule of execution has not been strictly followed for every task, and consequently, an updated schedule has been developed with the actual timings of the tasks that suffered a delay. As it can be seen, the tasks that were performed during my stay at IBEC were all executed as planned, each day doing what was supposed to be done, as the timings of the cells are very strict and are not suitable for modification. The only tasks that suffered a slight change in timings in IBEC where the analysis with the nanoparticles (during the characterization stage), where an unexpected error caused the

repetition of the experiments. But overall, the well-planned organization of the project has enabled a smooth development without major changes in the timings and without missing deadlines.

11. Legislation and Regulation

The whole development and execution of this project has been done in Barcelona. For this reason, the legislations and regulations that apply are those imposed by the Spanish Government.

The product developed in this project is under the group name of a type of 'organ-on-chip'. This product technology is relatively new, and as a consequence there are serious gaps in its legislation and regulation. One of the most pressing is that is there is still no specific standardisation[10] that regulates their characterisation (shape, size, tissue types, ways of use, material). There is also no uniform definition of OoC or its related vocabulary, nor any agreed requirements for their production.
Nevertheless, there are standards for other devices that can also be applied to OoCs, due to the similarity of their fields. For example, ISO-IWA 23 - 2016 is a standard that proposes a classification for microfluidic devices, and ISO 10991-2009 defines terms like 'microfluidics' and 'lab-on-chip'.

Considering the potential that these type of devices have in the revolutionization of biomedical research and drug development, standardization would facilitate OoC acceptance in regulatory contexts. For example, it would establish a clear and systematic characterization of the different types of OoC devices as a function of their structure and functionality, and it would regulate the qualification processes required to prove their technological and biological relevance. As a result, standardization would increase the advance of these technologies by placing them as commercial products and helping their implementation in healthcare industries.

OoC production involves the storage and use of human derived cells, and this gives rise to the ethical issue of legislation concerning the collection of cells in biomedical research. There are various legal rules that specify consent, authorization and licensing of research made with any type of human tissue or cell. These are stated in the Directive 2001/20/EC and Directive 2005/28/EC, and IBEC laboratories ensures its regulatory compliance.

[10] A standard is a document established by consensus and approved by a recognized body that provides, for a common and repeated use, rules, guidelines or characteristics for activities or their results, aimed at the achievement of the optimum degree of order in a given context. - ISO definition.

Moreover, concerning my stay at IBEC during the practical execution of the project, the regulation regarding the protection of personal data must also be considered. This regulation is stipulated in the 2016/679 reglament of the European Parliament.

12. Conclusions

The elaboration of this project has allowed the development of a functional microfluidic platform that models the human blood brain barrier. This platform has been designed in the form of an organ-on-chip, enabling the incorporation of three different types of human cells (astrocytes, pericytes and endothelial cells) to mimic the natural BBB with as much precision as possible.

The research of the literature surrounding the field has given a purpose to this project by shedding light into the necessity of developing precise models for the human BBB: most of the diseases of the CNS need their therapeutic drugs to target the tissue directly (the brain), and for this to happen these drugs have to be able to leave the blood vessel and enter the tissue, through the blood-brain barrier. For this reason, having been able to fabricate an *in vitro* model of the BBB has given the opportunity to test the permeability of bioengineered nanodrugs as they pass through the barrier.

The study carried out has been able to confirm that the use of a PDMS structure with an engraved 3D design of 2 side channels and a central chamber seeded with a tri-culture of HA-h, HBVP and hCMEC/D3, along with a hydrogel wall, is an appropriate and accurate model to the human BBB. Firstly, the tri-culture of cells has proven to mimic the organization and structure of the brain microcirculation *in vivo*. Secondly, the choice of PDMS as the 3D scaffold for the chip has allowed the outer structure to be rigid and at the same time be compatible with the survival of the cells up to the 7th day. Moreover, the simulation of the extracellular matrix has been accomplished due to the addition of hydrogel to the central chamber. Considering this, the initial objectives regarding the manufacturing of the chip, the choosing of the cells, and the assemble of the cells into the chip, have been met. However, some improvements can be made concerning the type of cells used, like for example incorporating neurons as a fourth cell type in the central chamber to obtain a model that is not only useful for proving that drugs can pass through the BBB, but that also has the added ability to test the effect these drugs have on neurons.

The assays carried out to evaluate the permeability performance of the modeled barrier have confirmed that the barrier is size restrictive, and therefore lets through smaller molecules easier that bigger ones. A remarkable conclusion of this project is the order of the permeability coefficient found in all permeability assays: it is much closer to the *in vivo* values than other *in vitro* models documented in literature, with an order of 10^{-6} cm/s. With this, the project has also fully reached the aim of evaluating the performance of the designed model.

The therapeutic application to the model of testing two different nanoparticles has shown that:

(i) The Ang2 peptide in the GNR-PEG-Ang2/D1 nanoparticle acts as a shuttling agent of the nanoparticle through the endothelial barrier,

(ii) The mechanism of entry to the endothelial barrier of the GNR-PEG-Ang2/D1 is mainly through dynamin-dependent endocytosis,

(iii) POM is an inhibitor of the aggregation of beta-amyloid and its addition to a nanosystem able to cross the BBB can be used as a possible treatment for Alzheimer's Disease,

(iv) The addition of the therapeutic agent POM into the AuNP-PEG-POM nanoparticle does not modify its permeability through the barrier,

(v) The AuNP-PEG-POM does not enter through the barrier by a dynamin guided endocytosis.

With these findings, the project has given new information on how these drugs enter the BBB and therefore they fulfill the aim of adding a clinical application to the model and give opportunity to future research for implementing these therapeutic drugs as treatments for Alzheimer's.

