



ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

**DEVELOPMENT OF *IN VITRO* THREE DIMENSIONAL
BLOOD-BRAIN BARRIER MODEL**

GÖZDE ERVİN KÖLE
Ph.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR

Assoc. Prof. DENİZ YÜCEL

SECONDARY SUPERVISOR

Prof. VASİF NEJAT HASIRCI

ISTANBUL-2025



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15.12.2025

Gözde Ervin Köle

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ABBREVIATIONS AND SYMBOLS

ACs	Astrocytes
α-SMA	α -Smooth muscle actin
AQP-4	Aquaporin-4
AJs	Adherens junctions
BBB	Blood brain barrier
BM	Basement membrane
BMECs	Brain microvascular endothelial cells
CFD	Computational Fluid Dynamic
Col	Collagen
CNS	Central nervous system
dH₂O	Deionized water
ECM	Extracellular matrix
ECs	Endothelial cells
EthD-1	Ethidium homodimer-1
FCS	Fetal calf serum
FTIR	Fourier transform infrared
HA	Hyaluronan/ Hyaluronic acid
HUVECs	Human umbilical cord vein endothelial cells
ICA	Internal carotid artery
IPN	Interpenetrating polymer network
JAM	Junctional adhesion molecule
LSCM	Laser Scanning Confocal Microscopy
MAP-2	Microtubule-associated protein 2
MeHA	Methacrylated hyaluronic acid
MCA	Middle cerebral arteries
NVU	Neurovascular unit
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PCs	Pericytes
PCL	Poly(ϵ -caprolactone)

PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
PNS	Peripheral nervous system
PFA	Paraformaldehyde
TEER	Transendothelial electrical resistance
TJs	Tight junctions
VFP	Viscous finger patterning
ZO-1	Zonula occludens-1



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ABSTRACT

Development Of *In Vitro* Three-Dimensional Blood-Brain Barrier Model

The neurovascular unit (NVU) plays a central role in maintaining blood–brain barrier (BBB) integrity; however, reproducing its multicellular organization and the complex intrinsic morphology of the native basement membrane remains a major limitation in current *in vitro* BBB models. This study aimed to develop a histologically relevant BBB–NVU platform by reconstructing the blood-side compartment using a fibrous membrane and the brain-side compartment using a hydrogel that recreates the neural microenvironment. First, a Transwell-like model using a flat fibrous membrane was established to evaluate endothelial monoculture and a multicellular co-culture obtained by integrating other cell types with a MeHA–collagen hydrogel beneath the membrane. The co-culture condition increased CD31 expression and resulted in higher TEER values compared to monoculture, demonstrating improved endothelial integrity. To better mimic the intrinsic morphology of the native capillaries, two microfluidic NVU-on-a-chip systems were developed: a basic chip model incorporating a two-branched tubular fibrous membrane, and a complex chip model featuring a further-branched tubular structure with increased curvature and effective surface area. Both tubular scaffolds were embedded in MeHA–collagen hydrogel and cultured for 14 days under continuous perfusion. Immunocytochemical analysis confirmed that endothelial cells, astrocytes, pericyte-like cells, and neurons preserved their immunophenotype and spatial organization in all systems. Barrier assessment demonstrated progressively reduced permeability to 0.4 kDa fluorescein from the Transwell model to the basic branched chip, reaching the lowest permeability in the complex branched NVU-on-chip, indicating improved barrier tightness with increasing architectural complexity. These findings highlight the potential of branched tubular fibrous membrane integrated with perfusion-based microfluidics as biomimetic NVU platforms for studying BBB physiology and drug transport *in vitro*.

Keywords: Blood-brain barrier, 3D *in vitro* model, NVU-on-a-chip, Fibrous membrane, Hyaluronic acid based hydrogel

ÖZET

In Vitro Üç Boyutlu Kan-Beyin Bariyeri Modelinin Geliştirilmesi

Nörovasküler ünite (NVU), kan ile beyin dokusu arasındaki etkileşimi düzenler ve kan-beyin bariyerinin (KBB) bütünlüğünün korunmasında kritik bir rol oynar. Ancak NVU'nun çok hücreli organizasyonu ile doğal bazal membranın karmaşık iç morfolojisinin *in vitro* ortamda yeniden üretilebilmesi, mevcut KBB modellerinin önemli bir sınırlamasıdır. Bu çalışmada, kan tarafı için fibröz bir membran ve beyin tarafı için nöral mikroçevreyi taklit eden bir hidrojel kullanılarak, histolojik olarak daha gerçekçi bir KBB–NVU platformu geliştirilmiştir. İlk olarak, düz fibröz membran kullanılan Transwell benzeri modelde endotel hücrelerin monokültürü ile bu membranların altına entegre edilen MeHA–kolajen hidrojel aracılığıyla diğer hücrelerin entegre edilmesi ile elde edilen çok hücreli birlikte kültür karşılaştırılmıştır. Birlikte kültür koşulları, monokültüre kıyasla CD31 ekspresyonu ve TEER değerlerinde artış göstererek endotelyal bütünlüğün iyileştiğini ortaya koymuştur. Doğal kılcal damarların yapısal özelliklerini daha yakın taklit etmek amacıyla iki farklı mikroakışkan NVU-on-a-chip sistemi geliştirilmiştir: iki dallı tübüler fibröz membran içeren temel model ve eğrilik ile etkin yüzey alanı artırılmış daha ileri dallanmış kompleks model. Her iki tübüler yapı da MeHA–kolajen hidrojel içinde 14 gün boyunca sürekli perfüzyon altında kültüre edilmiştir. İmmünohistokimyasal analizler, endotel hücreleri, astrositler, perisit-benzeri hücrelerin ve nöronların tüm modellerde immunofenotiplerini ve organizasyonlarını koruduğunu göstermiştir. Bariyer değerlendirmeleri, 0.4 kDa fluorescein geçirgenliğinin Transwell modelinden temel çip modeline ve en düşük değerlere ulaşılan kompleks çip modeline göre daha az olduğunu ortaya koymuştur. Bu bulgu, daha karmaşık geometrinin daha güçlü bir bariyer oluşturduğunu doğrulamaktadır. Elde edilen bulgular, dallanmış tübüler fibröz membranların perfüzyon temelli mikroakışkan sistemlerle entegrasyonunun, KBB fizyolojisi ve ilaç moleküllerinin geçiş dinamiklerinin *in vitro* incelenmesi için etkili bir biyomimetik NVU platformu sunduğunu göstermektedir.

Anahtar Sözcükler: Kan beyin bariyeri, 3B *in vitro* model, NVU-on-a-chip, Fiber membran, Hiyalüronik asit temelli hidrojel

1 INTRODUCTION

The human brain is composed of specialized neurons and glial cells, characteristic vascular structure, and a unique extracellular matrix (ECM) composition. The blood vessels responsible for vascularizing the central nervous system (CNS) play a key role in the blood-brain barrier (BBB), which is an active barrier and able to adapt in accordance with the needs of the CNS (1). The specialized and intricate network within the CNS that coordinates the relationship between neurons and blood vessels is known as the neurovascular unit (NVU). It consists of various cell types such as neurons, astrocytes, endothelial cells, pericytes, as well as the extracellular matrix providing structural and functional support. The NVU ensures the maintenance of cerebral homeostasis by coordinating cerebral blood flow, preserving BBB integrity, and supporting neuronal signaling and metabolism. This structural and functional synergy is essential for healthy brain function and plays a crucial role in the onset and progression of various neurological disorders, including stroke, neurodegenerative diseases, and neuroinflammatory conditions.

BBB and/or NVU studies have been conducted on animal models, which are often referred to as the gold standard because these organisms provide a native microenvironment for the BBB and immune system (2). However, it has been reported that 80% of drugs successful in animal tests fail in human trials because of the mismatch in cellular and molecular composition, as well as genetic variations between animals and humans. Therefore, the development of *in vitro* BBB models as innovative therapeutic strategies has become prominent, as 3D *in vitro* tissue models using human cells are promising tools to validate *in vivo* findings. The establishment of accurately replicated BBB model has tremendous potential to be used in a wide range of applications, such as CNS disease modeling, high-throughput drug screening, and neurotoxicology testing. Moreover, the NIH and FDA encourage scientist to develop “human-on-a-chip” models which provide a more realistic platform to perform experiments especially for visually invisible organs such as BBB or NVU *in vitro* condition.

Early BBB models in the literature were primarily based on conventional Transwell systems. Subsequently, more advanced 2D and 3D platforms utilizing microfluidic technologies have been developed to better replicate the structural and functional complexity of the native neurovascular environment. An ideal BBB-incorporated NVU model should possess the essential heterogeneous architectural features of the native tissue structure to achieve *in vivo* characteristics under *in vitro* conditions (3). These features can be listed as (i) integration of 3D vessel-like structures into the model, (ii) allowing co-culture of the relevant cell types, (iii) applying shear stress on endothelial cells (ECs) via perfusion to mimic the blood vessels, and (iv) the presence of a thin basement membrane (BM) in tubular form. Although co-culture and perfusion strategies have been incorporated into existing models, faithfully replicating the native BM, particularly in terms of its complex geometry and mechanical properties, remains a significant challenge. Moreover, many current models fail to simultaneously address both the neural tissue and vascular components, despite their closely coordinated roles within the NVU (4). The primary objective of constructing advanced models is to replicate the dynamic microenvironment of the BBB. Recent *in vitro* systems have been developed in the field of organ-on-a-chip technology, which integrates tissue engineering and microfluidic approaches (2). These BBB/NVU-on-chip platforms enable cell culture in a physiologically more realistic environment, better capturing the geometry, dimensionality, mechanical forces, and biochemical cues, thereby overcoming many limitations of the conventional Transwell systems.

The main objective of this study was to develop a physiologically relevant *in vitro* BBB-incorporated NVU model by integrating principles of tissue engineering and microfluidics. The proposed model included a branched, tubular electrospun fibrous membrane designed to imitate the structural, mechanical, and morphological features of the native vascular BM. This membrane enabled endothelial cell adhesion and monolayer formation on the luminal surface, while preventing cellular penetration and allowing controlled intercellular communication. The fibrous tubular scaffold was embedded within a PDMS-based microfluidic system, facilitating perfusion and spatial compartmentalization. The brain-side compartment was constructed using a hyaluronic acid-based hydrogel loaded with astrocytes, pericyte-like cells, and

neurons, thus replicating the biochemical and mechanical environment of the brain ECM. By recapitulating the dual-sided structure of the NVU, the model aimed to investigate how the neural compartment influenced barrier function of the tissue-engineered BBB under dynamic culture conditions. Ultimately, this platform is intended to serve as a versatile tool for modeling neurodegenerative and cerebrovascular diseases, as well as for studying cellular interactions, permeability dynamics, and disease mechanisms in a 3D culture, human-relevant context.



2 BACKGROUND

2.1 Blood Brain Barrier (BBB)

The human brain is defined as the most complex and complicated organ in the body since it is responsible for a wide range of roles starting from the fundamental motor functions such as eating, sleeping, and walking to the cognitive capabilities like, problem-solving, learning, and decision-making (5). Despite its known features, there are still many unknowns about the brain, which keep its mystery. Fundamentally, the human brain is composed of specialized neurons and glial cells, characteristic vascular structures, and a unique ECM composition. Anatomically, the nervous system is divided into two components as the CNS, composed of brain and spinal cord, and the peripheral nervous system (PNS) which includes nervous tissue outside the CNS (6). The blood vessels responsible for the vascularization of the CNS possesses distinctive properties that form the BBB. The concept of the BBB was first observed in the late 19th century by the Nobel Prize-winning scientist Paul Ehrlich (7). He worked on practicing tissue staining at the beginning of his career. He noted that when a dye was injected into the bloodstream of a mouse, it could infiltrate all the tissues except the brain and the spinal cord. The initial conclusion from this observation was that brain has a low affinity for the dye. Further studies performed by Goldman, an associate of Paul Ehrlich, showed that when the acidic dye trypan blue was injected into the brain ventricles, it stained only the CNS and not the other organs (8, 9). One of the pioneer in this field, Lewandowsky, proposed that cerebral capillaries have restrictive properties based on his latest studies involving toxins injections (10). Stern, a Russian scientist, was likely the first to coin the term “barrier” and referred to this concept as “Barrière hématoencéphalique” in her studies (11, 12). However, the limited magnification capacity of the light microscope hindered a better understanding of the BBB structure (10). Advancements in electron microscopy made it possible to investigate the structural characteristics of blood vessels throughout the body. In 1967, Reese and Karnovsky demonstrated for the first time that endothelial cells of cerebral capillary constitute a barrier structure (13). Subsequent studies using tracers and electrodes showed that the BBB has low permeability and high transendothelial

electrical resistance (TEER) (14). The main goal of the above-mentioned studies was to better understand the complex BBB and to reveal its structure and components.

BBB is an active barrier and able to change in accordance with the needs of the CNS (1). The specialized and intricate network within the CNS that coordinates the relationship between the multicellular and vascular components known as the NVU. It consists of various cell types such as neurons, astrocytes, endothelial cells, pericytes, and extracellular matrix as structural and functional support.

This collaborative assembly of cells and matrices works in harmony to regulate blood flow, maintain the integrity of the BBB, which selectively allows substances to pass from the blood to the brain, and support neuronal function. The NVU's functions are essential for ensuring proper nutrient delivery, waste removal, and response to changes in the brain's needs, contributing significantly to the overall health and function of the brain.

2.1.1 Native structure of NVU

The NVU is a concept established by the Stroke Progress Review Group of the National Institute of Neurological Disorders and Stroke in 2001 to emphasize the relationship between brain cells and brain vascularity (15). As the name indicates, NVU is comprised of both neuronal and vascular components. The interaction between the neuronal and vascular components varies across different segments of the cerebrovascular tree due to the structural diversity within the NVU. A typical vascular tree consists of arteries and arterioles, which transport blood to tissues, the capillary beds where gas and nutrient exchange occurs within tissues, and venules and veins that carry blood away from tissues (16). Anatomically, within the cerebrovascular tree, the internal carotid artery (ICA) enters the skull and then merges with the vertebral arteries to form a circle of Willis, which supplies blood to the cerebral cortex (Figure 1a) (15). Middle cerebral arteries (MCA) arise from the circle of Willis and branch into pial arteries and arterioles, which cover the brain surface. Arterioles form a highly interconnected network by penetrating deeper into the brain. These penetrated arterioles flow into the capillary bed, which connects to the venous system (Figure 1b). Histologically, the capillaries in the cerebrovascular tree consist of endothelial cells

surrounded by a complete basement membrane (BM), pericytes, and astrocyte end-feet that enclose the vessels; all of these components contribute to the formation of the BBB.

Nervous tissue side of the NVU mainly includes neurons, astrocytes, and microglia, while the vascular part consists of brain microvascular endothelial cells (BMECs), the basement membrane, and pericytes (Figure1c). The tight junctions between the BMECs and the presence of continuous BM are crucial for highly selective semipermeable BBB (Figure1d).



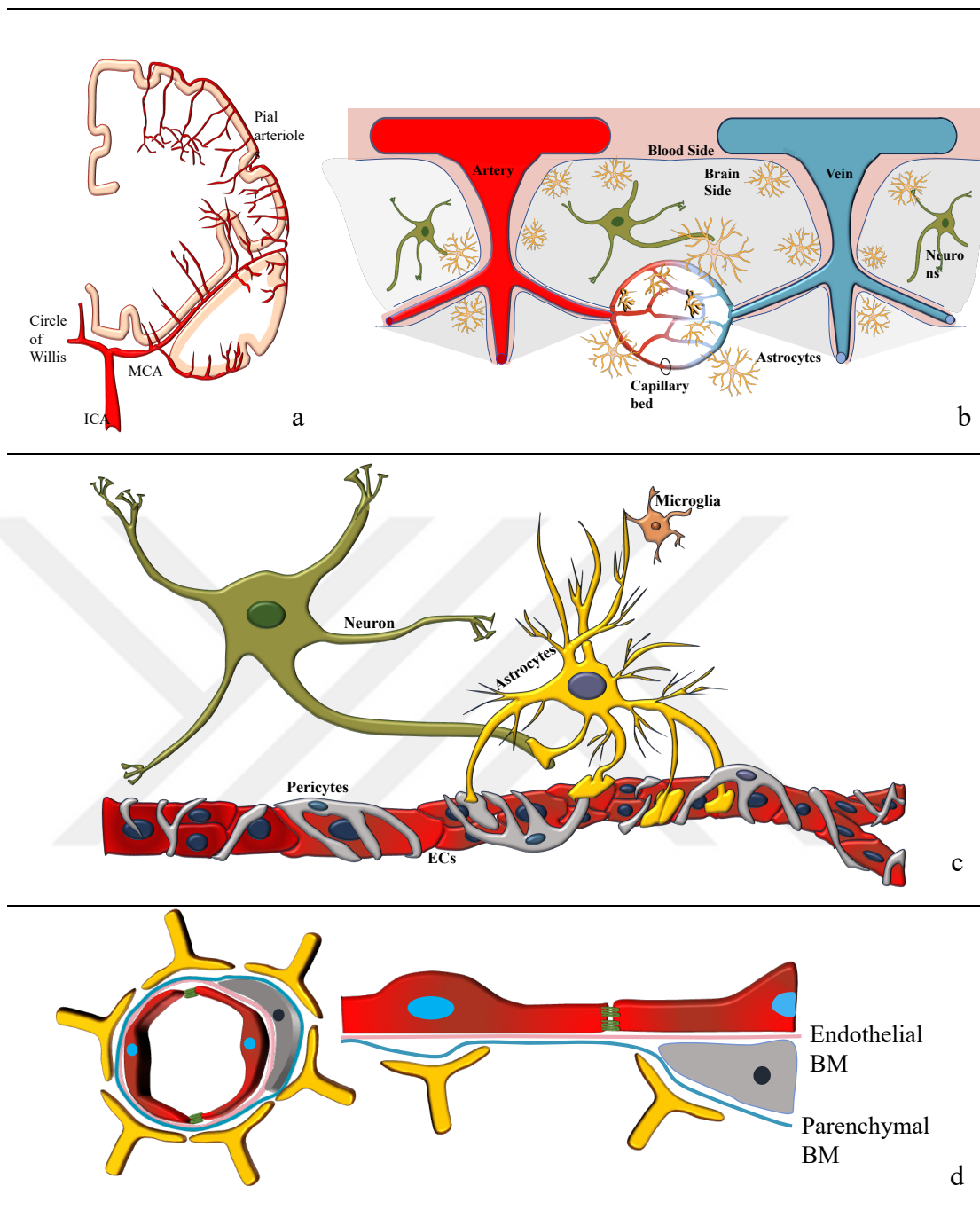


Figure 1. Illustrations of the anatomical and histological features of the NVU. (a) cerebrovascular tree, (b) structural and (c) cellular organization of NVU, and (d) components of BBB presented as a vessel and a cross section.

2.1.1.1 Structural constituents of NVU

The NVU is constituted of several types of cells and BM. The main cell types of NVU are BMECs, pericytes, astrocytes, and neurons. The two types of BMs are endothelial BM and parenchymal BM.