Consequently, the results of this project support the idea that a microfluidic model of the human BBB developed *in vitro* has the potential to act as a test for therapeutic drugs before their full development into the market, and that the model with the triculture of HA-h, HBVP, and hCMEC/D3 with specific culture protocols and a 3D environment of PDMS and fibrin hydrogel, mimics with precision the structure of the human BBB and acts as a great alternative to animal models.

12.1 Future work

It is convenient to consider that future works along this project line should contemplate the addition of shear stress simulation to the model with automatized medium pumps in order to provide an even more realistic environment for the cells. However, the increase in the cost of the project should probably be evaluated. Moreover, as mentioned previously, if a more clinical application of the model were to be achieved, the incorporation of neurons into the central chamber would give the possibility to test the effect that drugs have on these types of cells. Considering this, an even more ambitious modification would be the incorporation of patient derived cells to achieve a personalized model of the patient's BBB and therefore test how each of the possible treatments for that patient would work before trying them directly to the patient. This last improvement would require a more specific model and should consider the regularization process linked to its approval.

13. References

[1] A. Oddo, B. Peng, Z. Tong, et al., Advances in Microfluidic Blood-Brain Barrier (BBB) Models, CellPress, (**2019)**

[2] X.Wang, Y. Hou, X. Ai, J. Sun, et al., Potential Applications of Microfluidics Based Blood Brain Barrier (BBB)-on-chips for in vitro drug development, Elsevier, Biomedicine and Pharmacotherapy, **(2020**) 110822.

[3] M. Pardridge, Drug Transport across the Blood-Brain Barrier, Journal of Cerebral Blood Flow & Metabolsim, (**2012**) 1959-1972

[4], [5] G. McCaffrey, T.P. Davis, Physiology and Pathophysiologu of the Blood Brain Barrier, J Investig Med, (**2013**)

[6] L. Jiang, S. Li, J. Zheng, et al., Recent Progress in Microfluidic Models of the Blood-Brain Barrier, Micromachines, (**2019)**.

[7] L. Librizzi, L. Uva, L. Raspagliesi, M. Gionso, et al., Ultrasounds induce blood-brain barrier opening across a sonolucent polyofin plate in an in vitro isolated brain preparation, Scientific Reports, (Feb **2022**)

[8] B. Shah, X. Dong, Current Status of In vitro Models of the Blood Brain Barrier, PubMed, (**2022**)

[9] Linville, R.M., Searson, P.C. Next-generation in vitro blood–brain barrier models: benchmarking and improving model accuracy. Fluids Barriers CNS 18, 56 (**2021**).

[10] A. Appelt-Menzel, A. Cubukova, K. Günther, F. Edenhofer, J. Piontek, G. Krause, et al., Establishment of a human blood-brain barrier Co-culture model mimicking the
neurovascular unit using induced pluri- and multipotent stem cells, Stem Cell Rep.8 (**2017**) 894–906.

[11] E. Moutaux, B. Charlot, A. Genoux, F. Saudou, M. Cazorla, An integrated microfluidic/microelectrode array for the study of activity-dependent intracellular dynamics in neuronal networks, Lab Chip 18 (**2018**) 3425–3435.

[12] E. De Jong, D.S. Williams, L.K.E.A. Abdelmohsen, J.C.M. Van Hest, I.S. Zuhorn, A filter-free blood-brain barrier model to quantitatively study transendothelial delivery of nanoparticles by fluorescence spectroscopy, J. Control. Release 289(**2018)** 14–22.

[13]Patentimages.storage.googleapis.com. (**2022)** <https://patentimages.storage.googleapis.com/81/f7/16/794b67ab80313f/US8293495.pdf>.

[14] Patentimages.storage.googleapis.com. **2022**.

[15] M. Campisi, Y. Shin. T. Osaki, V. Chiono, R.D. Kamm, 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, perycites and astrocytes. Biomaterials Elsevier, (**2018)**.

[16] C.F.Cho,et al.,Blood-brain-barrier spheroids as an in vitro screening platform for brain-penetrating agents, Nat. Commun. 8 (**2017**).

[17] Ungrin, M. D., Joshi, C., Nica, A., Bauwens, C. & Zandstra, P. W. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One 3, e1565 (**2008**).

[18] Sakai, Y., Yamagami, S. & Nakazawa, K. Comparative analysis of gene expression in rat liver tissue and monolayer- and spheroid-cultured hepatocytes. Cells Tissues Organs 191, 281–288 (**2010**).

[19] Andrei, G. Three-dimensional culture models for human viral diseases and antiviral drug development. Antiviral Res 71, 96–107 (**2006**).

[20] T. Takano, et al., Astrocyte-mediated control of cerebral blood flow, Nat. Neurosci. 9 (2) (**2006**).

[21] Xingchi Chen Chang Liu , Laureana Muok , Changchun Zeng and Yan Li, Dynamic 3D On-Chip BBB Model Design, Development, and Applications in Neurological Diseases.