2.1.1.1.1 Cells of NVU

2.1.1.1.1.1 Brain microvascular endothelial cells (BMECs)

BMECs display flattened morphology, lack fenestrae, and contain a high number of mitochondria (4). Functionally, the distinguishing feature of brain endothelium compared to peripheral endothelium is its heavily restrictive barrier function (17). This highly selective, semipermeable membrane is primarily maintained by junctional complexes between endothelial cells in the brain microvasculature (1). The junctional complexes within the interendothelial space include adherens junctions (AJs), tight junctions (TJs), and gap junctions (Figure 2) (18). The role of the gap junctions is to mediate cell-cell communication, while AJs and TJs are responsible for the restrictive permeability of the endothelium. AJs regulate paracellular transport, initiate cell polarity, and mediate cell-to-cell adhesion. The main type of AJs is vascular endothelial (VE)-cadherin which is a Ca^{+2} -dependent cell-cell adhesion protein, and it is essential for barrier formation. Although disruption of AJs leads to an increase in permeability, TJs are primarily responsible for the barrier properties of endothelial cells, as they generate high TEER and restrict paracellular transport (17, 18). TEER value is typically 2-20 $\Omega\cdot\text{cm}^2$ in the peripheral capillaries, whereas it was $> 1000 \Omega\cdot\text{cm}^2$ in rat brain endothelium (19). TJs are protein complexes localized at the apical region of interendothelial cleft (Figure 2). The TJ protein complex provides the physiological function of BBB by completely sealing the intercellular cleft to limit paracellular transport, cooperating with active efflux transporter, and lacking fenestrae along the apical surface of the endothelium (18, 20, 21). At the molecular level, TJs form two distinct complexes of transmembrane proteins linked to the cytoskeletons of adjacent cells through cytoplasmic TJ proteins (Figure 2). TJ proteins include occludin, claudin and junctional adhesion molecule (JAM). Occludin is a primary transmembrane protein consists of four transmembrane domains, two extracellular

loops, and three cytoplasmic domains, and it acts as a paracellular barrier (18, 22). Characteristic charged structure of extracellular domains might promote cell-to-cell attachment and increase the electrical resistance of TJ-expressed tissues. Occludins are associated with zonula occludens (ZO)-1 via their cytoplasmic domains (Figure 2). This interaction maintains the cell surface polarity and regulates the permeability of endothelial cells (ECs). Occludins are continuously expressed along the cell membrane of cerebral endothelium, while they are sparsely expressed in peripheral endothelium. Claudins are integral transmembrane proteins consisting of four transmembrane domains and two extracellular loops, similar to occludins (22). One of the extracellular loops affects paracellular charge selectivity, while the other loop promotes claudin-claudin interactions. Claudins are responsible for regulating the selective permeability of ions and contribute to cell polarity. Their expression pattern vary among tissues, and claudin-1,-3 and -5 are expressed in the cerebral endothelium (18). JAMs are transmembrane proteins that belong to immunoglobulin superfamily proteins, and they are expressed by different cell types (22, 23). They are involved in cellular adhesion processes such as tight junction assembly, angiogenesis, and transmigration of leukocytes via their homophilic and heterophilic interactions. ZO-1 proteins are the cytoplasmic plaque types of TJ proteins (22). They are associated with TJs, AJs and gap junction proteins. Their interaction with TJs is important for the stability and function of TJs (18). Disruption of these proteins often associated with increased vascular permeability. Showing localization of TJs with immunostaining is widely used to validate cell characteristics. Continuous expression of TJs, such as ZO-1, at the cell membrane is one of the minimum criteria for *in vitro* BBB models.

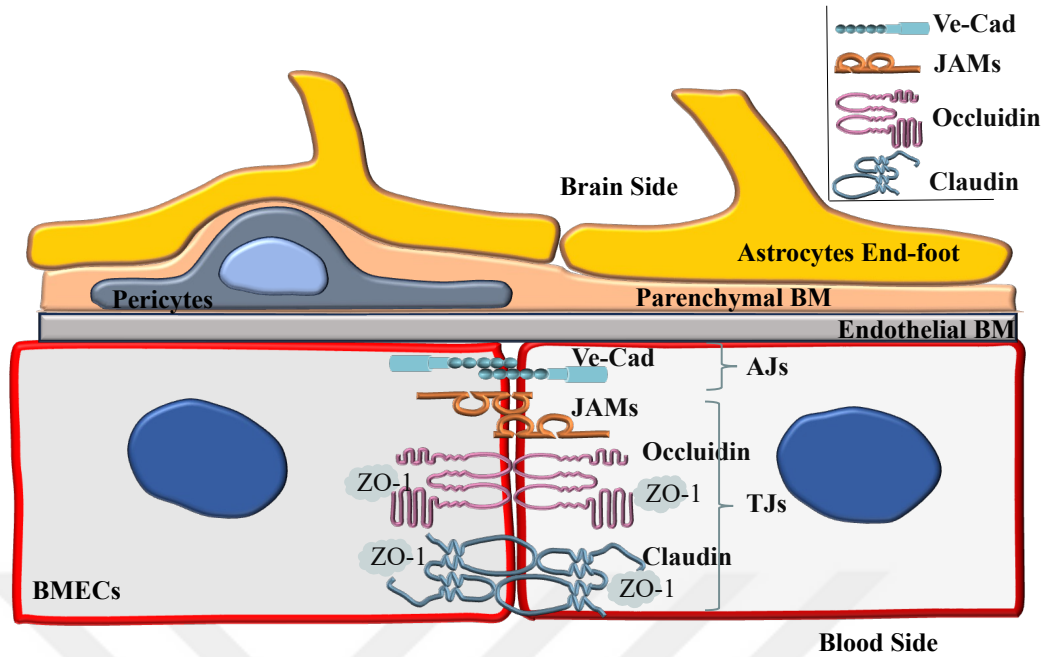


Figure 2. Illustration of junctional complexes (VE-Cad ; Vascular Cadherin, JAMs; Junctional Adhesion Molecule, AJs; adherens junctions, TJs; tight junctions)

2.1.1.1.2 Pericytes (PCs)

Pericytes (PCs) are multifunctional vascular mural cells embedded in basement membranes of small blood vessels, such as capillaries, precapillary arterioles, and post-capillary venules (24). They partially wrap ECs and provide support through direct physical contact and paracrine signaling. In addition, the close interaction of pericytes with ECs involves not only structural proximity but also functional support (25). They play a role in the regulation of cerebral blood flow, BBB permeability, angiogenesis and neuroinflammation (24). Pericytes express several contractile and cytoskeleton proteins, such as α -smooth muscle actin (α -SMA), vimentin, and myosin. Because they express contractile proteins, pericytes can modulate cerebral blood flow in response to neuronal activity and regulate capillary diameter. Pericytes-derived proteins can mediate the expression of TJs and AJs in BMECs, indicating their role in maintaining BBB integrity (18).

2.1.1.1.3 Astrocytes (ACs)

Astrocytes (ACs) are the predominant glial cells of the NVU. They communicate simultaneously with both neurons and capillaries, serving as a cellular bridge that enables physical and chemical interactions between these structures (17, 25). Astrocytes enclose the brain capillaries via their end-feet. Physical communication between neural tissue and brain capillary takes place through astrocyte end-feet. End-feet are highly specialized extensions unique to astrocytes, maintaining intimate contact with pericytes as well. These cellular extensions provide a large surface area for contact with pericytes and for the action of neurotransmitters and neuromodulators. These close associations of astrocytes and capillaries lead to the formation of a unique cellular microenvironment, thereby contributing to the specialized barrier properties of BBB (17, 18).

2.1.1.1.4 Neurons

Neurons reside in the neural compartment of the NVU alongside astrocytes and microglia, whereas ECs and pericytes are part of the vascular component (26). Neurons are often referred to as “the peacemaker of the NVU” since they take role in homeostatic mechanisms through dynamic nature of their neural activity (18, 25). Neurons can detect subtle changes like in nutrients and oxygen, and then convert these changes into signals that communicate directly with adjacent neurons or astrocytes, either through direct contact or signaling molecules. Thereby, neurons initiate regional vascular responses, such as activating mechanisms that adjust vascular tone (15).

2.1.1.1.2 Basement membrane

In addition to cellular components, another important structures that contribute the NVU formation are the BM for the blood side and extracellular matrix for the neuronal part (Figure 3a). BM surrounds and interact with the ECs, therefore they play an important role in the barrier function of the NVU (16). Capillaries located at the CNS segment of the vascular tree possess unique properties to contribute to restrictive barrier formation. Histologically, the microvasculature of the CNS belongs to the "continuous nonfenestrated type" of the three main structural classes of

capillaries. In these types of capillaries, cells are joined by tight junctions, they have a complete basement membrane with a lack of fenestra. Therefore, they restrictively regulate the movement of solutes from blood to the tissue. In addition to high expression of TJs, structure and function of BMs are among the reasons for heavily restrictive barrier feature. Anatomically, vascular structures of CNS were surrounded by two BMs, the endothelial BM and parenchymal BM (Figure3b). The endothelial BM were secreted by ECs and pericytes, while parenchymal BM were secreted by the astrocytes end feet. These BMs were mainly comprised of collagen type IV, laminin, nidogen, heparin sulfate proteoglycans, and other glycoproteins. Mechanically, biomechanical properties of the native structure is another important consideration, since BMECs are able to sense multiple mechanical cues and translate them into the biochemical signals which regulate the protein and gene expression of AJs (2). For instance, increase in matrix stiffness can lead the disruption in ECs barrier formation, and culture of ECs on the softer surface (in kPA range) can increase expression of AJs. In the development of *in vitro* BBB model, construction of the faithful mimicry of the native BMs remain as a challenge due to intrinsic complexity of the structure (3).

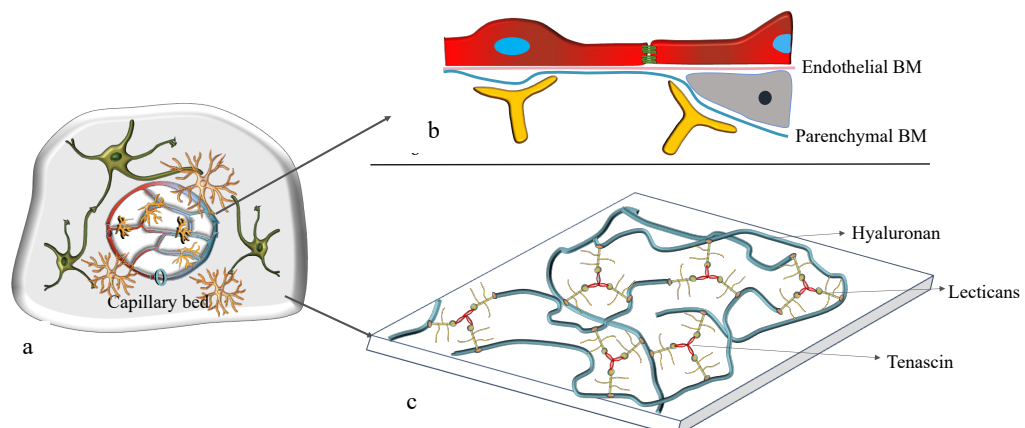


Figure 3. Representative images of (a) neurovascular unit, (b) basement membranes of brain capillaries and (c) brain extracellular matrix

Structural support of cells at the neuronal side is provided by the brain ECM. Brain ECM constitutes 20% of the total volume of the brain and is made up of a macromolecular network of glycosaminoglycans (hyaluronan), proteoglycans (lecticans), glycoproteins (tenascin) and low levels of fibrous proteins (collagen,

fibronectin) (27, 28). Mechanically, Young's modulus of the neuronal tissues is < 1 kPa and studies showed that mechanical properties of the ECM-like structure greatly affect the *in vitro* cell behavior, attachment, and cell growth (29, 30). Hydrogel having an elastic modulus of < 80 kPa provides a most successful condition for the neuronal cell culture. Structurally, hyaluronan (HA) also known as hyaluronic acid is the main structural component of the brain ECM and increases the hydration of the brain tissue. HA bonds to lecticans, and lecticans are linked via tenascins which act as crosslinkers and contain cell attachment side (Figure 3c). It acts as a physical barrier to regulate the diffusion of soluble and membrane-associated molecules, and cell migration (28). However, the brain side of NVU is generally overlooked in the development of *in vitro* NVU or BBB model studies. Therefore, a model that mimics the key features of both the vascular side and brain side in terms of the structure, topography, and mechanical properties, could be a promising approach to obtain a low permeable *in vitro* NVU model.

2.2 Integration Of Tissue Engineering And Microfluidics As An Emerging Approach: Organ-On-A-Chip Technology

Regenerative medicine and tissue engineering are promising fields that aim to address clinical challenges through the integration of biomaterials, engineering, biology and medicine (27). Tissue engineering is one of the major subfields in regenerative medicine that combines biomaterials, cells, and, optionally, bioactive agents to develop a 3D tissue-specific constructs. Biomaterials are used in the production of scaffolds that provide structural support and cues for cells to facilitate cell adhesion, proliferation, and differentiation, ultimately aiding in the reconstruction of tissue architecture in terms of morphological, biological, and mechanical features. These scaffolds thereby guide the organization and maturation of cells into functional tissue substitutes or tissue units under controlled *in vitro* conditions. These tissue equivalents can be used not only for tissue replacement in clinical applications, but also for the development of 3D *in vitro* models in translational research, and/or for pharmacological or toxicological screening. Microfluidics involves scientific and technological systems designed to handle and manipulate very small volumes of fluids, typically flowing through channels measuring in the range of ten to one hundred

micrometers (31). As a multidisciplinary field, it involves molecular biology and microelectronics, and it can be applied in various areas including physics, chemistry, biology, medicine, etc.

Organ-on-a-chip is an innovative and promising field that has emerged from the foundational areas of tissue engineering and microfluidics (32). It allows the incorporation of tissue engineering components such as cells, biomaterials, and signaling molecules into chip platforms fabricated using advanced microfabrication techniques. Thus, heterogeneous tissue architecture can be better mimicked, allowing for more accurate and functional *in vitro* tissue models to be developed within the chip platform. Moreover, the NIH and FDA have joined forces to encourage the development of “human-on-a-chip” platforms that provide more realistic environment to perform experiments (33). The development of “human-on-a-chip” project heavily depends on the establishment of various “organ-on-a-chip” platforms for different organs, supported by vascular structures and nerves. Another important aspect of these systems is their ability to enable the reconstruction of visually inaccessible organs, such as the BBB or NVU, under *in vitro* conditions. Until now, studies on the BBB and/or NVU have primarily been conducted using animal models, which are often referred to as the gold standard because they provide the native microenvironment of the BBB, and include an intact immune system (2). However, it has been reported that 80% of drugs that showed success in animal testing failed in human clinical trials, largely due to differences in cellular and molecular composition, as well as genetic variations between animals and humans. In light of this information, the development of *in vitro* BBB models as innovative therapeutic strategies has become increasingly important, since 3D *in vitro* tissue models using human cells serve as excellent tools to validate the *in vivo* findings. The establishment of an accurately replicated BBB model has tremendous potential for use in a wide range of applications, such as CNS disease modeling, high-throughput drug screening, and neurotoxicology testing (27).

An ideal NVU model should replicate the essential heterogeneous architectural features of the native structure to precisely mimic *in vivo* characteristics under *in vitro* conditions (3). These features include: (i) the integration of 3D vessel-like structures into the model, (ii) the provision of an appropriate environment for the co-culture of

all NVU cell types, (iii) the application of shear stress on ECs through perfusion to mimic blood flow, and (iv) the presence of a thin, tubular BM. Even though co-culture and perfusion have been successfully integrated into some models, the faithful replication of the native BM including its morphological and mechanical properties remains a significant challenge. Existing models also have major limitations, such as addressing neural tissue and brain vascularity separately, despite their interconnected roles (4).

NVU models that consider both the brain and vascular components such as mechanical/ morphological properties of BM and ECM, as well as cellular composition could serve as a promising alternative to *in vivo* models for advancing knowledge about disease mechanisms, intercellular communication, transport processes, and the development of new drugs.

2.3 Progression Of NVU Models From Early To Modern Approaches

The evolutionary path of *in vitro* BBB models shows that early models were based on simple Transwell systems, followed by the development of more advanced 2D and 3D models using microfluidic platforms (Figure 4). Transwell systems are commercially available and widely used systems in *in vitro* BBB models. Although these systems are inexpensive, accessible, and easy to replicate, they also have some drawbacks, such as oversimplicity, lack of physiological relevance, and difficulty in incorporating dynamic conditions. A standard Transwell system is composed of two parts, called the upper and lower compartments, separated by a porous membrane that mimics the BM of native tissue (Figure 4a). Models established using Transwell systems investigate the effect of monoculture and co-culture of cells, as well as different membrane characteristics including pore size, thickness, morphology, and mechanical properties on a permeability (34-36).

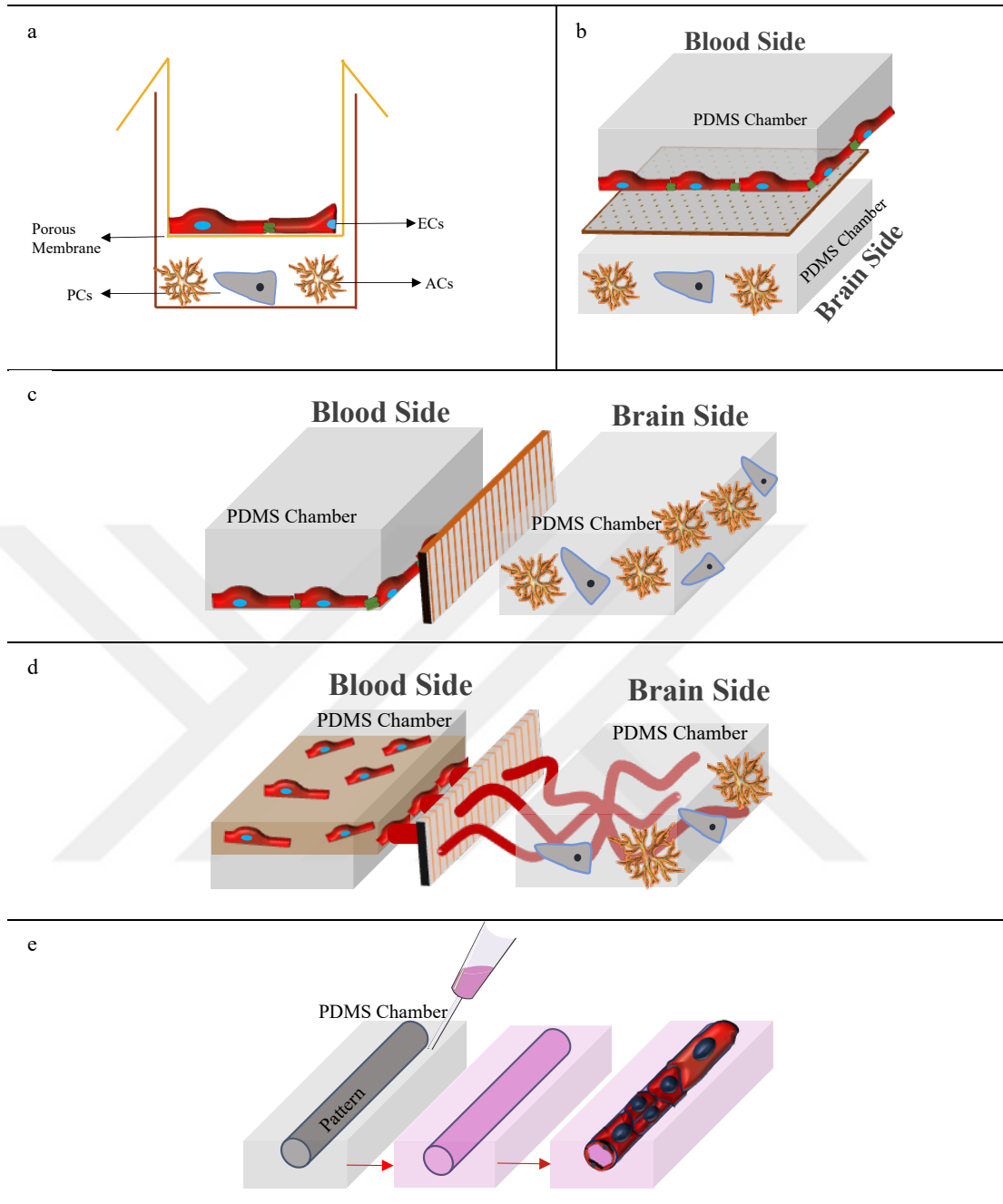


Figure 4. Schematic representation of various design approaches for the blood brain barrier (BBB) modelling; (a) Transwell systems, (b) sandwich design, (c) vertical design, (d) 3D horizontal design, (e) 3D patterned design.

The early and basic BBB models established on the Transwell system focused on the monoculture of ECs on traditional permeable PET membranes (37, 38). Later Transwell models incorporated co-cultures of ECs, astrocytes, pericytes, and neurons, as well as different membrane types and coatings (16, 35, 39-42). Hatherel et.al. compared the mono-, co- and tri-culture BBB models using traditional PET

membranes to identify the most effective configuration for BBB modeling, focusing on tight junction formation between ECs (39). They concluded that the most effective model for enhancing tight junction formation was the co-culture of astrocytes and ECs, as evidenced by TEER measurements. In one study, the effects of pore diameter, membrane material, manufacturer of PET membrane, and co-culture configuration on these membranes were evaluated (40). The researchers reported that a pore size of 0.4 μm and a non-contact co-culture configuration had a positive impact on barrier formation. Several studies using electrospun fibrous membranes or hydrogel membranes instead of PET membranes have been conducted to evaluate the impact of membrane physical and biochemical properties on barrier formation and performance (35, 36, 41, 42). The native BM is a fibrous network of proteins; therefore, the utilization of fibrous membranes could represent an effective strategy to imitate its native structure. Electrospinning is a widely used technique to obtain fibrous mesh structures, allowing control over fiber diameter, mechanical properties and degradation time by altering the polymer composition. In a recent study, Bischel et. al. fabricated a gelatin-based electrospun nanofibrous membrane and used it in a Transwell system to co-culture astrocytes and ECs (35). They found that cell-matrix secretion and tight junction proteins were upregulated when cells were cultured on an electrospun membrane compared to a traditional membrane. In another study, the biochemical characteristics of membrane surfaces were evaluated by comparing different coating conditions in relation to their barrier properties (42). They concluded that fibronectin and collagen IV coated membranes exhibited significantly higher TEER values compared to other coating conditions. Besides the aforementioned *in vitro* model construction studies and the investigation of various parameters affecting barrier formation, advanced models are being developed to achieve a more physiologically relevant *in vitro* model.

The main goal of constructing advanced models is to mimic the dynamic microenvironment of the blood-brain barrier (4). A new type of *in vitro* model has been developed in the field of organ-on-a-chip systems, which integrate tissue engineering with microfluidic systems (2). These *in vitro* BBB/NVU-on-chip models allow cells to be cultured in a physiologically more realistic environment in terms of geometry, dimensions, mechanical forces, and biochemical cues, thereby overcoming

the limitations of traditional Transwell systems. This is particularly important for endothelial cells, which need to be cultured under dynamic conditions and exposed to shear stress to improve barrier tightness. Dynamic BBB/NVU-on-a-chip models described in the literature can be classified as 2D and 3D models (3, 4). 2D models can be constructed using a sandwich design (43-48) or a parallel design strategy (49-53), while 3D models are typically developed based on either a patterned or self-assembly approach.

Throughout the development of *in vitro* BBB/NVU models, sandwich design models were the first dynamic systems introduced. The main difference between these models and the traditional Transwell system is the incorporation of perfusion into the model (Figure 4b). Resembling a sandwich structure, these models consist of two different polydimethylsiloxane (PDMS)-based compartments separated by a porous membrane (43-45). PC or PET membranes with varying pore sizes are commonly used as porous membranes. The upper chamber typically serves as the vascular side and is seeded with ECs, while the lower chamber represents the neuronal side and can contain various cell types, such as pericytes, astrocytes, neurons, and microglia. Perfusion is then incorporated into the vascular chamber. Research findings indicate that sandwich design models exhibit significantly higher TEER values compared to conventional Transwell systems (43, 44). However, these models have some limitations, such as difficulties in high-resolution imaging and in monitoring molecule transport, due to the chamber height and the poor transparency of membranes. Chamber height also affects cell-to-cell contact between ECs and nervous tissue cells. This contact was achieved by reversing the cell seeding configuration with endothelial cells cultured in the lower chamber and neuronal cells in the upper chamber. (46-48). In one of the most advanced NVU sandwich design chip models, Maoz et. al. connected three chips as coupled chips to study metabolic coupling between the vascular and nervous tissue sides (47). They suggested that the NVU system could serve as a valuable *in vitro* tool for investigating the transport, efficacy, mechanism of action, and toxicity of neuroactive drugs.

Another widely used approach to the 2D BBB/NVU chip model is the parallel design. In this configuration, two chambers are arranged horizontally and separated by

an array of PDMS microchannels (Figure 4c). These microchannels serve as the barrier, similar to the membrane in the sandwich design. In one study, 3 μm micropillars were used to separate the two compartments, the blood and brain sides, acting as a physical barrier between them (49). The blood side was seeded with ECs, while the brain side housed astrocytes. Cell-to-cell communication was provided through micropillars, and it was reported that the expression of tight junction proteins was upregulated compared to those in the Transwell system. Later, the same microfluidic system was applied to a neonatal BBB model to study neurodevelopmental injuries (50), as well as to a brain tumor barrier model to investigate changes in BBB in the presence of tumors (51).

Dynamic 2D models are generally based on rectangular microchannel systems due to the nature of the photolithography process. However, this restricted channel geometry leads to non-uniform flow patterns and shear stress distributions, resulting in variations in the morphology and behavior of the ECs along the rectangular channels (54). To address this issue, improved BBB models have incorporated 3D tubular geometries through the use of advanced microfabrication and fabrication techniques. These models are classified as self-assembled and 3D patterned microfluidic BBB models, each distinguished by the fabrication technique and the substrate used (Figure 4d, e).

In the patterned 3D in vitro BBB models, tubular structures were produced using various fabrication techniques (55-62) (Figure 4e). The first approach involved coating an ECM-based hydrogel over a microscale needle used as a mold, followed by removal of the needle to form a hollow tubular structure, which was subsequently endothelialized (55-57). In one study, a three-dimensional (3D) in vitro BBB model was microengineered within a microfluidic chip by creating a cylindrical collagen gel with a central hollow lumen inside a microchannel, Culturing brain microvascular endothelial cells on the inner surface,(56). This model was used to explore the individual contributions of primary human brain pericytes and astrocytes to the neuroinflammatory response. The molding technique is simple to reproduce; however, it requires expertise and carries a potential risk of damaging the structure during template removal. Additionally, it is limited to producing a single straight hollow

cylinder. Other microfabrication techniques include viscous finger patterning (VFP) and sacrificial templating. These methods offer alternative approaches for producing complex structures with distinct advantages and applications. VFP relies on viscosity gradient between two fluids to form hollow tubular structures. De Graaf et. al. optimized this technique to produce a collagen type 1 hydrogel with a consistent lumen diameter within a microfluidic system (58). Human induced pluripotent stem cell-derived ECs were seeded on the luminal side of the model and co-cultured with pericytes. The sacrificial template method is employed in vascularization studies, enabling the fabrication of complex vascular structures by temporarily embedding sacrificial materials within tissue-engineered constructs (59-62). The sacrificial templating method has been widely applied in various contexts, including thrombosis on-chip and breast tumor modeling (59-62). However, to the best of our knowledge, its application in blood-brain barrier (BBB) modeling has yet to be explored.

2.4 Proposed Model

An ideal BBB model should possess the essential heterogeneous architectural features of the BBB, reflecting both the blood side and the brain side. Such a model has to incorporate the following essential properties: (i) integration of 3D vessel-like structures, (ii) allowance for co-culture with other cell types, (iii) application of shear stress on ECs via perfusion mimicking blood flow, and (iv) the presence of a thin BM in tubular form (3). Developing an *in vitro* BBB model that meets all the requirements mentioned here is still a challenge. To date, co-culture and perfusion integration have been achieved within the BBB *in vitro* models. However, to the best of our knowledge, the integration of a tubular BM-like scaffolds into BBB models has not been reported in the literature. An artificial BM should ideally be made of biocompatible materials, have lumens of various diameters, and be fabricated as a perfusable branched network considering its mechanical, geometrical, and biochemical features. Incorporating these features into the design of *in vitro* BBB model would be valuable for advancing our understanding of disease mechanisms, cellular communication and transport processes, and the development of new drugs.

In this study, the aim was to develop a physiologically more relevant *in vitro* BBB-integrated NVU model using tissue engineering approaches. The model consisted of a tubular electrospun fibrous membrane designed to prevent cell penetration while allowing cell-to-cell communication. Two different designs were fabricated: a basic model with a simple two-branched structure and a complex model featuring a more elaborate branched network. The goal was to produce perfusable vessel-like networks of the electrospun fibrous membranes by using a 3D-printed sacrificial mold as a collector in the electrospinning setup. These branched tubular membranes were integrated into closed, controlled microenvironments composed of PDMS chambers with inlets and outlets. ECs were seeded onto the lumen side of the tubular membrane to mimic the blood side, while ACs, PCs, and neurons were loaded into the hydrogel on the outer side of the electrospun membrane to represent the brain side. The *in vitro* BBB/NVU models were assessed in terms of permeability, mechanical properties, expression of specific cell markers, and cell compatibility.

The ultimate goal of this study was to develop a structurally and functionally more relevant *in vitro* 3D BBB barrier model that closely mimics native tissue. The developed *in vitro* 3D BBB/NVU model could serve as an ideal platform for establishing *in vitro* models of neurodegenerative and cerebrovascular diseases to investigate disease mechanisms. In addition, this *in vitro* 3D BBB/NVU model, with its potential to be a disease model, could be promising tool for new drug development and patient-specific drug screening.

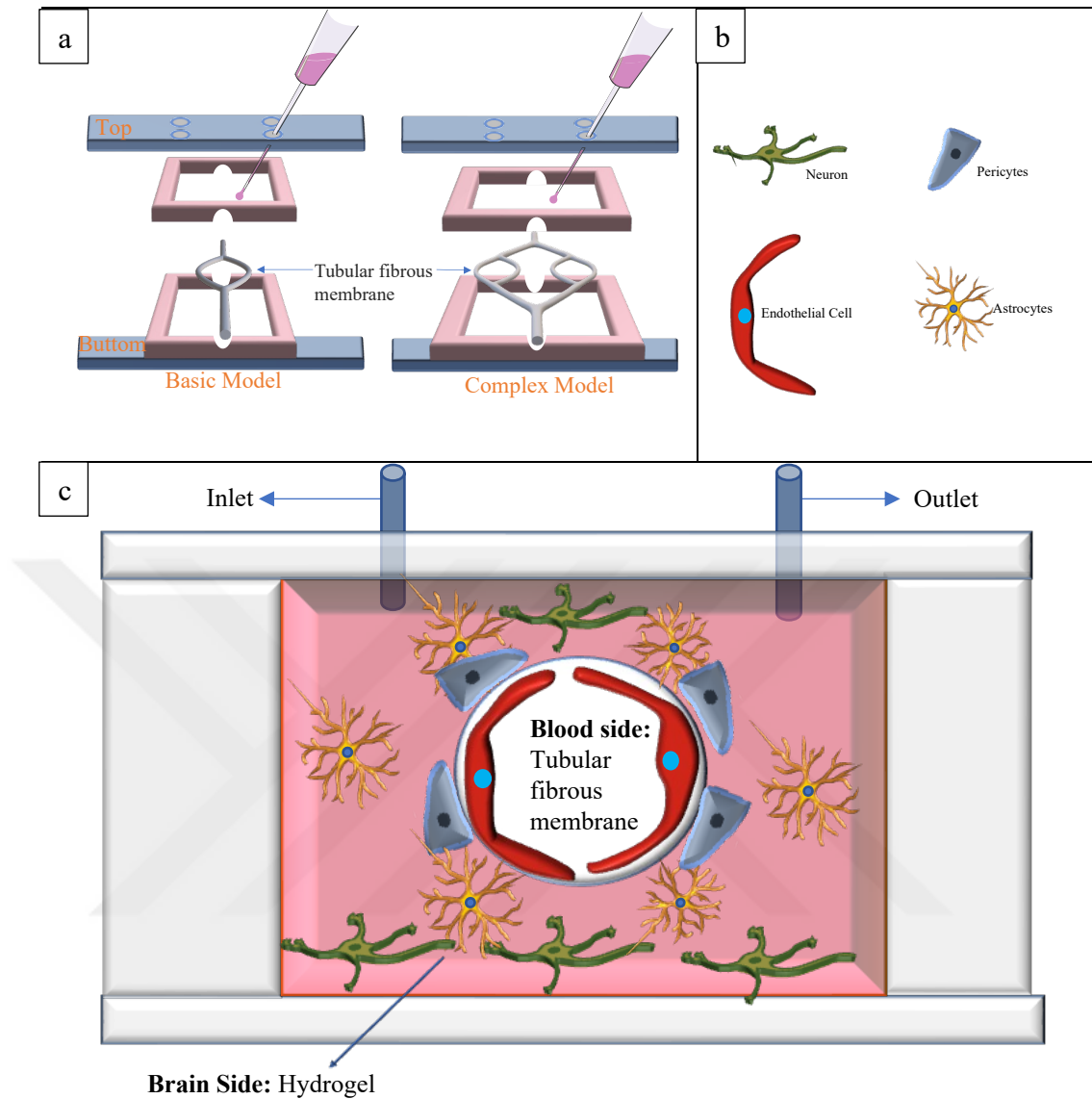


Figure 5. Representative images of (a) side view of basic and complex model components, (b) cell types of the BBB model, and (c) proposed tissue-engineered model

3 MATERIALS and METHODS

3.1 Materials

Polycaprolactone (PCL) (Mw 124000 g/mol) were obtained from Purac. Polyvinly alcohol (PVA) was purchased from Ultimaker. NaCl, DAPI, Trypsin-EDTA, FITC-Phalloidin, fibronectin, Hyaluronic acid, Methacrylic anhydride, Acetic Acid were obtained from Sigma. Thiol modified hyaluronic acid hydrogel kit (HyStem Kit, #GS310) were purchased from Advanced Biomatrix. EGM-2 was obtained from Lonza. Collagen was obtained from BuGa Med. PEG (Mw 1000) was obtained from Fluka, BioChemika. CD31 was obtained from Dako. Antibody against α -SMA was obtained from Syc-Tek. Antibody against AQP-4 was purchased from Proteintech. DMEM High Glucose, DMEM Low Glucose, FBS, Pen/Strep (Gibco), Live/Dead assay was obtained from Thermo Fisher.

3.2 Methods

3.2.1 Production and characterization of 3D branched tubular fibrous membrane

3.2.1.1 Modelling and fabrication of a sacrificial branched tubular construct

The proposed blood-brain barrier (BBB)/neurovascular unit (NVU) model consists of a branched tubular fibrous membrane and a hydrogel. In this study, it was planned to produce the branched tubular fibrous membrane via electrospinning, using a sacrificial cylindrical construct as a collector. This sacrificial construct was conceived to be fabricated by 3D printing. For this purpose, a branched, tubular sacrificial construct was designed through a biomimetic approach, using Murray's Law, which considers circulatory blood volume transfer.

In fluid dynamics, Murray's Law can be defined as the possible relationship between the radii at the junctions of a fluid-carrying tubular network. In this study, the sacrificial branched tubular construct was modeled by applying Murray's Law. A symmetrical system with two-branches, where the radii of the daughter branches are

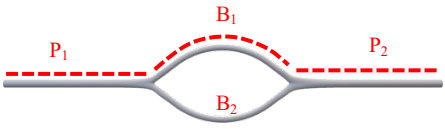
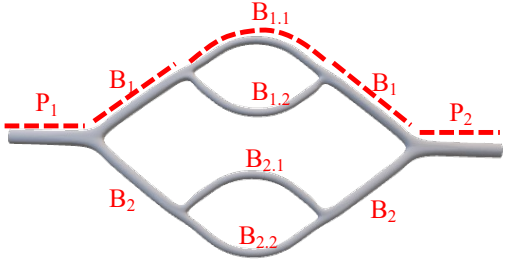
equal, was considered, and the lengths and diameters of the branches were calculated using the following formulas (I and II) (63):

$$\text{Diameter: } \frac{D_{\text{branched}}}{D_{\text{parent}}} = 2^{\frac{-2}{3\omega+3}} \dots \dots \dots I$$

$$\text{Length: } \frac{L_{\text{branched}}}{L_{\text{parent}}} = 2^{\frac{-3\omega+1}{3\omega+3}} \dots \dots \dots II$$

In the formula, ω represents the fluid behavior index, with water being a Newtonian fluid, having a value of $\omega = 1$ (64). Two different branched tubular models were designed using the Fusion360 program. In the basic two-branched tubular design, the parent tube splits into two branches. In the more complex model, further branching was applied to these two branches, resulting in a four-branched tubular construct. Based on Murray's Law, the radii of the tubes in our model were calculated as 800 μm , 630 μm , and 500 μm , from largest to smallest. The total length of the model was approximately 3 cm. In the basic model, the parent tube was 9.3 mm long, and the branched tubes were 11.8 mm each. In the complex model, the parent tube was 5 mm in length, and the branched tubes were 6.3 and 8 mm long (Table 1).

Table 1. Dimensions of the basic and complex models

Basic Model			Complex Model		
Vessel	Length (mm)	Diameter (μm)	Vessel	Length (mm)	Diameter (μm)
$P_1=P_2$	9.3	630	$P_1=P_2$	5	800
$B_1=B_2$	11.8	500	$B_1=B_2$	6.3	630
			$B_{1.1}=B_{1.2}=B_{2.1}=B_{2.2}$	8	500
					

The 3D printing parameters of both models were optimized by adjusting the settings on the Ultimaker Cura software, and the structures were printed using polyvinyl alcohol (PVA) filament (Table 2 and 3). 3D printing optimization studies were mainly conducted with the basic sacrificial model. Initially, the 3D printing parameters were adjusted by altering the basic settings such as layer height, layer width, and print speed, while keeping the remaining parameters at their default values (Table 2-a). Given that the smallest part was 500 μm , these basic parameters were insufficient to achieve high print quality. Therefore, additional parameters such as print acceleration, print jerk, fan speed, and minimum layer time were also adjusted and optimized.

Table 2. Optimization of 3D printing parameters for the basic model

Basic Mold	a	b	c	d	e	f (optimized)
Layer Height (mm)	0.2-0.4	0.2	0.2	0.2	0.1	0.1
Layer Width (mm)	0.2-0.4	0.4	0.35	0.35	0.25	0.25
Print Speed (mm/s)	10	10	5	5	5	3
Print Acceleration (mm/s²)	2500	2000	500	500	500	500
Print Jerk (mm/s)	20	20	10	5	5	5
Fan Speed (%)	80	80	100	85	50	30
Minimum Layer Time (s)	20	20	50	50	50	50

Table 3. Optimization of 3D printing parameters for the complex model

Complex Mold	g	h	i	j (optimized)
Layer Height (mm)	0.2	0.1	0.1	0.1
Layer Width (mm)	0.35	0.25	0.25	0.25
Print Speed (mm/s)	5	5	5	3
Travel Speed (mm/s)	500	500	500	150
Print Acceleration (mm/s²)	500	150		
Print Jerk (mm/s)	10	5	5	5
Fan Speed (%)	100	85	50	30
Minimum Layer Time (s)	50	20	50	50

3.2.1.2 Fabrication of 3D branched tubular electrospun fibrous membrane

It was planned to obtain 3D branched tubular fibrous membrane via electrospinning using poly(ϵ -caprolactone) (PCL), and a blend of PCL and collagen (PCL-Col) solutions. The optimization of electrospinning process was carried out by adjusting parameters using a metal plate collector before depositing fibers onto the sacrificial construct. Optimization studies were conducted using polymers with varying molecular weights and concentrations, while also altering other parameters such as applied voltage, the distance between the needle tip and the collector, and the flow rate (Table 4). The optimized conditions for electrospinning to obtain bead-free, uniform PCL fibers were determined to be 30 % PCL (50 000 g/mol) concentration, 0.2 mL/h flow rate, 10 kV voltage, and 24 cm distance. For the blend of PCL and collagen, the same electrospinning parameters were used, with the polymer concentration adjusted to 22.5%.