[22] B. Srinivasan, A.R. Kolli, M.B. Esch, H.E. Abaci, M.L. Shuler, J.J. Hickman, TEER measurement techniques for in vitro barrier model systems, J Lab AUTOM. (**2015**)

[23] E. Urich, C. Patsch, S. Aigner, M. Graf, R. Iacone, P. Freskgard. Multicellular Self-Assembled Spheroidal Model of the Blood Brain Barrier. Scientific reports (**2013**)

[24] Barrile R, van der Meer AD, Park H, Fraser JP, Simic D, Teng F, Coregliano D, Nguyen J, Jain A, Zhoul, Karalis K, Ingber DE, Hamilton GA and Otieno MA. Organ-on-Chip Recapitulates Thrombosis induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems; Clin Pharmacol Ther. (**2018)** Dec; 104(6):1240-1248. https://doi.org/10.1002/cpt.1054

[25] Song J, Miermont A, Lim CT and Kamm RD. A 3D microvascular network model to study the impact of hypoxia on the extravasation potential of breast cell lines; Sci Rep. **2018**, 8(1): 17949. https://dol.org/10.1028/54159g-018-36381-5

[26] Vormann MK, Gijzen L, Hutter S, Boot L, Nicolas A, van den Heuvel A, Vriend J, Ping Ng C, Nieskens TE, van Duinen V, de Wagenaar B, Masereeuw R, Suter-Dick L, Trietsch Su, Wilmer M, Joore J, Vulto P and Lanz HL. Nephrotoxicity and Kidney Transport Assessment on 3D Perfused Proximal Tubules; AAPS J. (**2018)** Aug 14;20(5):90. https://doi.org/10.1208/512248-018-0248-Z

[27] Wevers N, van Vught R, Wilschut K, Nicolas A, Chiang C, LanzHL, Trietsch, Su, Joore J and Vulto P. High-throughput compound evaluation on 3D networks of neurons and glia in a microfluidic platform; Scil Rep. (**2016)** Dec 9;6:38856. https://doi.org/10.1038/srep38856

[28] Zhang M, Jiang L and Qin J. A 3D human lung-on-a-chip model for nanotoxicity testing; Toxicol Res (Camb).6. (**2018)** ;7(6):1048-1060. https://doi.org/10.1039/c8tx00156a.

[29] Osaki T, Uzel SGM and Kamm RD. Microphysiological 3D model of amyotrophic lateral sclerosis (ALS) from human iPS-derived muscle cells and optogenetic motor neurons; Sci. Adv. (**2018)**; 4 : eaat5847 10 October 2018. https://doi.org/10.1126/sciadv.aat5847.)

[30] Baglietto-Vargas, D. et al. Generation of a humanized AB expressing mouse demonstrating aspects of alzheimer's disease-like pathology, Nature News. Nature Publishing Group. (**2021**)

[31] Maider Badiola-Mateosa, Davide Di Giuseppee, RobertoPaolia, Maria Jose Lopez-Martinez, Arianna Mencattinie, Josep Samitier et al. A novel multi-frequency trans-endothelial electrical resistance (MTEER) sensor array to monitor blood-brain barrier integrity, Sensors and Actuators B: Chemical. Elsevier. (**2021**)

[32] Paoli, R. et al. Rapid manufacturing of multilayered microfluidic devices for organ on a chip applications, MDPI. Multidisciplinary Digital Publishing Institute. (**2021**)

[33] Menaceur, C. et al. The blood-brain barrier, an evolving concept based on technological advances and cell-cell communications, Cells. U.S. National Library of Medicine. (**2021**)

[34]Louise Delsing, Anna Herland, Anna Falk, Ryan Hick, Jane Synnergren, Henrik Zetterbergaghi, et al. Models of the blood-brain barrier using IPSC-derived cells, Molecular and Cellular Neuroscience. Academic Press. (**2020)**

[35] Wang YI, Abaci HE, Shuler ML. Microfluidic blood-brain barrier model provides in vivo-like barrier properties for drug permeability screening. Biotechnology and Bioengineering; (**2017)** 114:184–94. https://doi.org/10.1002/bit.26045.

[36] Ines Hammami, N. Alabdhallah et al. Gold nanoparticles: Synthesis properties and applications, Science. Elsevier. (**2021**)

Annexes

ANNEX 1: FABRICATION OF BBB-oC PDMS REPLICAS

a. Mixing, degasification and curing

- Take the two reagents needed to obtain the PDMS replicas, the silicon elastomer and the curing agent (Sylgard 184).
- Go to the weighting scale and pour them in a proportion of 10:1 (elastomer:curing agent). (35g in each master)
- Then stir the mix with a glass stick for around 1 min to homogenize the content and start the crosslinking process.
- Place the glass inside the desiccator, connect it to the vacuum system and leave it for around 30 min.
- After that, break the vacuum to remove all the bubbles. If some small bubbles are left, remove them with a plastic pipette.
- Pour the PDMS mix inside the petri dish with the designed mold.
- Leave the Petri dish with PDMS in the bench for 10 min and afterwards place it in the oven at 65 ºC for about 2 hours.
- After that, cut the borders with the scalpel and gently peel off the PDMS from the wafer and cut the PDMS block into single devices. The cutting in this step must be a bit larger than the final one, leaving more space in the edges so that when the holes are punched, the chips don't break.
- Punch access holes in the PDMS with a 1 mm biopsy punch for the hydrogel chamber inlets and 4mm for the media reservoirs.
- Cut the borders of the PDMS in the final size of approximately 6x11 mm each single device.
- Cut the edges of each device at 45º with the scalpel to remove irregularities in the borders.

b. Cleaning and bonding PDMS – glass slides

- Wash the slides following the next steps:

1. Put the slides into water/soap solution in slide chamber and then sonicated for 5 minutes at high intensity.
2. Then, rub the glass and wash with water
3. Put the slides into acetone and sonicated for 5 minutes at high intensity.
4. Immediately, put the slides into IPA and sonicated for 5 minutes at high intensity.
5. Dry them with N_2.

1. Wash the PDMS chips with ethanol with the wash bottle and dry it with hot plate at 85ºC for 10 min to evaporate any trace of ethanol .