Table 4. Optimization of parameters for electrospinning

Composition of Polymer Solution (w/v)	Distance (cm)	Voltage (kV)	Flow Rate (mL/h)
32 % PCL (50 000 g/mol)	25-30	12-20	0.2-0.4
30 % PCL (50 000 g/mol)	17-25	10-20	0.2-0.8
30 % PCL (124 000 g/mol)	24	14	0.4
22.5 % PCL-Col (50 000 g/mol) (4:1; w:w)	24	14	0.4
22.5 % PCL-Col (50 000 g/mol) (1:1;w:w)	24	14	0.4
25 % PCL-Col (50 000 g/mol) (4:1; w:w)	24	14	0.4

Electrospinning under optimized conditions was carried out using a 3D-printed sacrificial branching tubular mold as the collector. Nevertheless, due to the poor electrical conductivity of PVA compared to the commonly used metal collectors in electrospinning, only a few fibers were collected on top of the mold. It was demonstrated that coating the sacrificial mold with an electrolyte-containing polymer solution enhanced fiber collection efficiency on the mold surface. Therefore, this approach was tested in the present study to further improve fiber deposition and obtain a uniform nanofibrous mesh (65). Therefore, this approach was tested in the present study to further improve fiber deposition and obtain a uniform nanofibrous mesh. Different concentrations and ratios of NaCl and KBr as electrolytes, along with varying concentrations of poly (ethylene glycol) (PEG), were tested to promote the efficiency of fiber deposition (Table 5). During the coating optimization, it was observed that fibers at the connection points of the branching tubular mold were not properly collected, leading to leaky points in the perfusable tubular network. To address this issue, the viscosity of the polymer solution was increased by using PCL with a high molecular weight (124,000 g/mol), and the electrospinning parameters were optimized at a flow rate of 0.2 mL/h, an applied voltage of 10 kV, and a tip-to-collector distance of 15 cm. Following the electrospinning process over sacrificial molds, constructs for basic and complex models were incubated in ddH₂O on a shaker overnight to facilitate removal of the sacrificial molds from the fibrous membranes. Afterwards, the samples were freeze-dried and examined with SEM. In addition, flat electrospun membranes on a metal plate collector were fabricated under the same

conditions for a couple of characterization studies, including degradation and mechanical testing.

Table 5. Optimization of coating solution for sacrificial branched tubular molds

Salt Solution (in dH ₂ O)	PEG (%) (in EtOH)	Ratio (Salt:PEG)
1M NaCl	20	9:1
		8:2
		5:5
2M NaCl	20	9:1
		5:5
6M NaCl	20	9:1
	10	
5M NaCl	20	9:1
	10	
4M NaCl	20	9:1
5 M KBr	20	9:1
4 M KBr	20	9:1

3.2.1.3 Characterization of 3D branched, tubular, electrospun fibrous membrane

3.2.1.3.1 Morphological analysis of fibrous membranes

The surface morphology of fibrous membrane was investigated using scanning electron microscopy (SEM). Samples were coated with gold for 2 min at a current of 120 mA, then imaged under high vacuum at 5 kV using a scanning electron microscope (ZEISS Evo10, Germany) at Acıbadem Mehmet Ali Aydınlar University Biomaterials Center.

3.2.1.3.2 Mechanical analysis of fibrous membranes

The mechanical properties of PCL and PCL-Col (8:2) based fibrous membranes were evaluated with mechanical test (Shimadzu, AGS-X Series Universal Test Machine)). Fibrous membranes of PCL and PCL-Col (8:2) were fabricated on a metal

plate and cut into samples measuring 1 cm x 4 cm. The membranes were attached to the grips of the mechanical tester, and tensile stress tests were performed at a test speed of 1 mm/min using 20 N load cell. Stress-strain curves were plotted for each sample, and Young's modulus was calculated from the slope of stress-strain curve.

3.2.1.3.3 Degradation profile of fibrous membranes

The degradation profiles of fibrous membranes were analyzed using a gravimetric method. Fibrous membranes were cut into 3 x 3 cm squares. Samples were weighed on day 0 and then incubated in phosphate-buffered saline (PBS) containing 0.1% sodium azide at 37°C on a shaker to simulate physiological conditions. Every 6 days for a period of 4 weeks, samples were removed from the incubation solution, rinsed with deionized water (dH₂O) for 30 min to remove residual salts, freeze-dried overnight, and then reweighed. The degradation profile was calculated as the percentage of remaining weight according to the following equation (III), and the weight change over time graphs were plotted.

$$\text{Weight Remained (\%)} = 100 - ((W_i - W_f) / W_i) * 100 \dots \dots \dots \text{(III)}$$

where W_i is the initial weight, W_f is the dry weight after incubation.

the dry weight after incubation. Change in weight by time graphics were plotted.

3.2.1.3.4 Analysis of surface properties of the polymeric membranes

Surface properties of the PCL and PCL/Collagen blends were studied using a goniometer (Krüss Scientific DSA25, Germany). Film forms of PCL (30%, w/v) and PCL-Col (25%, w/v, 8:2, w/w) were prepared on glass slides by pouring the polymer solutions, followed by air-drying. Droplets of dH₂O were placed at five different points on the polymeric films, and contact angles were measured using ADVANCE software. The contact angle (θ) was calculated according to Young's equation (IV)

$$\sigma_{sg} = \sigma_{sl} + \sigma_{lg} \times \cos \theta \dots \dots \dots \text{IV}$$

where the σ_{lg} is the surface tension between liquid and gas, σ_{sl} is the interfacial tension between the solid and the liquid, σ_{sg} is the solid–gas surface energy.

3.2.1.3.5 Fluid flow modelling and lumen flow tracking in tubular nanofibrous membranes

Computational Fluid Dynamic (CFD) analysis was employed to simulate fluid behavior within the basic model (BM) and the complex model (CM). First, the 3D model file was exported as .step file and opened in Autodesk CFD Program. The material type was selected as fluid-water. The boundary conditions were defined as volume flow rate (cm^3/s) at the inlet and pressure (atm) at the outlet. CFD results for both models were presented as static pressure, which represents the pressure exerted by the fluid on the walls and provides valuable information about the fluid's behavior, pressure distribution, and flow characteristics.

Beside the CFD analysis, flow behavior within the BM and CM fabricated as described in Section 3.2.1.2 was monitored in real time using blue dye injection. Blue dye was injected into the inlets of the BM and CM tubular nanofibrous networks to assess suitability of channels for perfusion. The process was video recorded and presented as a time-lapse image sequence.

3.2.2 Preparation and characterization of the hydrogel

3.2.2.1 Optimization of the hydrogel composition

The final goal of this study is to obtain hyaluronic acid and collagen-based IPN hydrogel to mimic the native brain ECM environment with this hydrogel composition. Two different types of hyaluronic acid-based hydrogel sources were employed in the optimization procedure. The first was a thiol-modified hyaluronic acid hydrogel kit (Table 6), and the second was methacrylated hyaluronic acid (MeHA) (Table 7). Different types of proteins, including collagen and fibronectin, were incorporated into the hyaluronic acid-based hydrogel solution as cell attachment sites. *In vitro* evaluation of various hydrogel compositions was conducted using the Live/Dead assay on pericyte-like cells, based on cell morphology within the hydrogel. Subsequently, the optimized hydrogel composition was further characterized.

For the thiol-modified hyaluronic acid hydrogel, 0.5% (w/v) polyethylene glycol diacrylate (PEGDA) was mixed with 1% (w/v) thiol-modified hyaluronic acid at a 1:4

volume ratio, as suggested by the manufacturer. Additionally, ratios of 1:8 and 1:2 were also tested to determine the optimal condition. The collagen hydrogel solution was prepared by mixing 10X DMEM, 2.8 M NaOH, 7.5% NaHCO₃, 1X DMEM, 5 mg/mL atelocollagen, and the cell suspension, following the specified pipetting order. Three different hydrogel solutions were prepared: (i) hyaluronic acid-only (HA), (ii) collagen-only (Col), and (iii) collagen-hyaluronic acid mixture (HA-Col). The solutions were incubated at 37°C for gelation. Stereomicroscopy images were taken and examined to evaluate the gelation process. Cell viability and behavior within the 0.5% HA- 3% Col hydrogel was asseesed using the Live/Dead assay, following the detailed protocol described in Section 3.2.3.5.

Table 6. Hydrogel compositions using HyStem® thiol-modified hyaluronic acid hydrogel kit

Hyaluronic acid-only (HA) Solution	Collagen-only (Col) Solution	HA-Col Mixture (v:v)
0.5 % (w/v) polyethylene glycol (PEGDA)	10X DMEM	1:2
1 % (w/v) thiol-modified hyaluronic acid	2.8 M NaOH	1:1
(Ratios of PEGDA-HA)	7.5 % NaHCO ₃	2:1
(1:2)	1X DMEM	
(1:4)	5 mg/mL atelocollagen	
(1:8)	Cell suspension	
	(pericytes-like cells, neurons, astrocytes)	

To obtain hyaluronic acid-based hydrogel from methacrylated hyaluronic acid (MeHA) to mimic the brain ECM microenvironment, MeHA was synthesized through a chemical reaction between hyaluronic acid and methacrylic anhydride (66). Briefly, 1 g of HA was dissolved in 100 mL of dH₂O overnight at RT. DMF was then added to the 1% HA solution to achieve a dH₂O:DMF ratio of 2:3. The HA solution was incubated at +4°C for 1 h. Methacrylic anhydride (5 mL) was slowly added to the HA solution, and the pH was maintained between 8 and 9 for 6h at +4°C, then allowed to react at 4°C for 24 h. The solution was dialyzed against dH₂O for 3 days, with the dH₂O changed every 12 h for purification. The MeHA solution was filtered at 4°C, and freeze-dired.

To achieve the characteristic spindle-shaped morphology of pericyte-like cells within the hydrogel, different MeHA concentrations, protein types and concentrations, MeHA:protein ratios, and crosslinking conditions were tested (Table 7). Hydrogels were prepared by dissolving MeHA at different concentrations in phenol red-free DMEM containing 0.5% Irgacure and penicillin/streptomycin. The MeHA solutions were then mixed with either collagen-based hydrogel solution (Table 6) or fibronectin solutions at specific ratios, as summarized in Table 7. Fibronectin–MeHA hydrogels were crosslinked under UV light, whereas MeHA–collagen-based hydrogels underwent dual crosslinking — first under UV light for MeHA and subsequently at 37 °C to crosslink collagen. Cell morphology and viability within these hydrogels were evaluated using the Live/Dead assay, as described in Section 3.2.3.5. The optimum hydrogel composition was determined as 3% MeHA with 6% collagen (1:3, v:v), crosslinked under UV light at 0.160 J for 2 min, followed by incubation at 37 °C for 1 h.

Table 7. Hydrogel compositions using methacrylated hyaluronic acid

MeHA Concentration (% w/v)	Protein Type/ Concentration (% w/v)	MeHA: Protein Ratio (v:v)	Crosslinking UV Dose and Duration (J, min)
1%	Collagen 3%	1:1 2:3 1:2	1.6 J, 1.5 min
1%	Fibronectin 2.5% 5% 10% 15%	1:1	1.6 J, 1.5 min
1.5%	Collagen 3%	1:2 1:1 2:1	0.160 J, 2 min
2.25 %	Collagen 3%	1:2 1:1	0.160 J, 2 min
3%	Collagen 3%	1:2	0.160 J, 2 min
2.25 %	Collagen 6%	1:2	0.160 J, 2 min
3%	Collagen 6%	1:3	0.160 J, 2 min

3.2.2.2 Preparation and characterization of the hydrogels

3.2.2.2.1 Preparation of the hydrogels

Solutions of MeHA (3%, w/v) and collagen (Col) (6%, w/v) were prepared separately. The solutions were mixed at a ratio of 1:3 to achieve final composition of 0.75% MeHA and 4.5% Col (w/v) (Table 8). The MeHA-Col hydrogel solution was sequentially crosslinked to achieve complete gelation and structural stability of the hybrid hydrogel. First, it was subjected to UV light exposure (0.160 J, 2min) to photocrosslink MeHA, then incubated at 37°C to induce thermal crosslinking.

Table 8. Compositions of MeHA and collagen solutions for hydrogel preparation

MeHA Solution	Collagen Solution
3% MeHA dissolved in 0.5 % Irgacure containing phenol red free DMEM containing pen/strep	10X DMEM 2.8 M NaOH 7.5 % NaHCO ₃ 1X DMEM 5 mg/mL atelocollagen Cell suspension (pericyte-like cells, Neuron, Astrocytes)

3.2.2.2.2 Characterization of the hydrogel compositions

3.2.2.2.2.1 Morphological analysis

The morphology of the MeHA-Col hydrogels was analyzed using SEM. Hydrogels were prepared as described in Section 3.2.2.2.1. Following fabrication, the samples were freeze-dried, and sputter-coated with gold for 2 min at a current of 120 mA. The samples were then examined at 5 kV using a scanning electron microscope (ZEISS Evo10, Germany).

3.2.2.2.2.2 Chemical characterization of hydrogel

Three experimental groups were designed: (i) 0.75% MeHA-based hydrogel, (ii) 4.5% Col-based hydrogel, and (iii) interpenetrating polymer network (IPN) hydrogel composed of 0.75% MeHA and 4.5% Col. Freeze-dried hydrogel samples were analyzed using Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer, Spectrum Two, USA), and their FTIR spectra were recorded in the 4000–400 cm^{-1} range. The analysis was performed to identify the characteristic absorption bands of both hyaluronic acid and collagen in the hybrid hydrogel.

3.2.2.2.2.3 Degradation profile of hydrogels

The degradation profile of the MeHA (3%, w/v) and collagen (Col) (6%, w/v) hydrogels was analyzed using a gravimetric method, as described in Section 3.2.1.3.3. Hydrogels were prepared by dispensing 400 μL of hydrogel solution into each well of a 48-well plate. The initial weight of each sample was recorded on day 0. Samples were then incubated in PBS containing 0.1% sodium azide at 37°C under constant agitation on a shaker to simulate physiological conditions. At 6-day intervals over a 4-week period, samples were removed from the incubation solution, rinsed with dH_2O for 30 min to eliminate residual salts, freeze-dried overnight, and subsequently reweighed.

The degradation profile was determined as the percentage of remaining weight, calculated using the following equation (V), and the weight change over time was plotted graphically.

$$\text{Weight Remained \%} = 100 - ((W_i - W_f) / W_i) \times 100 \dots \dots \dots \text{(V)}$$

where W_i is the initial weight and W_f is the final dry weight after incubation.

3.2.2.2.2.4 Determination of mechanical properties of the hydrogels

The mechanical properties of the MeHA (3%, w/v) collagen (Col) (6%, w/v) (1:3) hydrogels were evaluated using a compression test performed with a universal testing machine (Shimadzu, Shimadzu AGS-X Universal Test Machine, Japan). Hydrogels were prepared by dispensing 400 μ L of hydrogel solution into each well of a 48-well plate, forming samples with a height of 5 mm. Compression test was conducted at a speed of 1 mm/min using a 20 N load cell. Stress-strain curves were obtained for each sample, and Young's modulus was calculated from the initial linear region of the stress-strain curve, representing the elastic behavior of the hydrogel.

3.2.2.2.2.5 Swelling degree of the hydrogels

Three experimental groups were designed: (i) 0.75% MeHA-based hydrogel, (ii) 4.5% Col-based hydrogel, and (iii) IPN hydrogel consisting of 0.75% MeHA and 4.5% Col. The samples were incubated in PBS at 37°C overnight to achieve equilibrium swelling degree. Samples were weighed in both the hydrated (wet) and dry states. The swelling degree was calculated using the following equation:

$$\text{Degree of Swelling \%} = (W_h - W_d) / W_d \times 100 \dots \dots \dots \text{(VI)}$$

where W_h is the wet weight and W_d is the dry weight.

3.2.3 *In Vitro* Studies: cell culture and their incorporation into the fibrous membrane and hydrogel

This study utilized endothelial cells to model the blood side and astrocytes, pericyte-like cells, and neurons to represent the brain side in *in vitro* studies. All experiments were conducted in accordance with the principles of the Declaration of Helsinki and local ethical regulations for human-derived cells, with the approval of the Acibadem University and Acibadem Healthcare Institutions Medical Research Ethics

Committee (Approval No: 2023-21/771), and in compliance with institutional ethical guidelines for the use of established cell lines.

3.2.3.1 Culture of endothelial cells

Previously isolated human umbilical cord vein endothelial cells (HUVECs) (P2-P5) were used as EC source in this study (67). Cryopreserved HUVECs were thawed and cultured on T75 flasks coated with 50 µg/mL fibronectin. Cells were cultured in Endothelial Growth Medium-2 (EGM-2; Promocell, C22111) containing 5 ng/mL Epidermal Growth Factor (EGF), 10 ng/mL basic Fibroblast Growth Factor (bFGF), 20 ng/mL Insulin-like Growth Factor (IGF), 0.5 ng/mL Vascular Endothelial Growth Factor (VEGF), 1 µg/mL ascorbic acid, 22.5 µg/mL heparin, 0.2 µg/mL hydrocortisone, 2% fetal calf serum (FCS) and 100 units/mL-100 mg/mL penicillin/streptomycin. The medium was changed every two days. When the cells reached approximately 80–90% confluency, they were detached via trypsinization by incubating them in 0.05% trypsin-EDTA solution for 5 min, followed by centrifugation at 1500 rpm for 5 min. The pellet was resuspended in EGM-2 growth medium and the cells were sub-cultured. For characterization studies, ECs were stained for CD31 following the protocol described in Section 3.2.3.6.2.

3.2.3.2 Culture of pericytes-like cells

Previously isolated Wharton's Jelly Mesenchymal Stem Cell (WJ MSC) were used instead of pericytes (68). The substitution of WJ-MSCs for pericytes was based on their suitability, strong similarity in the expression of specific cell surface markers (CD146⁺/CD34⁺/CD45⁺), shared multipotent characteristics, and comparable contribution to barrier function (69). Pericyte-like cells (P3) were thawed, resuspended in MSC growth medium, and transferred into a T75 cell culture flask. The cells were cultured in MSC growth medium composed of DMEM/F12 medium supplemented with 10% FBS, 100 units/mL-100 µg/mL penicillin/streptomycin. Upon reaching 80–90% confluency, the cells were detached via trypsinization and sub-cultured. For characterization studies, the cells were seeded onto fibronectin-coated glass slides at a density of 5,000 cells/ slide, and cultured for three days before fixation with 4% paraformaldehyde (PFA) for 30 min at room temperature. Cells were characterized

using Phalloidin-DAPI staining (Section 3.2.3.6.1) and immunocytochemistry (Section 3.2.3.6.2).

3.2.3.3 Culture of astrocytes

SVG p12 (P2-P4) (ATCC- CRL-8621) were used as the astrocyte source in this study. Astrocytes were kindly provided as a gift by the Middle Technical University Center of Excellence in Biomaterials and Tissue Engineering (METU- BIOMATEN). The astrocytes (SVG p12, P2-P4) were thawed and cultured in DMEM high glucose medium containing 10 % FBS and 100 units/mL-100 µg/mL penicillin/streptomycin. After 80-90% confluency, the cells were detached via trypsinization and sub-cultured. For characterization, the cells were seeded onto fibronectin coated glass slide at a density of 10 000 cells/ slide and cultured for 3 days. The cells were then fixed with 4% PFA for 30 min at room temperature. Cells were characterized using Phalloidin-DAPI staining (Section 3.2.3.6.1) and immunocytochemistry (Section 3.2.3.6.2).

3.2.3.4 Culture of neurons

SH-SY5Y (P20-P23) (ATCC- CRL-2266) were used as neuron cell source in this study. Neurons were kindly provided as a gift by the Middle Technical University Center of Excellence in Biomaterials and Tissue Engineering (METU- BIOMATEN). Cells were thawed and cultured in DMEM: F12 medium containing 10 % FBS and 100 units/mL-100 µg/mL penicillin/streptomycin. After 80-90% confluency, cells were seeded onto fibronectin coated glass slide at a density of 10,000 cells/slide and cultured for 3 days. The cells were then fixed and characterized using Phalloidin-DAPI staining (Section 3.2.3.6.1) and immunocytochemistry (Section 3.2.3.6.2).

3.2.3.5 Live/Dead assay

The Live/Dead assay, a common method to assess cell viability and cytotoxicity, was used to evaluate the behavior of cells on fibrous membrane or in hydrogels. Cells were incubated in a solution containing the fluorescent dyes, calcein-AM and ethidium homodimer-1 (EthD-1) for 30 min at room temperature. Calcein-AM is a cell-permeant dye that is converted by intracellular esterases into a green-fluorescent

calcein derivative in live cells, while EthD-1 is a cell membrane-impermeant dye that stains the nuclei of cells with compromised membrane integrity, such as dead cells. Following staining, samples were examined using Laser Scanning Confocal Microscopy (LSCM).

3.2.3.6 Characterization of Cells

For morphological characterization of cells, Phalloidin staining was used to visualize actin filament organization, while immunocytochemistry was performed to assess the expression of cell type-specific markers.

3.2.3.6.1 Phalloidin and DAPI Staining

Cells were first permeabilized with 0.1% Triton X-100 for 5 min. After washing with PBS, they were incubated in the blocking solution of 1% BSA at 37°C for 30 min, followed by incubation with Phalloidin (1:100, diluted in PBS) at 37°C for 1 h. Finally, the cells were counterstained with DAPI at RT for 10 min to assess the organization of the actin cytoskeleton and to visualize the cell nuclei.

3.2.3.6.2 Immunocytochemistry

For the immunocytochemistry studies, cells were first incubated in 100 mM glycine at RT for 15 min, followed by three washes with PBS. Permeabilization was performed using 0.1% Triton X-100 for 10 min, then the cells were washed again with PBS. After blocking with 1% BSA at 37°C for 30 min, cells were incubated overnight at +4°C with the appropriate primary antibodies (Table 9). The following day, cells were treated with the secondary antibody solution at 37°C for 1 h and counterstained with DAPI at RT for 10 min. The samples were examined and imaged using LSCM.

Table 9. Antibody list used in this study

Primary Antibodies	Cell Type	Catalog Number	Dilution
Anti-Human CD31	ECs	Dako- M082301-2	1:40
Anti-Human α -SMA	Pericyte-like cells	Syc Tech- A00002-0007	Ready to use
Anti-Human GFAP	Astrocytes	Thermo Fisher, PA1-10004	1:1000
Anti-Human AQP-4	Astrocytes	Protein Tech 16473-1-AP	1:250
Anti-Human MAP-2	Neurons	Thermo Fisher, 131500	1:200
Anti-Human β -Tubulin	Neurons	Abcam, Ab18207	1:200
Secondary Antibodies		Catalog Number	Dilution
Anti-Mouse Alexa Fluor 555		Thermo Fisher A21424	1:200
Anti-Rabbit Alexa Fluor 488		Thermo Fisher AP132JA4	1:100
Anti-Chicken Alexa Fluor 488		Thermo Fisher A-11039	1:500

3.2.3.7 Assessment of cell viability on fibrous membrane and hydrogels

3.2.3.7.1 Viability of cells on the fibrous membranes for the blood side

To evaluate monolayer formation of HUVECs on the fibrous membranes, the membranes were treated with three different protein coating compositions: fibronectin, gelatin, and a fibronectin:gelatin mixture (1:1, v/v). First, PCL nanofibrous membranes were cut into 1.5 cm $\times$ 1.5 cm pieces, sterilized under UV light for 30 min on each side, and placed into inserts. The membranes were then coated with 50 μ g/mL fibronectin, 1% gelatin, or a mixture of 50 μ g/mL fibronectin and 1% gelatin (1:1, v/v) for 2 h at 37°C in a CO₂ incubator. After coating, the membranes were dried in a biosafety cabinet. ECs, cultured in EGM-2 medium, were detached via trypsinization, and counted using a hemocytometer. Cells were seeded onto the protein coated membranes at a density of 7.5×10^4 cells/membrane. Cell viability was assessed on day 3 of culture using the Live/Dead assay. Cell coverage on the membrane was quantified using ImageJ and expressed as a percentage.

3.2.3.7.2 Viability of cells within hydrogels for the brain side

For the *in vitro* assessment of the brain side, pericyte-like cells, astrocytes, and neurons were separately incorporated into the MeHA-Col-based hydrogel solution, according to the protocol given in Section 3.2.2.2.1. The cells loaded in the hydrogels were incubated in their respective growth media for 7 days. Cell viability and morphology within the hydrogels were assessed using the Live/Dead assay on the 3rd and 7th days of culture, following the procedure described in Section 3.2.3.5. Viability analysis was performed on 3D z-stack images, where live (green) and dead (red) cells were counted in three images using ImageJ (version X.X.X) and expressed as a percentage (%).

3.2.4 Establishment of NVU model

3.2.4.1 Optimization of NVU model assembly

A custom-designed setup, composed of a main body part that holds fibrous membrane, and a ring to secure the membrane, was fabricated with 3D printing using polylactic acid (PLA). It was designed to enable hydrogel integration and facilitate TEER measurements. Both sides of the fibrous membranes ($1.5 \times 2 \text{ cm}^2$) were sterilized under UV light for 30 min. The sterilized membranes were inserted into the 3D-printed setup and coated with 50 $\mu\text{g/mL}$ fibronectin at 37°C in a CO₂ incubator for 2 h (Figure 6b). After the fibronectin solution was removed and the membranes were allowed to dry under sterile conditions, the hydrogel layer was subsequently applied to the opposite side of the membrane

The model assembly was optimized by incorporating a cell-free hydrogel on the basolateral side of the membrane at varying thicknesses (Figure 6b). Hydrogel thickness was controlled by adjusting the hydrogel volume to 100 μL , 200 μL , and 400 μL , resulting in final thicknesses of 2.5 mm, 5 mm, and 10 mm, respectively. Endothelial cells (5×10^4 cell/membrane) were seeded on the apical side of the membrane, and the effect of hydrogel thickness on monolayer endothelium formation was evaluated using immunocytochemistry, following the procedure described in Section 3.2.3.6.2.

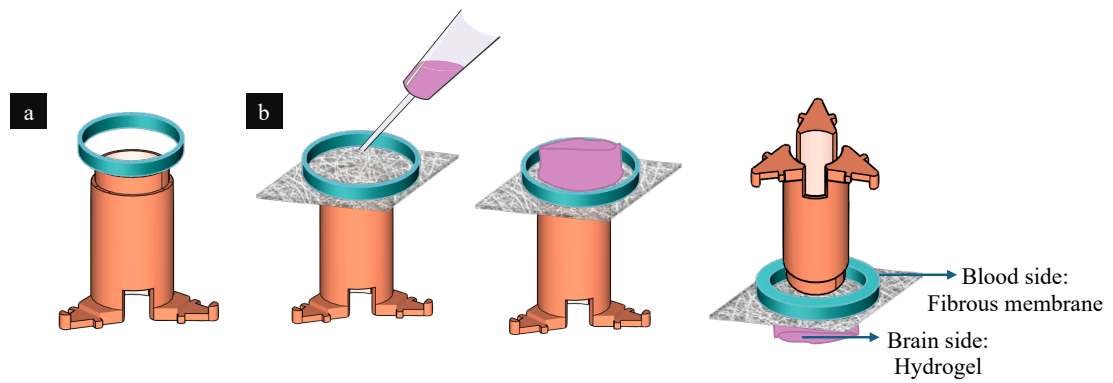


Figure 6. 3D Printed system a) Schematic representation of the custom-designed 3D printed system, including the main body part and the securing ring. (b) Stepwise assembly of the NVU Model. First, the membrane was placed onto the main body part and secured with a ring. Next, the hydrogel solution was added over the fibrous membrane on the ring side of the system, and gelation was performed. Then, the setup was inverted, and ECs were seeded on the opposite side of the membrane.

3.2.4.2 Construction of multicellular NVU model

For the construction of the multicellular NVU model, ECs were seeded on the apical side of the membrane, representing the blood side. Astrocytes, neurons and pericyte-like cells were integrated over the opposite side of the fibrous membrane within the hydrogel solution to mimic the brain side. Initially, two separate hydrogel solutions were prepared, one for astrocytes and pericyte-like cells, and another for neurons. These solutions were obtained by mixing Col-MeHA hydrogel formulations at a 1:3 (w/v) ratio (Table 8).

Astrocytes and pericyte-like cells were resuspended in the hydrogel solution at concentrations of 5×10^6 cells/mL and 1×10^6 cells/mL, respectively, maintaining a 5:1 ratio (Figure 7a). Neurons were separately incorporated into the hydrogel solution at a concentration of 2.5×10^4 cells/hydrogel (Figure 7b). The sequential integration of the hydrogel solutions was designed to mimic cellular microenvironment of the brain side, where astrocytes and pericytes are positioned near the basal membrane, and neurons interact with astrocytes. Endothelial cells (5×10^4 cell/membrane) were then seeded on the apical side of the membrane, and the cells were co-cultured in the endothelial growth medium for 21 days within the NVU model (Figure 7c).

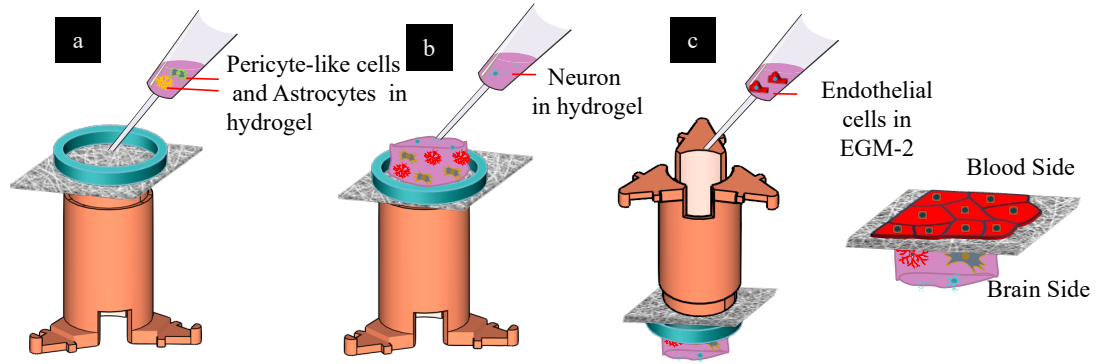


Figure 7. Schematic representation of the multicellular NVU model assembly. Sequential integration of (a) astrocyte- and pericyte-like cell- loaded hydrogel, and (b) neuron- containing hydrogel on the basolateral side of the model, and finally, (c) seeding of ECs on the apical side of the fibrous membrane.

3.2.4.3 Investigation of the barrier function in the tissue-engineered NVU model

3.2.4.3.1 Investigation of cell behavior in NVU model

The cells in the model were fixed in 4% PFA for 30 min at RT. The cells were immunostained against cell-specific antigens such as α -SMA for pericyte-like cells, aquaporin-4 (AQP-4) for astrocytes, and microtubule-associated protein 2 (MAP-2) and β -Tubulin III for neurons. Briefly, the cells in the NVU model were incubated in 100 mM glycine for 15 min at RT and rinsed with PBS. Then, the cells were permeabilized with 0.1% Triton-X-100 solution for 10 min at RT. After permeabilization, the samples were treated with blocking solution of 1% BSA for 30 min at 37°C. Models were sequentially stained with primary antibodies, anti-CD31 (Dako- M082301-2, 1:40) for endothelial cells, anti- α -SMA (Syc Tech- A00002-0007) for pericyte-like cells, anti-AQP-4 (Abcam, Ab18207, 1:250) for astrocytes, and anti- β -Tubulin III and MAP-2 (1:200) for neurons, at +4°C for overnight. Then, the samples were incubated with appropriate secondary antibodies, anti-mouse Alexa Fluor 555 (Thermo Fisher A21424, 1:200), anti-rabbit Alexa Fluor 488 (Thermo Fisher AP132JA4O, 1:100), and anti-chicken Alexa Fluor 488 (Thermo Fisher A-11039, 1:500), at 37°C for 1 h. The cells were counterstained with DAPI (1:5000 in PBS) for 10 min at RT and examined under LSCM (Zeiss LSM 900). The monoculture model was used for comparison. For quantification, both monoculture and multicellular models (n=3) were imaged using

the same configurations of LSCM. The total fluorescence intensity was divided by the number of cells to calculate the intensity per cell.

3.2.4.3.2 Trans-endothelial electrical resistance measurement

The barrier integrity of the model was evaluated using Trans-Endothelial Electrical Resistance (TEER) measurement with an EVOM-3 Epithelial Volttohmmeter (World Precision Instruments, USA). A cell-free model was used as the blank. TEER measurements were performed in PBS, in triplicate for each sample, over a period of 14 days (n=3) for both the multicellular and monoculture models. The resistance values obtained from the cell-free blank model were subtracted from the resistance of the test models (n=3) and then normalized to the cell culture area, corresponding to the area of the fibrous membrane (0.45 cm.). Results were expressed in $\Omega\cdot\text{cm}^{-2}$.

3.2.4.3.3 Permeability assesement

The permeabilities of the multicellular and monoculture models were assessed using two solutes, fluorescein disodium salt (0.376 kDa; 50 $\mu\text{g/mL}$) and fluorescein isothiocyanate (FITC)-labeled dextran (20 kDa; 1 mg/mL), on days 14 and 21 of culture (n=4) (35). The models were transferred into new 24-well plates, and 950 μL of PBS was added to the basolateral side. Then, 50 μL of each solute, fluorescein and FITC-labelled dextran, was added to the apical side. At time points 0, 30, 60, 90 and 120 min after solute addition, 10 μL samples were collected from the basolateral side and diluted to 200 μL with PBS. Florescent intensities of the samples were measured using a multimode plate reader (Perkin Elmer Victor Nivo, USA).

Additionally, further permeability studies of the multicellular model were performed using drugs such as ceftriaxone (554.6 Da) and amikacin (585.6 Da) on day 14 of culture. For both drugs, the models were transferred to new wells containing 950 μL of fresh endothelial growth medium prior to drug administration. For ceftriaxone, 50 μL of a 5 mg/mL ceftriaxone solution (prepared in endothelial growth medium) was added to the apical side. After 48 h, 200 μL of the basolateral medium was collected, and absorbance was measured at 300 nm. Since amikacin lacks a

characteristic absorbance peak, its concentration was determined using a ninhydrin-based assay. Specifically, 50 μL of the amikacin solution (50 mg/mL in endothelial growth medium) was added to the apical side. After 48 h, 50 μL of the basolateral sample was collected, mixed with 50 μL of PBS, and incubated with 100 μL of 0.5% ninhydrin solution at 37°C for 15 min. Absorbance was then measured at 570 nm.

Absorbance values were converted into mass with calibration curves for each solute, and the change in mass over time in the basolateral chamber was plotted. The apparent permeability coefficient (P_{app}) was calculated with following equation (VII).:

$$P_{app} = \frac{dQ}{dt} \frac{1}{AC_0} \dots\dots\dots (VV)$$

where P (cm/s) is the apparent permeability coefficient, dQ/dt (mg/s) is the rate of solute mass transfer over time, A (cm^2) is the surface area of the membrane, and C_0 is the initial concentration of solute on the apical side.

The average and standard deviation were calculated for each group, and a cell-free model was used as a control.

3.2.5 Construction of NVU-on-chip system

3.2.5.1 Design and fabrication of NVU- on-a-chip system

The NVU-on-a-chip model was designed to contain a branched, fibrous tubular membrane mimicking a basic vascular network, positioned between PDMS-based microfluidic chambers (Figure 8a). The system consisted of a lower PDMS layer that was first bonded to a glass slide (Figure 8b-d). This bottom layer featured a narrow channel matching the dimensions of the tubular branches, providing adequate space between the glass surface and the fibrous membrane. This configuration enabled the integration of cell-loaded hydrogels and prevented direct contact between the membrane and the glass.

The upper section of the model consisted of a PDMS layer composed of two identical parts, precisely aligned to form a central cavity for the placement of the branched fibrous membrane. These layers were bonded using a thin layer of uncured

PDMS to ensure sealing. The design also allowed co-culture with astrocyte- and pericyte-like cells in direct proximity to the fibrous membrane structure.

Each PDMS layer was fabricated using a 3D-printed mold. PDMS prepolymer (Sylgard 184, Dow Corning) was mixed with its curing agent at a 10:1 (w/w) ratio and degassed under vacuum to remove air bubbles. The mixture was then poured into the molds and cured at 100 °C for 1 hour. After curing, the PDMS layers were successfully demolded. The thicknesses of the bottom and upper PDMS layers were measured as 430 μ m and 2.5 mm, respectively.

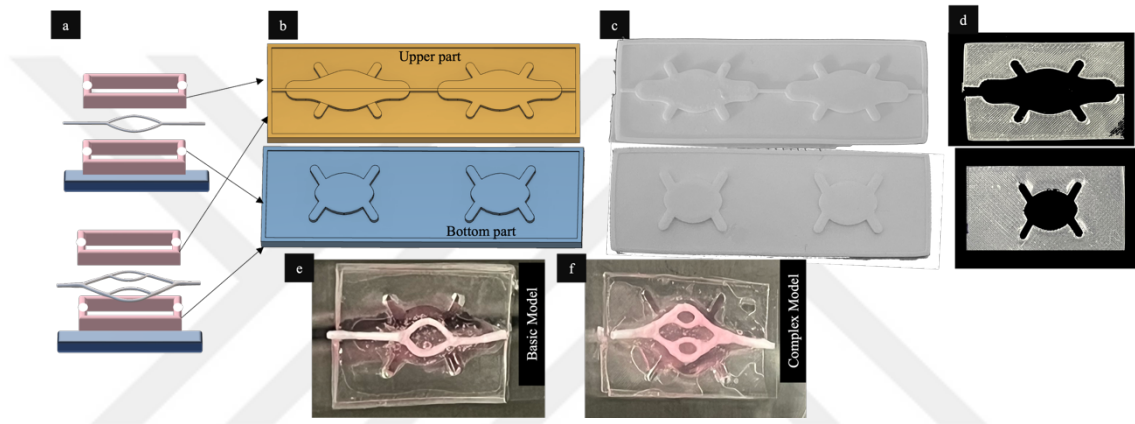


Figure 8. Layered chip structure (a) Schematic representation of the layered chip structure incorporating fibrous tubular membrane between PDMS microfluidic chambers. (b) Computer-aided design (CAD) of the upper and bottom parts. (c) 3D-printed molds used for casting PDMS layers and (d) resulting PDMS replicas representing the upper and bottom layers. Assembled (e) basic and (f) complex- NVU-on-a-chip models.

3.2.5.2 Cell culture in the NVU- on-a-chip System

To construct the multicellular NVU-on-a-chip model, ECs were first seeded onto the branched, fibrous tubular membrane. Subsequently, neuron-loaded and astrocyte/pericyte-like cell loaded MeHA-Col hydrogels were sequentially incorporated around the tubular membrane to mimic the layered organization of the neurovascular unit.