2. Remove the replicas from the hot plate a few minutes before putting them into the plasma machine, to cool them down.

c. Bonding

- Check if the chips and coverslips have any dust (if so, blow them with N2)
- Place them in the plasma cleaner for 30 seconds 0,8 torr at constant pressure.
- Take the coverglass with the activated side facing upwards and place the activated side of the PDMS chips on top to bond them together. Press the PDMS to promote the stacks bonding, especially in the central part.
- Place the bonded chips to the hot plate at 85°C for 5 minutes.
- Place the bonded chips to the oven at 85ºC overnight to hydrophobized them.

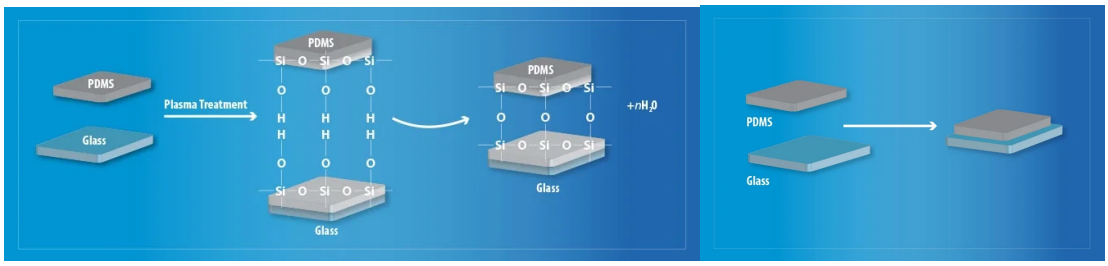


Figure A1. Representative scheme of the chemical explanation for the plasma treatment.

ANNEX 2: CELL CULTURE

2.1. Culture of Blood-Brain Barrier hCMEC/D3:

(Cat. No. SCC066, Millipore)

(Max. 10 passages)

Medium preparation

EndoGRO MV complete media kit = basal medium (2-8ºC) + supplements (-20ºC) (Cat. No. SCME004, Millipore)

supplemented with:

- 1 ng/ml FGF-2*(Cat. No. GF003, Millipore) (for 500 ml of medium, 5 µl of stock solution 1 mg/ml)
- Antibiotics (1% Pen Strep)

*FGF-2 is reconstituted in 0,1M phosphate buffer, pH 6.8 to a concentration of 0.1-1.0 mg/ml (stock of 1 mg/ml= 50 µl buffer for 50 µg of lyophilized FGF-2)

ECM Coating of flask

- Thaw collagen type I, rat tail at room temperature (C3867, Sigma)
- Dilute 1 ml of collagen type I with 19 ml 1XPBS. Mix gently.
- Coat flask with 1:20 diluted collagen type I solution. Use 5-10ml for T75 flasks and 15-25ml for T225 flasks. Incubate in 37ºC incubator for at least one hour before use. (Note: flasks may be coated 5-6 days in advance and stored at 2-8ºC in the coating solution)
- Aspirate de coating solution just before plating the cells.

Thawing of cells

- Remove the vial of hCMEC/D3 cells from liquid nitrogen and incubate at 37ºC water bath.
- As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol
- In a laminar flow hood, transfer the vial content to a conical tube and add dropwise 9 ml of pre-warmed complete medium.
- Mix the cell suspension by slow pipetting up and down.
- Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
- Decant as much of the supernatant as possible.
- Resuspend the cells in a total volume of 10 ml of medium.
- Plate the cell mixture onto a pre-coated culture flask.
- Incubate the cells at 37ºC in a 5% CO_2 humidified incubator.
- Change the medium the next day and exchange every two to three days thereafter.
- When the cells are approximately 80% confluent (3-4 days) they can be dissociated with trypsin-EDTA.

Subculturing of cells

- Carefully remove the medium of the flask
- Apliy 3-5 ml of trypsin-EDTA and incubate in a 37ºC incubator for 3-5 minutes.

- Add 8 ml of pre-warmed complete medium and mix the cells suspension.
- Transfer the dissociated cells to a conical tube.
- Centrifuge at 300 x g for 3-5 minutes
- Discard the supernatant
- Resuspend in medium and count the cells.
- Plate the cells to the desired density

Cryopreservation of cells

hCMEC/D3 medium with 10 % DMSO, using a Mr. Frosty container.

2.2. Culture of human astrocytes-hippocampal HIPPOCAMPAL (HA-h):

(Cat. No. 1830, ScienCell)

Caution: Cryopreserved primary cells are very delicate. Thaw the vial in a 37ºC water bath and return the cells to culture as quickly as possible with minimal handling! Do not centrifuge the cells after thawing as this can damage the cells.

Defreeze

1. Prepare a poly-L-lysine-coated culture vessel (2 µg/cm^2). For a T-75 flask add 10 ml of sterile water and 15 µl of poly-L-lysine stock solution (10 mg/ml Cat. No. 0413, ScienCell) and leave in the 37ºC incubator for a minimum of one hour or overnight.
2. Prepare complete medium:

   Astrocyte Medium (Cat. No. 1801)

   500 ml basal medium. Add the supplements:

   10 ml FBS (Cat. No. 0010)

   5 ml Astrocyte Growth Supplement (Cat. No. 1852)

   5 ml Penicillin/streptomycin solution (Cat. No. 0503)

   Storage in the dark at 4ºC. Reconstituted medium is stable for one month.
3. Rinse the poly-L-lysine coated vessel twice with sterile water and add 15 ml of complete medium.
4. Loose the cap of the frozen vial inside the biosafety cabinet to release the pressure inside. Tight the cap again and place the frozen vial in a 37ºC water bath until thaw the cryopreserved cells. Wipe it down with 70% ethanol and transfer to the biosafety cabinet.
5. Carefully remove the cap and gently resuspend and dispense the contents of the vial into the culture vessel previously coated with poly-L-lysine.
6. Return the culture vessel to the incubator.
7. Do not disturb the culture for at least 16 hours after initation. Refresh culture medium the next day to remove residual DMSO and unattached cells. Change the medium every 2-3 days until they reach 90% confluency.

Subculture

1. Prepare poly-L-lysine coated culture vessel on day before.
2. Warm complet medium, trypsin/EDTA solution 0,25% (Cat. No. 0103), T/E neutralization solution (TNS) (Cat. No. 0113) and DPBS (Ca^{++}- and Mg^{++}- free, Cat. No. 0303) to room

temperature. (when TNS finishes we can go without it. When DPBS from ScienCell finishes we use DPBS cat. No. 21600 Life Technologies. When Trypsin/EDTA finishes we can use Trypsine/EDTA cat. No. 25200 Life Technologies)

3. Rinse the cells with DPBS
4. Add 9 ml DPBS and 1 ml of 0,25% Trypsine/EDTA. Gently rock the flask to ensure coverage of the cells and use a microscope to monitor the change in morphology until cells detach.
5. Add 5 ml of FBS to a centrifuge tube.
6. Transfer the cells in suspension to the centrifuge tube containing the 5 ml of FBS.
7. Add TNS solution to the flask to collect the residual cells and transfer to the centrifuge tube.
8. Examine the cells being left behind in the flask, there should be less than 5%.
9. Centrifuge the tube at 1000 rpm for 5 min (attention: in the cell culture room centrifuge we use 500 rcf per 5 min and Deceleration: 1)
10. Discard the supernatant (by decantation or by aspiration, carefully) and resuspend the cells in culture medium.
11. Count and plate the cells in a new poly-L-lysine coated culture vessel. A seeding density of 5000 cells/cm^2 is recommended.

Freezing:

Cryopreservation of primary cells is not recommended. Refreezing cells may damage them and affect cell performance.If we want to freeze: 1 million cells per 1 ml in FBS 10% DMSO.

2.3. Culture of human brain vascular (HBVP)

(Cat. No. 1201, ScienCell)

Caution: Cryopreserved primary cells are very delicate. Thaw the vial in a 37ºC water bath and return the cells to culture as quickly as possible with minimal handling! Do not centrifuge the cells after thawing as this can damage the cells.

Defreeze

1. Prepare a poly-L-lysine-coated culture vessel (2 µg/cm^2). For a T-75 flask add 10 ml of sterile water and 15 µl of poly-L-lysine stock solution (10 mg/ml Cat. No. 0413, ScienCell) and leave in the 37ºC incubator for a minimum of one hour or overnight.
2. Prepare complete medium:

   Pericyte Medium (Cat. No. 1801)

   500 ml basal medium. Add the supplements:

   10 ml FBS (Cat. No. 0010)

   5 ml Pericyte Growth Supplement (Cat. No. 1852)

   5 ml Penicillin/streptomycin solution (Cat. No. 0503)

   Storage in the dark at 4ºC. Reconstituted medium is stable for one month.
3. Rinse the poly-L-lysine coated vessel twice with sterile water and add 15 ml of complete medium.

4. Loose the cap of the frozen vial inside the biosafety cabinet to release the pressure inside. Tight the cap again and place the frozen vial in a 37ºC water bath until thaw the cryopreserved cells. Wipe it down with 70% ethanol and transfer to the biosafety cabinet.
5. Carefully remove the cap and gently resuspend and dispense the contents of the vial into the culture vessel previously coated with poly-L-lysine.
6. Return the culture vessel to the incubator.
7. Do not disturb the culture for at least 16 hours after initiation. Refresh culture medium the next day to remove residual DMSO and unattached cells. Change the medium every 2-3 days until they reach 90% confluency.

Subculture

8. Prepare poly-L-lysine coated culture vessel on day before.
9. Warm complet medium, trypsin/EDTA solution 0,25% (Cat. No. 0103), T/E neutralization solution (TNS) (Cat. No. 0113) and DPBS (Ca^{++}- and Mg^{++}- free, Cat. No. 0303) to room temperature. (when TNS finishes we can go without it. When DPBS from ScienCell finishes we use DPBS cat. No. 21600 Life Technologies. When Trypsin/EDTA finishes we can use Trypsine/EDTA cat. No. 25200 Life Technologies)
10. Rinse the cells with DPBS
11. Add 9 ml DPBS and 1 ml of 0,25% Trypsine/EDTA. Gently rock the flask to ensure coverage of the cells and use a microscope to monitor the change in morphology until cells detach.
12. Add 5 ml of FBS to a centrifuge tube.
13. Transfer the cells in suspension to the centrifuge tube containing the 5 ml of FBS.
14. Add TNS solution to the flask to collect the residual cells and transfer to the centrifuge tube.
15. Examine the cells being left behind in the flask, there should be less than 5%.
16. Centrifuge the tube at 1000 rpm for 5 min (in the cell culture room centrifuge we use 300 rcf per 5 min)
17. Discard the supernatant (by decantation or by aspiration, carefully) and resuspend the cells in culture medium.
18. Count and plate the cells in a new poly-L-lysine coated culture vessel. A seeding densitit of 5000 cells/cm^2 is recommended.

Freezing:

Cryopreservation of primary cells is not recommended. Refreezing cells may damage them and affect cell performance.