To enable EC seeding, the PVA-based sacrificial template used as the collector during electrospinning was removed by immersing the branched tubular fibrous membranes in dH₂O overnight. Following template removal, the membranes were

sterilized by incubation in 70% EtOH for 2 h at +4 °C. After sterilization, the membranes were coated with a 3% collagen solution (w/v, prepared in 0.03% acetic acid) and subsequently lyophilized to stabilize and preserve the internal lumen structure prior to cell seeding. Endothelial cells were then seeded on the luminal surface of the branched tubular fibrous membranes at a density of 10^6 cells/mL. A total volume of 50 μ L and 100 μ L cell suspension was applied for the basic and complex models, respectively. The models were incubated at 37°C for 2h to allow cell attachment and then incubated under static condition for 24 h.

For the construction of the brain- side of the model, two distinct MeHA-Col hydrogels were prepared, one for neurons and one for astrocytes/pericyte-like cells. The preparation protocol for each hydrogel was described in detail in Section 3.2.2.2.1. The hydrogels were sequentially incorporated into the device, with the neuron-loaded hydrogel introduced first, followed by the astrocyte-/pericyte-like cell loaded hydrogel. After hydrogel incorporation, the assembled NVU-on-a-chip models were placed on a perfusion rocker and incubated for 14 days to enhance endothelial tight junction formation and barrier integrity.

3.2.5.3 Investigation of cellular behavior within the NVU-on-a-chip model

The NVU-on-a-chip models were fixed in 4% PFA for 2 h at RT, then embedded in optimal cutting temperature (OCT) compound. The samples were cryosectioned at a thickness of 50 μ m to allow visualization of the inner surfaces of the branched tubular structures and the surrounding hydrogel. Cell behavior in both the basic and complex NVU-on-a-chip models was evaluated using immunocytochemistry, as described in detail in Section 3.2.4.3.1. The antibodies used for these analyses were listed in Table 9.

3.2.5.4 Evaluation of barrier integrity and functionality of the NVU-on-a-chip model

The permeabilities of the basic and complex NVU-on-a-chip models were assessed using the solutes, fluorescein disodium salt (0.376 kDa; 50 μ g/mL) and Fluorescein and FITC-labeled dextran, on days 14 of culture (n=4). Fluorescein and

FITC-labeled dextran were injected into the luminal side of the branched fibrous membranes at volumes of 50 μL and 100 μL for the basic and complex models, respectively. Correspondingly, 950 μL and 900 μL of PBS were added into the PDMS chamber. Samples of 10 μL were taken from the chamber at 0, 30, 60, 90 and 120 min after solute addition. The sample volumes were completed to 200 μL with PBS, and the florescent intensity values were measured using a Multimode Plate Reader (Perkin Elmer Victor Nivo, USA). The apparent permeability coefficient (P_{app}) was calculated as described in Section 3.2.4.3.3.



4 RESULTS

4.1 Fabrication And Characterization Of 3D Branched Tubular Fibrous Membrane

4.1.1 Modelling and fabrication of sacrificial branched tubular construct

Murray's Law is observed in the circulatory and respiratory system of animals, the respiratory system of insects and the xylem in plants. The physiologist who gave Murray's law its name, Dr. Cecile D. Murray argued for the first time that efficient transportation could explain the structure of the human vascular system. Therefore, this law also incorporated to the biomimetic engineering approaches (70). According to Murray's Law, the relationship between parent and daughter branches should be considered in terms of three main aspects: angle, length and diameter (71, 72). A branched, tubular sacrificial construct was designed through a biomimetic approach using Murray's Law considering the circulatory blood volume transfer. Two models were designed, the basic model with two-branched tubular design and the more complex model with four-branched tubular construct (Figure 9). Based on the Murray's Law the radii of the model tubes were calculated as 800 μm , 630 μm , and 500 μm from the largest to the smallest diameter. The total length of the model was determined approximately 3 cm. Length of the basic model was determined as 9.3 mm for the parent tube and 11.8 mm for the branched tubes (Figure 9a). While, lengths of branches in the complex model were determined as 5 mm for the parent tube and 6.3 and 8 mm for the branched tubes (Figure 9b). 3D designs of the basic and the complex models with calculated dimensions in length and diameter were constructed and the sacrificial molds for each model were printed using polyvinyl alcohol (PVA) filament with Ultimaker 3D printer (Figure 10).

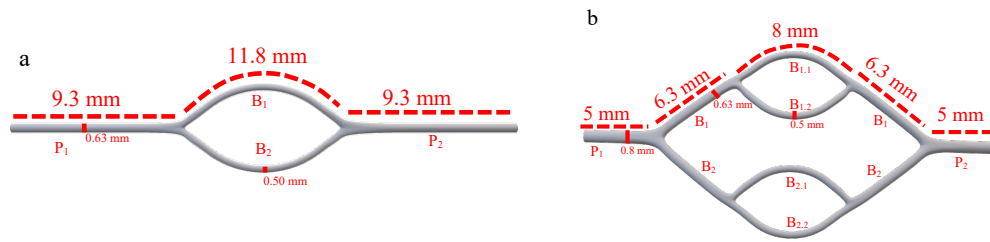


Figure 9. Design and dimensions of (a) the basic model and (b) the complex model (P:parent, B:branches).

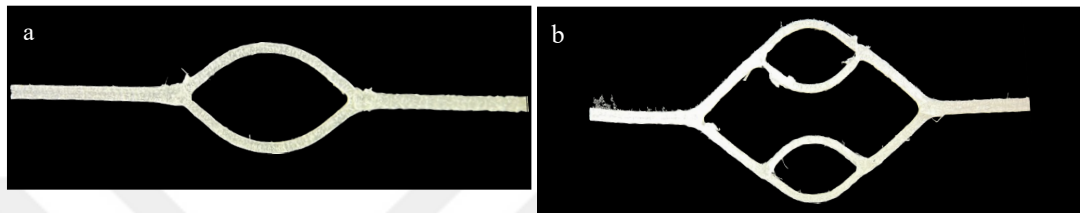


Figure 10. 3D printed PVA-based sacrificial molds for (a) the basic model and (b) complex model.

4.1.2 Fabrication of 3D branched tubular electrospun fibrous membrane

It is widely known that mechanical properties of the tissue affect the tissue function, therefore the mechanical properties of tissue substitute are important for its performance. It was revealed that when endothelial cells were cultured on softer surfaces (with Young's modulus in kPA range), the expression of adhesion molecules at the junctional complex was reinforced (73, 74). Therefore, considering the importance of cell-cell adhesion and cell-matrix interaction in the BBB, it was aimed to obtain fibrous membrane which has kPA range Young's modulus in this thesis. It was shown that PCL solution prepared in acetic acid decreases both fiber diameter from micro to nanoscale and the Young modulus from mPA to kPA range (75, 76). Therefore, PCL-Col polymer solution was prepared in 90% acetic acid since the incorporation of collagen introduces bioactive binding sites that promote endothelial cell attachment and support ECM-like interactions. Optimization studies were conducted using different polymer concentrations, and altering process parameters.

For electrospinning optimization, firstly 32% PCL was prepared and electrospinning was carried out with 0.2 mL/h flow rate, 12 kV applied potential, and 30 cm distance between the tip of the needle and the collector. The electrospun

membrane was collected with a low number of fibers, and there were large beads (Figure 11a). Afterwards, the polymer concentration was reduced from 32% to 27.5%, the flow rate was increased to 0.3 mL/h, and the voltage kept constant while the tip-to-collector distance were decreased to 25. It was observed that more fibers at nanoscale in diameter were collected but they were non-uniform and there were still numerous large beads on the membrane (Figure 11b). Then, using the same polymer concentration and keeping the distance constant, the flow rate and the voltage were reduced to 0.2 mL/h and 10 kV, respectively. It was observed that there was a decrease in the number of beads, but the fiber morphology should be improved (Figure 11c). Therefore, the polymer concentration was reduced to 22.5% but the fibrous membrane could not be obtained. Therefore, in order to improve viscosity of polymer solution and enhance cell adhesion, a blend of PCL and collagen type 1 solution (4:1 and 1:1, w:w; 22.5%, w/v%) was prepared and electrospun at a flow rate of 0.2 mL/h , an applied potential of 14 kV, a distance of 24 cm. It was observed that uniform, bead-free fibrous membranes were successfully obtained in both PCL-Collagen (4:1) and PCL-Collagen (1:1) polymer compositions (Figure 11d, e). Average fiber diameters of PCL, PCL-Collagen (4:1) and PCL-Collagen (1:1) membrane were calculated as 168.42 ± 49.91 nm, 211.44 ± 42.28 nm and 296.52 ± 124.86 nm, respectively. Among all fibrous membranes, the electrospun fibers produced from the PCL-Collagen (4:1) blend were more uniform, bead-free, and exhibited small nanoscale diameters.

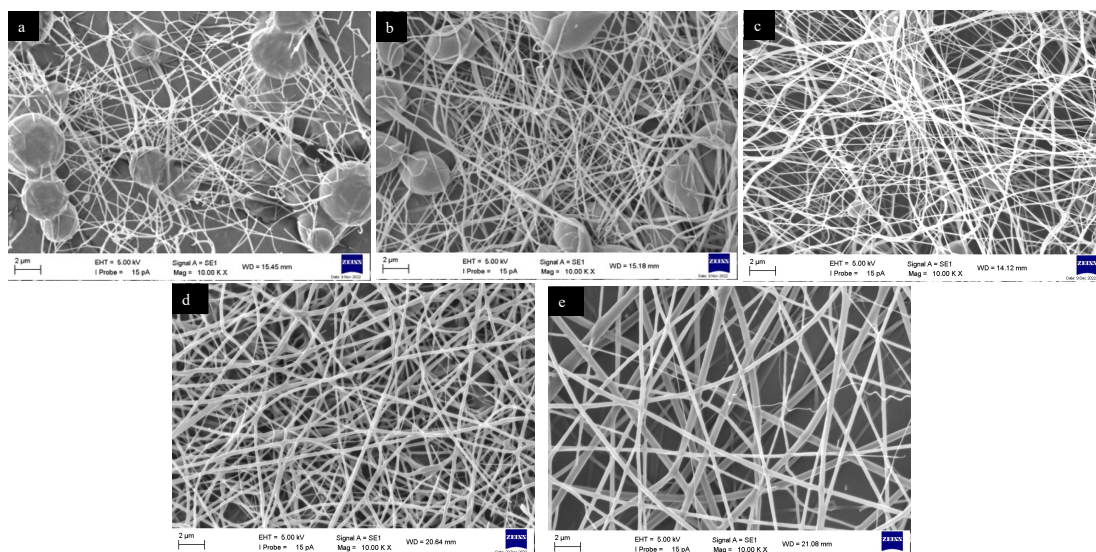


Figure 11. SEM images of electrospun fibrous membranes obtained during electrospinning optimization. The electrospun membranes were fabricated using different polymer solutions (32%-22.5%) altering the flow rate (0.2-0.3 mL/h), the applied potential (10-14 kV) and the distance between the needle tip and the collector (24-30 cm). (a) 32% PCL, 0.2 mL/h, 12 kV, 30 cm; (b) 27.5% PCL, 0.3 mL/h, 12 kV, 25 cm; (c) 27.5% PCL, 0.2 mL/h, 10 kV, 25 cm, (d) 22.5% PCL-Collagen (w:w, 4:1), 0.2 mL/h, 14 kV, 24 cm; (e) 22.5% PCL-Collagen (w:w, 1:1), 0.2 mL/h, 14 kV, 24 cm.

Fiber deposition over PVA-based sacrificial molds could not be achieved due to the poor electrical connectivity of PVA. According to a study, complex configurations of 3D nanofibers can be achieved by hydrogel-assisted electrospinning when a 3D Y-shaped hydrogel structure containing 1 M NaCl as an electrolyte is used instead of a metal collector (65). They found that the efficiency of fiber deposition in the presence of the electrolyte was comparable to that achieved with conventional metal collectors. Considering this knowledge, in our study, different protocols were applied to coat the sacrificial molds in order to obtain a 3D branched tubular electrospun fibrous membranes (Table 10). Firstly, a PVA-based sacrificial branched tubular mold was coated with a 20% PEG and 1 M NaCl (9:1, v/v) solution. It was seen that more fibers were deposited on the coated branching mold than on the uncoated one. However, it was observed that a significant improvement was still required to obtain a perfusable lumen of tubular fibrous membranes. Therefore, different concentration of NaCl with varying ratios and concentration of PEG was tested to improve fiber deposition. Despite the coating of the mold with a 20% PEG and 4 M NaCl (9:1, v/v) solution,

which promoted the fiber deposition, uneven and inconsistent fiber collection was still observed (Figure 12c). It was reported that KBr has higher conductivity than NaCl, and PEG containing KBr also exhibited higher conductivity than pure PEG (77, 78). Considering these information, KBr was used to increase conductivity and improve fiber collection. It was observed that better fiber deposition was achieved by coating the 3D printed branched mold with a solution of 20% PEG and 4 M KBr (9:1, v/v) (Figure 12d). However, the fibers at the connection points of the model's branches were not properly collected, leading to leak points in the perfusable tubular network. This problem was solved by increasing the viscosity of the solution by using PCL with high molecular weight (124,000 g/mol). Consequently, it was found that the optimized protocol for the fiber deposition over the 3D printed branched tubular mold was firstly coating the sacrificial mold with 20% PEG and 4 M KBr (9:1, v/v) solution, followed by electrospinning with 30% PCL solution at 0.2 mL/h flow rate, 10 kV applied voltage, 15 cm distance (Figure 12e, g). For collagen-containing electrospun membranes, the polymer concentration of 22.5% PCL-Col (8:2, w/w) was increased to 25% to enhance fiber deposition over the sacrificial models, while the coating conditions (20% PEG and 4 M KBr, 9:1 v/v) were kept constant.

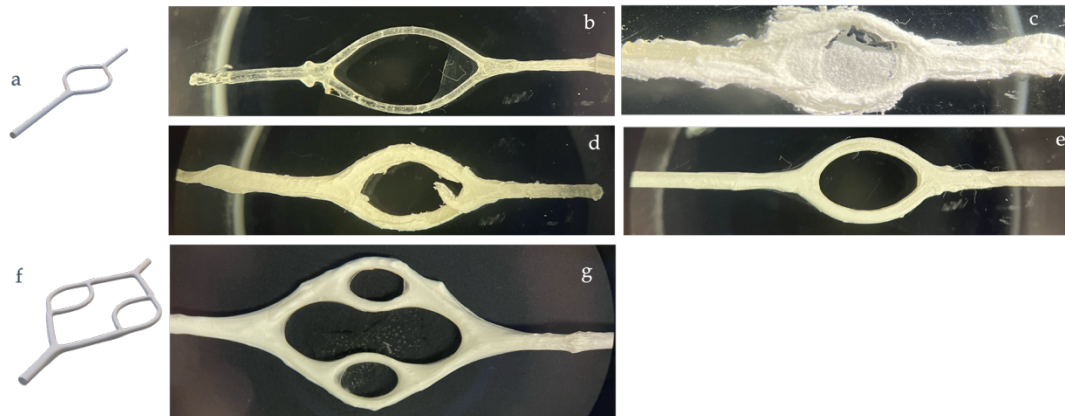


Figure 12 Schematic representation of the proposed (a) basic and (f) complex model, and stereomicrographs of fiber deposition over the basic model: (b) without coating, (c) coating 20% PEG with 4M NaCl (9:1, v/v), (d) 20% PEG with 4M KBr (9:1, v/v). 3D branched tubular electrospun membranes of 30% PCL were deposited over the sacrificial molds for (e) the basic model and (g) the complex model, which were coated with 20% PEG with 4M KBr (9:1, v/v).

Table 10. Optimizing the coating protocol of the sacrificial mold using PCL to achieve a 3D branched tubular electrospun fibrous membrane.

Salt Solution (in dH₂O)	PEG (%) (in EtOH)	Ratio (PEG: salt)	Observation
1M NaCl	20	9:1	Few fibers were collected over sacrificial mold
		8:2	Collection of fibers was not improved
		5:5	PVA based sacrificial mold became sticky
2M NaCl	20	9:1	Collection of fibers was not improved
		5:5	PVA based sacrificial mold became sticky
6M NaCl	20	9:1	NaOH was precipitated upon mixing with PEG solution
	10		
5M NaCl	20	9:1	NaOH was precipitated upon mixing with PEG solution
	10		
4M NaCl	20	9:1	Collection of fibers was improved (Figure 12c)
5 M KBr	20	9:1	NaOH was precipitated upon mixing with PEG solution
4 M KBr	20	9:1	Best condition for collection of fibers over sacrificial mold (Figure 12d,e)

4.1.3 Characterization of 3D branched tubular electrospun fibrous membrane

4.1.3.1 Morphology of the tubular fibrous membranes

Fiber deposited basic model (BM) and complex model (CM) were examined with SEM (Figure 13-16). SEM images showed that fibers of 30% PCL, and 25% PCL-Col (8:2, w/w) were effectively collected over both models' sacrificial molds, including the connection points between the branches. (Figure 13-16, b-d). The images of the fiber-deposited BM and CM at high magnification showed that the fiber diameter was remained within the nanometer range (data not given). SEM results showed that sacrificial molds were effectively removed, resulting in the formation of tubular structure with perfusable lumens (Figure 13-16, e-f).

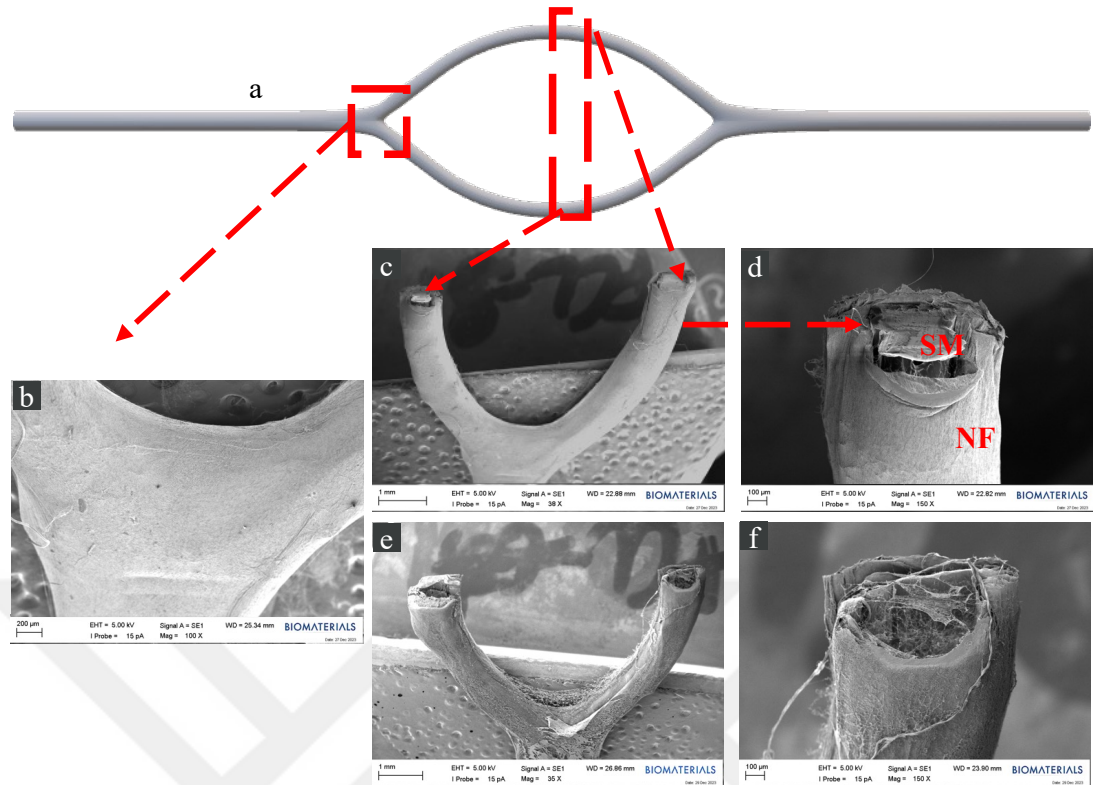


Figure 13. Electrospinning of 30% PCL over the basic branched tubular sacrificial model. (a) Computer-aided design of the model. SEM images of electrospun nanofibrous membrane (NF) collected over the sacrificial model: (b) branch connection part, (c,d) branches before, and (e,f) after removal of sacrificial mold (SM).