If we want to freeze: 1 million cells per 1 ml in FBS 10% DMSO.

ANNEX 3: TRYPSINIZATION

3.1. Trypsinization of HA and HP:

Reagents:

- PBS (Fridge. Third shelf on the left)
- Suero
- Polyglicine (Fridge. Third shelf on the left. In a centrifuge tube)
- Trypsine Scientific Cell (Freezer. First shelf)
- HA and HP Medium: (Fridge. Third shelf on the left. Prepare a reduced quantity on a centrifuge tube to prevent contaminating the whole bottle of medium)

Methodology:

- Prepare the coating of the flask: Resuspend PBS with Polyglicine in a centrifuge tube.
- With a pipette, add to a new flask and incubate for 40 min (minimum) at 37 ºC and 5 pCO2

  During the incubation time of the coated flask:
- Add 5 ml of trypsin Scientific to the flask containing the cells
- 2 min in the incubator (temperature and pCO2)
- Add 5 ml of suero to stop the enzymatic activity of the trypsin
- Resuspend the cells in the flask to disaggregate the cells and place the 10ml volume in 15mL centrifuge tube
- Centrifuge 5 min at 1000 rpm acceleration 9 (put another tube with the exact same volume to equilibrate)
- Remove the supernatant carefully pouring it into a precipitate glass and controlling that the pellet does not detach
- Resuspend the pellet (cells) in 10 ml of HA / HP medium respectively

- Once the incubation time of the coated flask is up, remove the medium of the flask with an aspirating pipette
- Wash 2 times with 5 ml PBS and also with water, with the help of a pippete and making sure to wash all the surface of the flask
- Remove the water using aspirating pipette
- Pour 5ml of the cells into the coated flask and add 5ml of HA /HP medium repectively. If it is not necessary for the cells to be confluent the next day, count with a Neubauer chamber the exact number of cells that need to be added.
- Mark the flask with the passage number, date and your name and put it in the incubator at 37 ºC and 5 pCO2.

Cabinet cleaning process:

- Remove all reagents and place them in their correspondant place (fridge, freezer, etc.)
- Remove the waste bag and throw it in the biological waste bin
- Clean the aspirating tube with bleach and water, inserting the first the bleach and aspirating, and then doing the same with water. Leave the aspiration open for a bit so that the tube dries.
- Throw away all used paper covers for pippetes and stack the unused ones in their specific place.
- Once the cabinet only has its essential materials (3 manual pippetes and one automatic pippete), clean with ethanol.

- Close the light and the glass and put UV light

3.2. Trypsinization of hCMEC/D3:

Reagents:

- Innoprot™ Dulbeccos's Phospate-Buffered Solution (DPBS): Fridge, third shelf on the left
- Sigma-Aldrich™ Collagen Type I Solution from rat tail (C3867-1VL, PCode: 1003233209) Fridge, third shelf on the left. In a centrifuge tube
- Trypsine (Freezer. First shelf)
- EndoGRO™ Basal Medium (Catalog #SCME-BM). Fridge. Third shelf on the left. (Prepare a reduced quantity on a centrifuge tube to prevent contaminating the whole bottle of medium)

Methodology:

- Prepare the coating of the flask: Resuspend 4.75mL PBS with 250µl de Collagen in a centrifuge tube of 20ml.
- With a pippete, add to a new flask and incubate for 40 min (minimum) at 37 ºC and 5 pCO2
- Remove the medium of the flask (containing the cells) with an aspirating pipette
- Wash 1 time with 5 ml PBS
- Remove the PBS using aspirating pipette and add 5 ml of trypsin
- 2 min in the incubator (temperature and pCO2)
- Add 5 ml of EM medium to stop the enzymatic activity of the trypsin
- Resuspend the cells in the flask to disaggregate the cells and place the 10ml volume in 15mL centrifuge tube
- Centrifuge 5 min at 1000 rpm acceletarion 9 (put another tube with the exact same volume to equilibrate)
- Remove the supernatant carefully pouring it into a precipitate glass and controlling that the pellet does not detach
- Resuspend the pellet (cells) in 10 ml of EM medium if you have a big pellet
- Aspirate the remaining liquid from the coated flask once it is out of the incubator (after the 40 min)
- Pour 5ml of the cells into the coated flask and add 5ml of EM medium. If it is not necessary for the cells to be confluent the next day, count with a Neubauer chamber the exact number of cells that need to be added.
- Mark the flask with the passage number, date and your name and put it in the incubator at 37 ºC and 5 pCO2.
-
- Cabinet cleaning process:
- Remove all reagents and place them in their correspondant place (fridge, freezer, etc.)
- Remove the waste bag and throw it in the biological waste bin
- Clean the aspirating tube with bleach and water, inserting the first the bleach and aspirating, and then doing the same with water. Leave the aspiration open for a bit so that the tube dries.
- Throw away all used paper covers for pippetes and stack the unused ones in their specific place.
- Once the cabinet only has its essential materials (3 manuak pippetes and one automatic pippete), clean with ethanol.
- Close the light and the glass and put UV light

ANNEX 4: CELL SEEDING PROTOCOL IN MICROFLUIDIC DEVICES

4.1 HA, HP cell seeding

Materials:

- Trypsine 0.05% Science cell: Freezer, first shelf.
- Eppendorf tubes
- Aspirating pipettes
- Neubauer chamber: second shelf in culture room.
- 10ml, 25ml, and 5 ml pipettes
- Yellow and blue micropipette tips
- Fibrinogen: freezer -20ºC outside the culture room. Box 3.
- Thrombin: freezer -20ºC outside the culture room. Box 3.
- Medium AM:EM (1:1), ScienCell™ Astrocyte Medium (AM) (Cat #1801), EndoGRO™ Basal Medium (Catalog #SCME-BM) . Culture room fridge. Third shelf
- Trypsinisation reagents:
- Innoprot™ Dulbeccos's Phospate-Buffered Solution (DPBS) (Fridge. Third shelf on the left)
- Suero
- Polyglicine Fridge. Third shelf on the left. In a centrifuge tube)
- Trypsine Scientific Cell (Freezer. First shelf)
- ScienCell™ Astrocyte Medium (AM) (Cat #1801), ScienCell™ Pericyte Medium (PM) (Cat #1201): Fridge. Third shelf on the left. Prepare a reduced quantity on a centrifuge tube to prevent contaminating the whole bottle of medium)

Methodology

- Disinfect the chips and scissors inside the class II cabinet during 15 min with UV light
- To prepare the hydrogel: Take the thrombin and fibrinogen from the fridge and weigh the fibrinogen 3mg/ml (prepare at least 3 mL, thus weight 9mg). Keep them in ice (Almost better if you do this before starting because the thrombin takes time to unfreeze).
- When resuspending, avoid the formation of bubbles by keeping the pippet close to the bottom of the tube without air entering.
- Trypsinyze HA and HP to detach the cells (see protocol trypsinization of HA,HP)
- Count the cells with the neubauer chamber. Each chip needs 40000 cells of each type.
- In a small eppendorf tube, add the volume that corresponds to 40000 cells of each type and mix them (label it to found it easily in the ice box)
- Centrifuge the eppendorf tube for 5min 1000g outside the culture room.
- Discard the almost complete supernatant with a yellow tip and keep the pellet.
- Put each eppendorf tube with the pellet in ice.
- Homogenized fibrinogen and thrombin suspension before use them.
- Take 50µL of fibrinogen and resuspend it the pellet. After, add 1µL of thrombin. Resuspend in eppendorf tube. This step needs to be fast to avoid the polymerization due to heat. Thrombin and fibrinogen need to be kept in ice during the whole process.
- Take 15µL of the resuspended pellet and insert it in the chip
- The cells+hydrogel need to be inserted into the small hole (Central chamber) with delicacy to avoid breaking the chip. It is recommended to hold the lateral parts of the chip with the hands so that it is stable.
- Check with the optical microscope that the cells+hydrogel have stayed in the central chamber and have not filtered to the side channels. If so, throw the chip away.

- If the filtration has only happened in one of the side channels, keep the chip because it still works in one channel.
- Incubate for 15min at 37ºC and 5 pCO2
- Once the chips are out of the incubator, add medium: EM:AM (1:1)
- Add the medium into the larger holes of the chip. The medium must only be added to one hole of each side.
- Cut a p1000 tip and insert it to the hole that does not have medium, slightly aspirate so that the medium flows to both holes.
- Remove the medium of all holes, to discard any debris
- Add new medium to all holes
- Incubate and change medium every day

4.2 HCMECs cell seeding

Materials:

- Innoprot™ Dulbeccos's Phospate-Buffered Solution (DPBS): fridge, third shelf
- EM:AM medium :ScienCell™ Pericyte Medium (PM) (Cat #1201), ScienCell™ Astrocyte Medium (AM) (Cat #1801): fridge. Third shelf
- Scissors: Sujey's drawer
- 1000p, 200p and corresponding tips: culture room. Second shelf
- Tip boxes
- Neubauer chamber: culture room. Second shelf
- Eppendorf tubes: culture room. Second shelf. In a closed container.

Trypsinization Reagents:

- Innoprot™ Dulbeccos's Phospate-Buffered Solution (DPBS): Fridge, third shelf on the left
- Sigma-Aldrich™ Collagen Type I Solution from rat tail (C3867-1VL, PCode: 1003233209) Fridge, third shelf on the left. In a centrifuge tube
- Trypsine (Freezer. First shelf)
- EndoGRO™ Basal Medium (Catalog #SCME-BM). Fridge. Third shelf on the left. (Prepare a reduced quantity on a centrifuge tube to prevent contaminating the whole bottle of medium)

Methodology:

1. Prepare the coating of collagen in one of the channels: in a centrifuge tube, add 4.75mL PBS and 250µL collagen
2. Remove the medium from the chips and add new medium only to the channel that will NOT be seeded with ECs.
3. With a marker, make a signal on the glass beside the channel that will be seeded with ECs, for easy recognition.
4. Add the collagen coating into one of the holes of the channel that will be seeded with ECs
5. Cut with scissors the p1000 tip and carefully place it in the hole that doesn't have collagen. Slightly aspirate with the pipette until the collagen flows through the channel and it is visible that both holes have more or less the equal amount of collagen.
6. With the p200, remove the collagen from the holes, as remove previous cell debris.