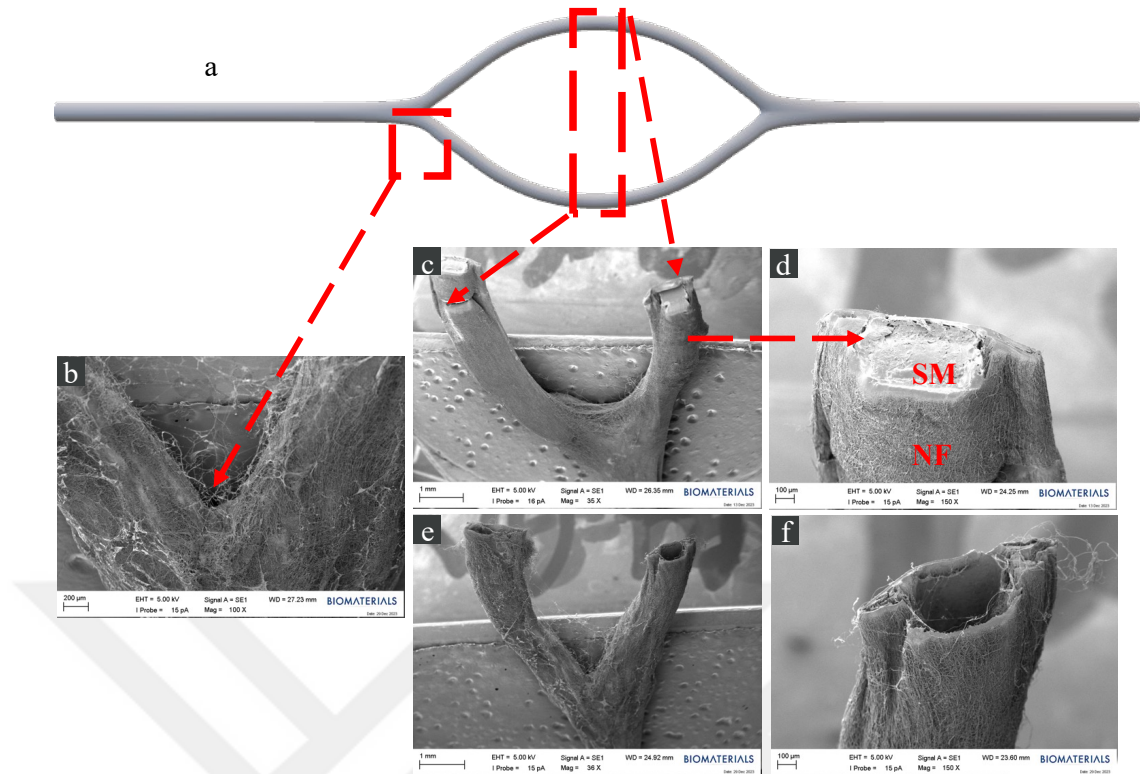


Figure 14. Electrospinning of 25% PCL-Col (8:2, w/w) over the basic branched tubular sacrificial model. (a) Computer-aided design of the model. SEM images of electrospun nanofibrous membrane (NF) over the sacrificial model: (b) branch connection part, (c,d) branches before, and (e,f) after removal of sacrificial mold (SM).

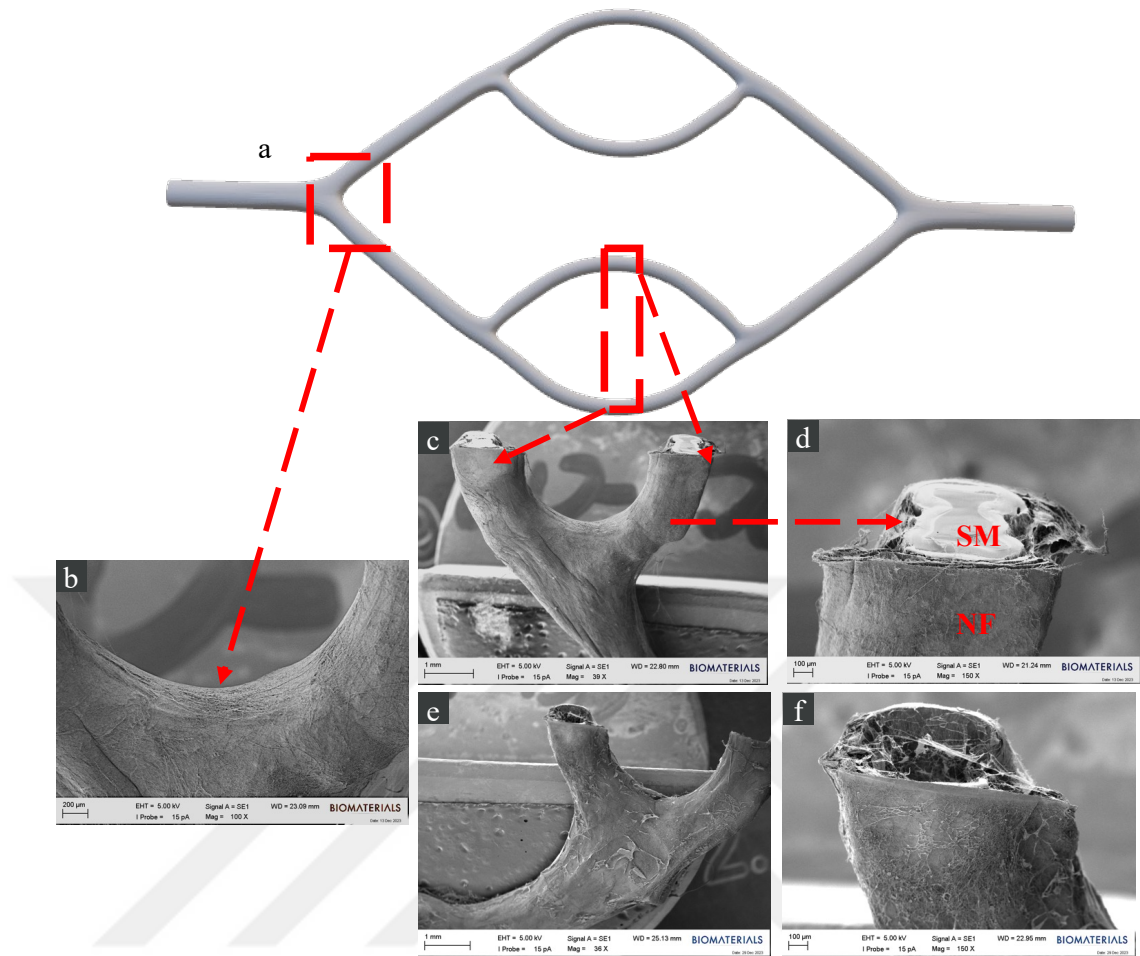


Figure 15. Electrospinning of 30% PCL solution over the complex branched tubular sacrificial model. (a) Computer-aided design of the model. SEM images of electrospun nanofibrous membrane (NF) over the sacrificial model: (b) branch connection part, (c,d) branches before, and (e,f) after removal of sacrificial mold (SM).

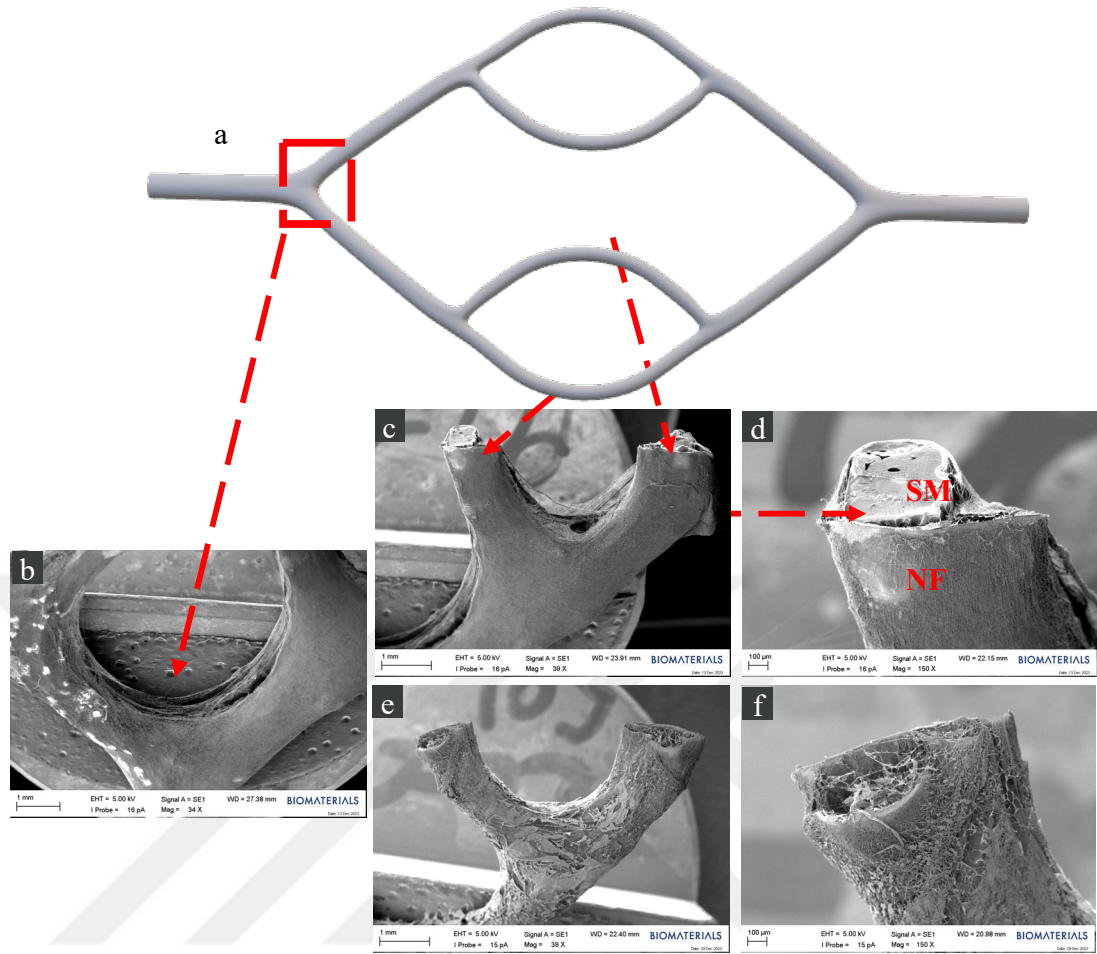


Figure 16. Electrospinning of 25% PCL-Col (8:2, w/w) over the complex branched tubular sacrificial model. (a) Computer-aided design of the model. SEM images of electrospun nanofibrous membrane (NF) over the sacrificial model: (b) branch connection part, (c,d) branches before, and (e,f) after removal of sacrificial mold (SM).

4.1.3.2 Mechanical properties of the fibrous membranes

Mechanical properties of tissues are critical in determining their biological behavior. As mentioned earlier, the stiffness of a substrate, particularly in the kPa range, significantly influences endothelial cell function, particularly the reinforcement of adhesion molecules at cell junctions (73, 74). The mechanical properties of the fabricated fibrous membranes were investigated in this study. Mechanical testing was performed, and the Young's Modulus of the electrospun fibrous membranes were determined from the stress-strain curves. The Young's Modulus of the PCL membrane was $0.74 \pm 0.15 \text{ N/mm}^2$ ($740 \pm 150 \text{ kPa}$), while that of the PCL-Col (8:2, w/v) membrane was $0.75 \pm 0.10 \text{ N/mm}^2$ ($750 \pm 100 \text{ kPa}$) (Figure 17). These results

demonstrate that the desired Young's modulus in the kPa range was achieved. Additionally, the incorporation of collagen into the fibrous membranes did not significantly affect the mechanical properties, as the Young's Modulus remained within the same range for both PCL and PCL-Col membranes.

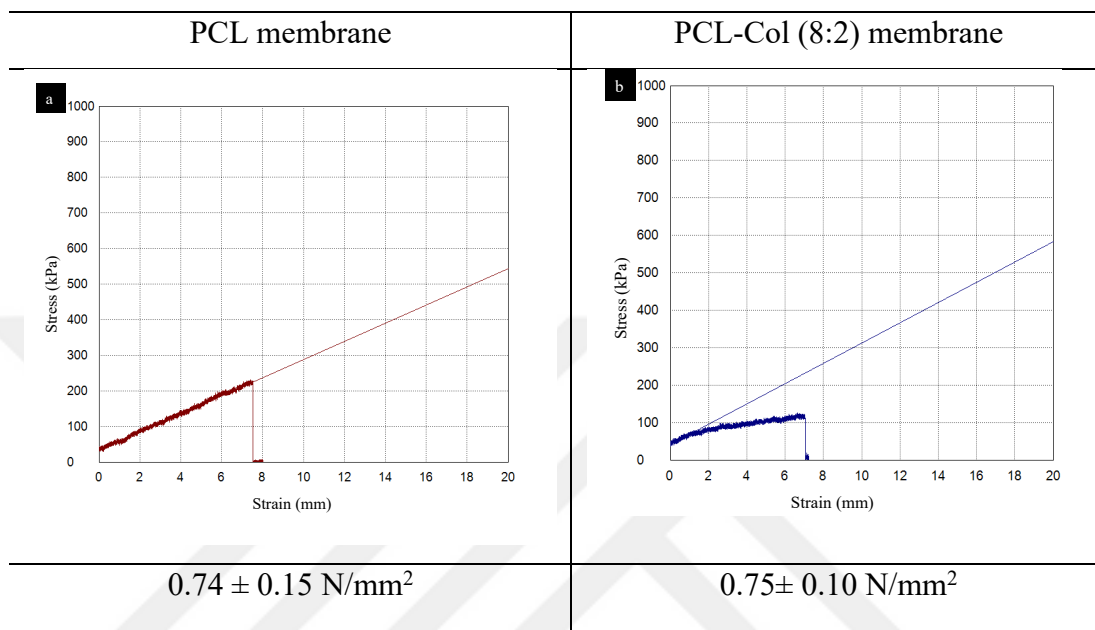


Figure 17. Representative stress-strain curves for (a) PCL and (b) PCL-Col (8:2, w/v) fibrous membranes.

4.1.3.3 Degradation profile of fibrous membranes

The degradation profile is a critical consideration in barrier modeling, as it ensures the maintenance of barrier integrity and stability. The long-term degradation profile of a membrane allows the model to achieve permeability control, physiological accuracy, and reproducibility. The degradation profile of the fibrous membranes showed that the PCL membrane remained stable over 24 days, whereas the PCL-Col-based membrane lost 20% of its initial weight within the first 10 days (Figure 18). The degradation rate confirms that the weight loss observed in the PCL-Col (8:2) membrane primarily results from the collagen component, which undergoes rapid degradation in an aqueous environment. Both membranes exhibited a prolonged degradation profile over time, maintaining their structural integrity throughout the 24-day degradation period, demonstrating their suitability for long-term applications.

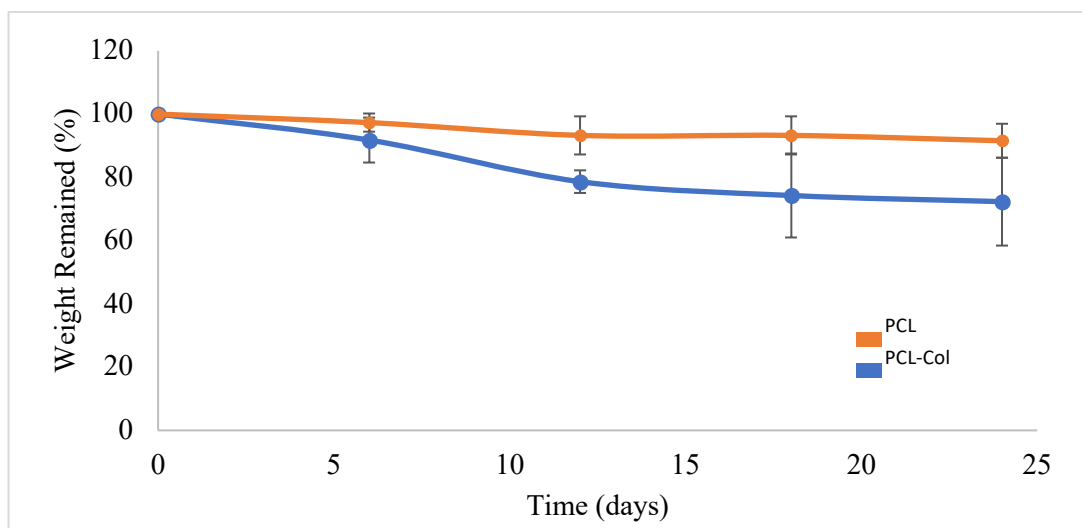


Figure 18. Degradation profile of fibrous PCL and PCL-Col membranes over time

4.1.3.4 Surface properties of the polymeric membranes

The wettability properties of the PCL and PCL-Col (8:2, w/v) membranes were assessed through contact angle measurements. Surfaces with contact angles between 90° and 180° are generally considered hydrophobic, whereas those with contact angles between 40° and 70° are classified as moderately hydrophilic, which is more favorable for endothelial cell attachment. The contact angles were determined as $85.12^\circ \pm 1.76^\circ$ for PCL and $57.50^\circ \pm 1.66^\circ$ for PCL and PCL-Col (8:2, w/v) films (Figure 19). Based on these results, the PCL-Col (8:2, w/v) films exhibited moderate hydrophilicity, indicating its potential suitability for promoting endothelial cell adhesion. However, cell attachment on the more hydrophobic PCL films could be enhanced by applying a protein coating, such as fibronectin or gelatin. These observations reflect only a comparative characterization of the two materials, and the membrane composition will be determined based on cell behavior in the subsequent studies

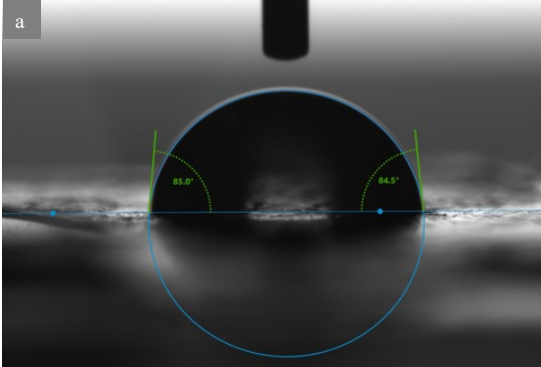
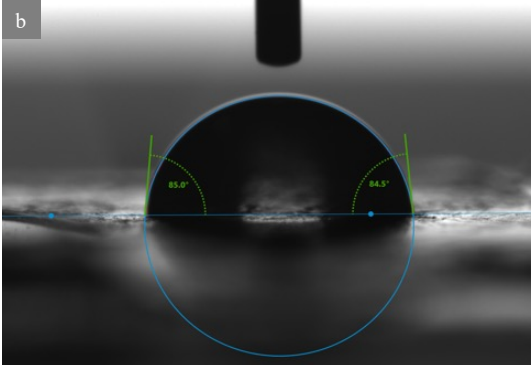
PCL films	PCL-Col (8:2) films
	
$85.12^{\circ} \pm 1.76^{\circ}$	$57.50^{\circ} \pm 1.66^{\circ}$

Figure 19. Representative images of contact angle measurements for (a) PCL, and (b) PCL-Col (8:2, w/v) films.