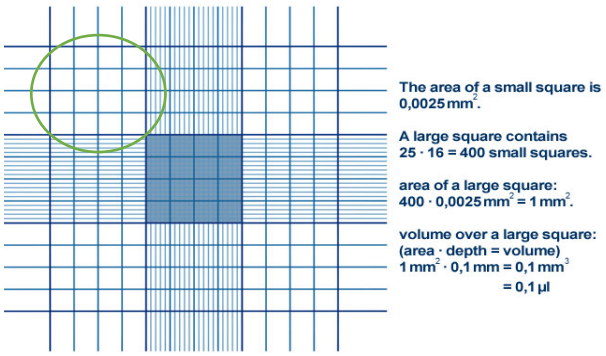
7. Add new collagen from the prepared centrifuge tube into both of the holes from the seeded channel.
8. Place the coated chips in the incubator at 37ºC 5pCO2 for at least 40 min
9. For the preparation of the EC cells, once they have been trypsinized and centrifuged (see protocol Trypsinization HCMEC/D3), and they are in a tube with their medium, count them.
10. For the counting of cells, place 10µL of cells into the neubauer chamber (with a p50) and count how many cells are in each quadrant. Sum the total number of cells (of each of the four quadrants) and divide it by 4, to average the number of cells per quadrant. Multiply this average by 10^4 to obtain the nº cells /ml. Each chip needs 100.000 ECs, so divide this number by the nº cells/ml to obtain the exact quantity (in µL) to insert in each chip.
11. Put the computed quantity corresponding to 100.000 cells into individual eppendorf tubes
12. Centrifuge the eppendorf tubes during 5min at 1000rpm outside the culture room.
13. With a pipette, remove the supernatant and keep the pellet.
14. After 40 min, remove the chips from the incubator and remove the remaining liquid collagen from the holes (it has already coated the channel)
15. Resuspend the pellet of cells with 30µL of EM:AM medium
16. Add the resuspended cells+medium into one of the holes of the marked channel. Avoid the formation of bubbles.
17. With scissors, cut the tip of the p1000 and carefully place it on the hole with no seeded cells. Aspirate slightly until the cells flow through the channel. It is reccomended to insert the tip until the bottom of the chip to put more pressure.
18. Place the chips vertically (the endothelial channel facing downwards to the hydrogel, so that the ECs can adhere to the hydrogel by gravity) in sterilized tip boxes. To keep the chips from sliding, use a soft adhesive tape to stick them to the wall of the box.
19. Place the boxes with the vertical chips in the incubator at 37ºC 5pCO2 for 1h30min.
20. After this time is up, change the medium of the chips.
21. Place them into the incubator at 37ºC 5pCO2 overnight

Cabinet Cleaning process:

- Remove all reagents and place them in their correspondant place (fridge, freezer, etc.)
- Remove the waste bag and throw it in the biological waste bin
- Clean the aspirating tube with bleach and water, inserting the first the bleach and aspirating, and then doing the same with water. Leave the aspiration open for a bit so that the tube dries.
- Throw away all used paper covers for pippetes and stack the unused ones in their specific place.
- Once the cabinet only has its essential materials (3 manual pippetes and one automatic pippete), clean with ethanol.
- Close the light and the glass and put UV light

ANNEX 5: CELL COUNTING PROTOCOL WITH NEUBAUER CHAMBER

- Clean the chamber and the coverslide.
- Fill the chamber with 18-20 µl of cell suspension with a micropipette (place the tip between the coverslide and the chamber and the liquid enter by capillarity)
- Count the cells on the microscope using the 10X objective. Calculate the average of cells in the 4 fields (4x4 little squares marked in the image with an orange circle)



- Use this formula to calculate the number of cells.

c = n x v

c = concentration of cells in 1 ml of cells suspension

n = number of counts

v = volume of the chamber

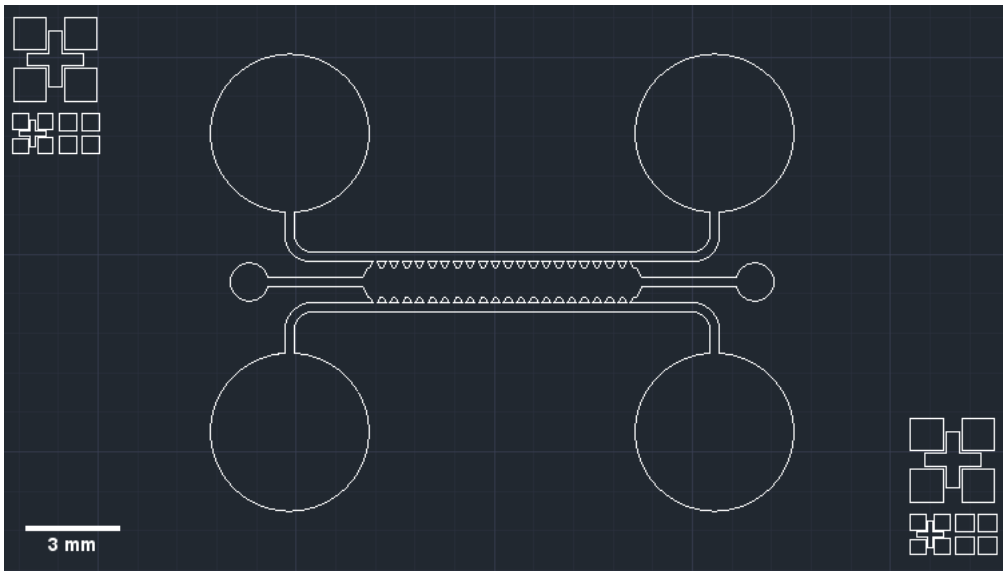
Depth: 0, 1 mm

Area: 1 mm^2

Volume: 0,1 mm^3 (10^{-4} ml)

c = n x 10^{-4} (=cels/ml)

ANNEX 6: DIMENSIONS OF MICROFLUIDIC BBB-oC DESIGN



Design of microfluidic BBB-oC

Main chamber: 1300 µm wide, 8800 µm long, 150 µm high
Lateral channels: 300 µm wide, 150 µm high
Trapezoidal pillars: 300 µm base and separated 100 µm

Designed by CAD software (AutoCAD 2019). Master moulds fabricated in a cleanroom environment using standard photolithography techniques with 4-inch silicon wafers as substrates and the SU8-2100 as photoresist.