4.1.3.5 Modelling and tracking fluid flow in BM and CM tubular fibrous membranes

CFD involves using numerical methods and computer simulations to analyze and predict the behavior of fluids, including blood, within microfluidic systems and complex geometries, and it plays a significant role in the study of blood flow (79). It aids in the investigation of hemodynamics, assessment of vascular diseases, design of medical devices, surgical planning, and drug delivery optimization, ultimately contributing to advancements in vascular research and clinical practice. In this study, CFD was employed to simulate fluid behaviour within the basic and complex models. The static pressure within the blood vessels varies along the circulatory system (80). It is highest in the arteries and gradually decreases as blood flows through arterioles, capillaries, venules, and veins. CFD analysis of the first version of the both models revealed that the static pressure has the highest at the inlet and near the bifurcation points, and exhibits an alternating waveform fluid behavior (Figure 20a,c). However, it was aimed to gradually decrease the static pressure profile in the models to comply with the CFD analysis of native blood vessels (81). Consequently, the design approaches at the bifurcation points were altered. It was demonstrated that the pressure of the fluid remained highest at the starting point, indicated by the color red. As the fluid progressed through the model, the pressure gradually decreased, causing the color to change from red to yellow, green, and eventually blue. This fluid behaviour in the

models was intentional, aiming to replicate the static pressure profile of native blood vessels (Figure 20b,d).

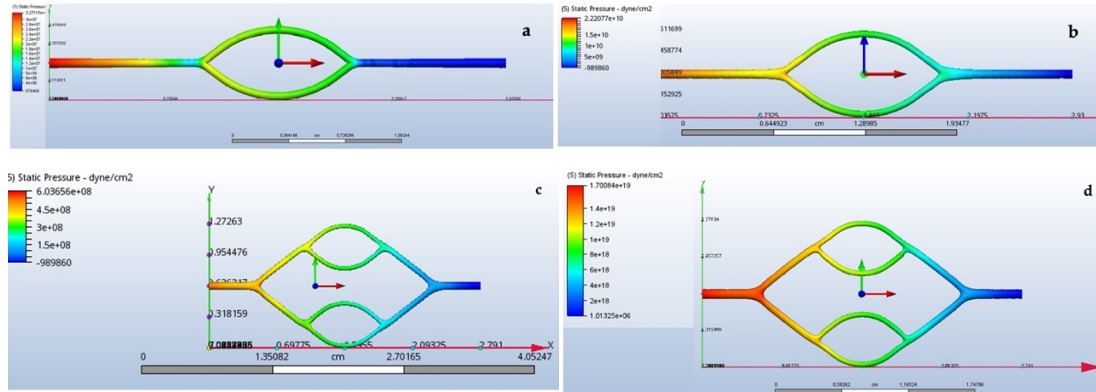


Figure 20. Computational fluid dynamics (CFD) analysis showing the fluid behavior of the basic and the complex branched models. (a,c) Initial versions (first version), and (b,d) improved versions of the basic and complex models at the bifurcation point.

Images of the 30 % PCL nanofiber network of BM and CM showed that after injection of ink from the inlet, ink was split simultaneously into the branches by following the channels and met at the connection point near to outlet of the model, then left (Figure 21-22a-d). Images of the 30 % PCL-based tubular fibrous membranes of BM and CM showed that, after ink was injected through their inlets, it split simultaneously into the branches by following the channels, converged at the connection point near the outlet of each model, and then exited. (Figure 21-22a-d). These results showed that the even transport of fluid within the tubular membrane for both model can be provided with the sacrificial branched tubular mold developed using a biomimetic approach, in which the branch angles, lengths, and diameters were designed according to Murray's Law.

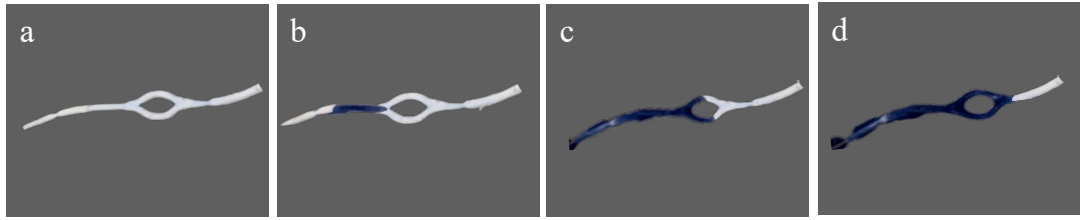


Figure 21. Photographs of ink flow in the 30% PCL-based tubular fibrous membrane of the basic model after removal of sacrificial branched tubular mold: (a) without ink, (b) ink injected into inlet, (c) ink splitting into the branches, (d) ink converged at the connection point near the outlet.

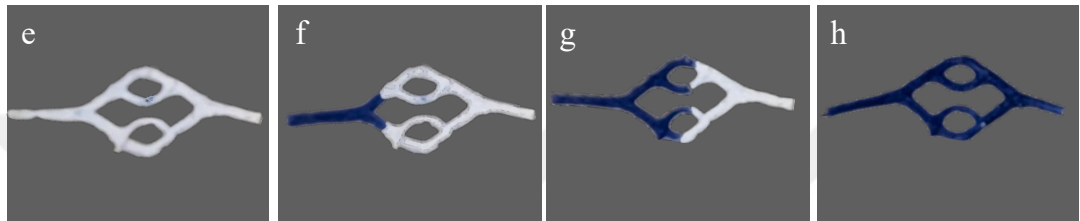


Figure 22. Photographs of ink flow in the 30% PCL-based tubular fibrous membrane of the complex model after removal of sacrificial branched tubular mold: (a) without ink, (b) ink injected into inlet and split into the first branches, (c) ink splitting into the smallest branches, (d) ink converged at the connection point near the outlet.

4.2 Hydrogel Preparation And Characterization

4.2.1 Determination of hydrogel composition

The brain side of NVU is composed of unique brain ECM. Healthy brain ECM mainly composed of hyaluronic acid, proteoglycans (lecticans) and tenascins (27). The lecticans serve as organizational components, and tenascins are function as linkers and also provide cell attachment site. Hyaluronic acid is main structural component of brain ECM, and increases the hydration due to it carry high negative charge which attract the sodium and charged proteins. It is hypotized that the primary reason for brain's low stiffness is their high water content. Important design requirements for the succesfful neuronal tissue engineering is to biological activity, apropaite stiffness, and degradation profile. The brain side of this model was designed to be hyaluronic based hydrogel as a biomimetic approach.

Stereomicroscopy images showed that individual collagen (Col)- and hyaluronic acid (HA)-based hydrogel solutions were effectively crosslinked at 37°C, forming stable hydrogels (Figure 23a, c). Similarly, the Col-HA (1:1) hydrogel solution formed a uniform and homogeneous hydrogel (Figure 23e). The cell viability within these hydrogels was also investigated with Live/Dead assay using pericyte-like cells to determine the hydrogel composition. LSCM images indicated that pericyte-like cells were viable and exhibited their characteristic fibroblastic morphology in the collagen-based hydrogel (Figure 23b). In contrast, the cells remained viable in the HA-based hydrogel, however; they did not exhibit a fibroblastic morphology, and the loading capacity was lower compared to the collagen-based hydrogel (Figure 23b,d). Cells were not effectively incorporated into HA-Col hydrogels, and those that were loaded did not remain viable within the hydrogel (Figure 23f). These findings suggest that HA-Col (1:1) hydrogels do not support cell survival and spreading.

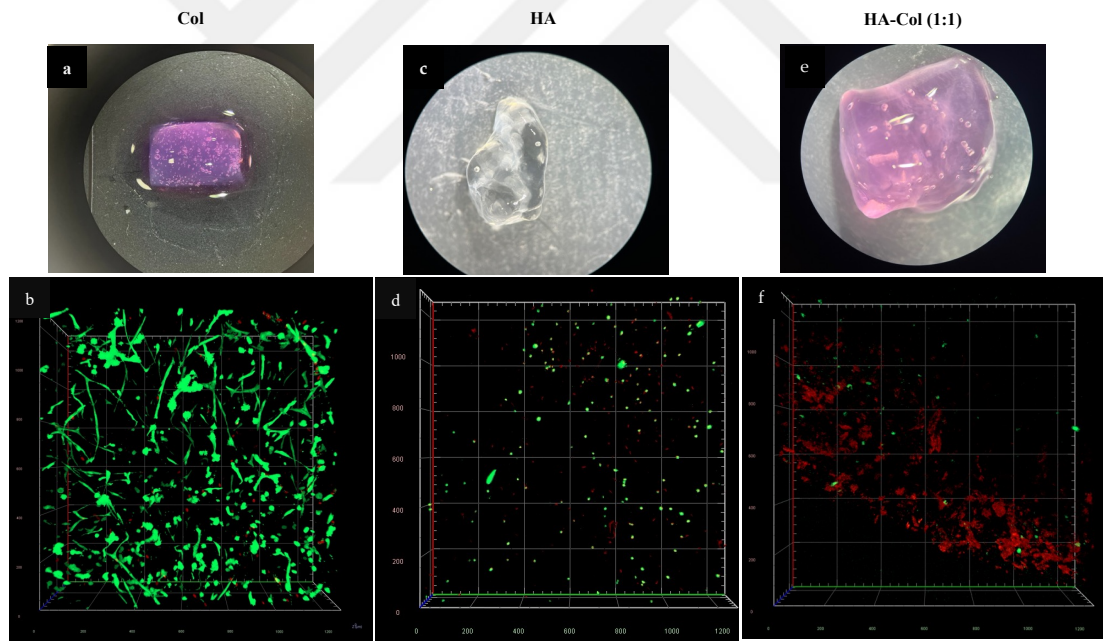


Figure 23. Stereomicrographs of hydrogels and LSCM images of the cells after Live/Dead assay on day 3 of culture: (a, b) Col-based hydrogel, (c, d) HA hydrogel, and (e, f) HA-Col (1:1) hydrogel. (green: viable cells, red: dead cells).

Therefore, optimization studies were planned for hydrogel formation by varying the HA/ Col concentration, incubation time, and the concentration of the cross-linker (PEGDA) provided in the HA kit. In the initial optimization step, the PEGDA content

was kept constant according to the kit protocol (PEGDA:HA 4:1, v/v) to isolate the effect of HA and Col concentrations on gelation behavior. First, 0.66% HA–1% Col and 0.5% HA–1.5% Col hydrogel solutions were prepared and incubated at 37 °C for 2 h to investigate the effect of the HA:Col ratio on gelation. Uniform hydrogel structures were not obtained with either composition after 2 h of incubation; therefore, the hydrogels were incubated overnight to assess the effect of extended incubation time on gelation. The 0.66% HA–1% Col formulation generated more hydrogel volume than the 0.5% HA–1.5% Col formulation from the same starting volume (Figure 24a,b). Thus, 0.66% HA- 1% Col solution was used for further hydrogel optimization studies. Effect of PEGDA concentration on the hydrogel formation was evaluated by altering the ratio of PEGDA in the solution. HA hydrogel solutions were prepared by combining thiol-modified hyaluronan with the crosslinker PEGDA at ratios of 8:1 and 2:1 (v:v), which were twofold higher and lower than the manufacturer's recommendation, respectively, and subsequently mixed with Col solution (Figure 24c,d). The final solution was incubated for 2 h at 37°C to allow gelation, after which incubation was continued overnight. There was no noticeable difference between the hydrogels after 2h. After overnight incubation, HA-Col IPN hydrogel formation was greater for the solution prepared with HA:PEGDA (8:1) than for HA:PEGDA (2:1). Due to inconsistent hydrogel formation and insufficient support for cell survival with the HyStem Kit, it was decided to prepare hyaluronic acid-based hydrogel from MeHA to mimic the brain ECM microenvironment and to incorporate a component, primarily a protein, to facilitate cell attachment.



Figure 24. Stereomicrographs of hydrogels in spatula prepared with (a) 0.5% HA-1.5% Col HA-Col (1:1), (b) 0.66% HA- 1% Col solutions, and 0.66% HA- 1% Col hydrogel prepared with thiol-modified hyaluronan using PEGDA ratios of (c) 8:1 and (d) (2:1).

Initially, although some of the pericyte-like cells remained viable on day 3 within the 0.5% MeHA-1.5% Col hydrogel, a significant proportion of them were dead (Figure 25a). On the 7th day of culture, viable cells outnumbered the dead cells, displaying a slightly expanded morphology, while only a few cells exhibited cell death (Figure 25b). The MeHA component provides structural stability to the hydrogel, while collagen serves as a site for cell attachment. However, pericyte-like cells did not exhibit their characteristic fibroblastic morphology in this hydrogel composition. Therefore, further optimization studies were conducted, including increasing concentrations of collagen or MeHA and exploring different protein configurations, to enhance cell-to-cell and cell-to-matrix interactions, thereby improving signaling within the 3D microenvironment (Table 7).

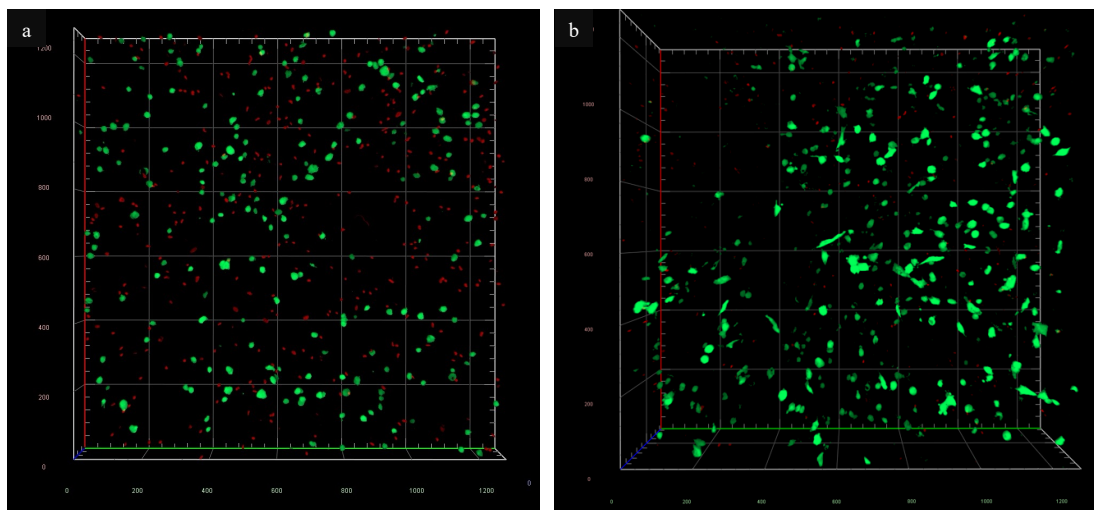


Figure 25. LSCM images of pericytes-like cells in 0.5% MeHA-1.5% Col (1:1) hydrogel after Live/Dead assay on (a) day 3 and (b) day 7 of culture (green: viable cells, red: dead cells).

In order to achieve the characteristic spindle-shaped morphology of pericyte-like cells in hydrogel, the hydrogel composition was changed as 0.33% MeHA-2% Col (Figure 26a-b). Additionally, fibronectin was added to the hydrogel solution to promote cell expansion (Figure 26c,d). The results indicate that pericyte-like cells in varying hydrogel compositions proliferated and stayed viable for 7 days of culture; however, increasing the protein content of the hydrogel did not change cell spreading (Figure 26).

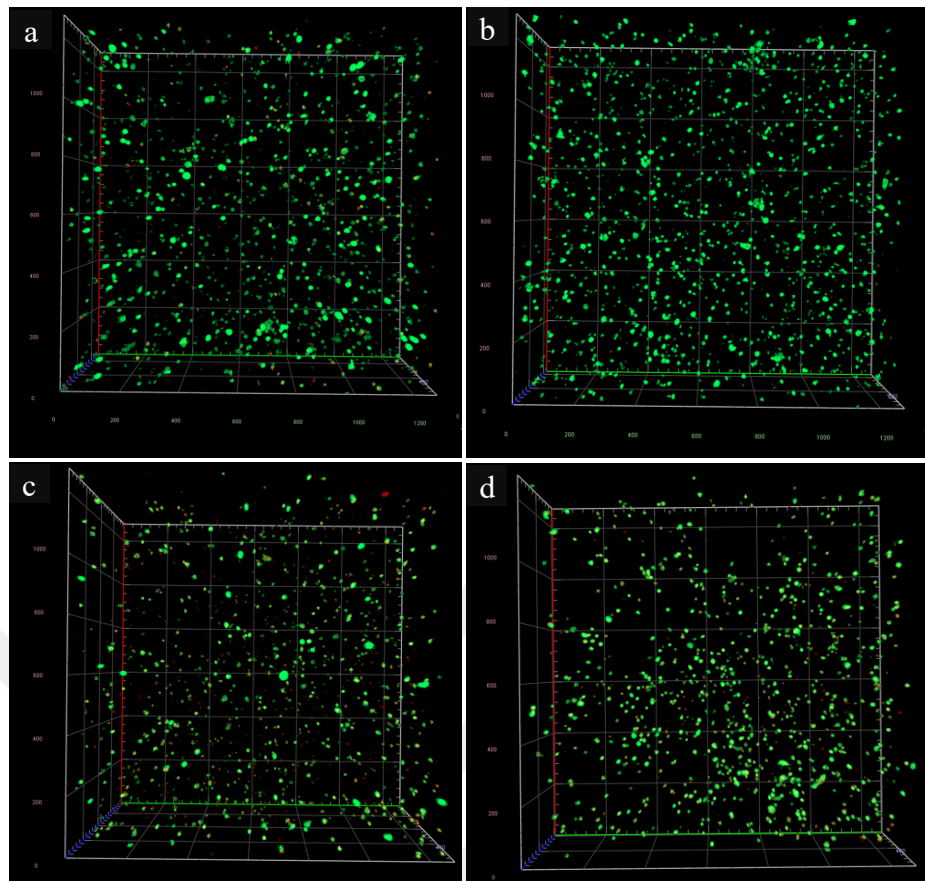


Figure 26. LSCM images of pericyte-like cells in (a,b) 0.33% MeHA - 2% Col, (c,d) 0.4% MeHA - 1.8% Col - 5% Fnc hydrogels after Live/Dead assay on (a,c) day 3 and (b,d) day 7 of cell culture (green: viable cells, red: dead cells).

Fibronectin (Fnc) is a glycoprotein that contains the arginine-glycine-aspartate (RGD) peptide sequence, which provides strong cell attachment and spreading (82). Therefore, the effect of fibronectin on the viability and morphology of pericyte-like cells was evaluated by incorporating varying concentrations of fibronectin (1.25%, 2.5%, 5%, and 7.5%) into a 0.5 % MeHA solution (Figure 27). Hydrogels prepared with all fibronectin concentrations showed a high density of viable pericyte-like cells, indicating good initial cell attachment and viability (Figure 27a, c, e, g). During the culture, the morphology of cells was examined using a light microscope, and the culture period was extended to 14 days to assess the effect of time on the spindle-shaped morphology of pericyte-like cells in the hydrogels (Figure 27 b,d,f,h). It was observed that pericyte-like cells exhibited a high viability, and showed a slight increase in density within the hydrogels. However, there was no significant improvement in their cell morphology. Compared to the cell viability results in collagen-MeHA

hydrogels, it was concluded that fibronectin, as a water-soluble molecule, in the hydrogel might be released over time. Even though it slightly increased cell viability in the hydrogel, it did not stay in the hydrogel and could not promote cell spreading. Therefore, another strategy—the formation of an interpenetrating network (IPN) hydrogel—was implemented to enhance cell behavior. Although collagen–MeHA hydrogels were tested previously, it was considered that an efficient IPN network could not be formed in those initial formulations because the MeHA–collagen composition was not optimized. For this reason, temperature-responsive collagen was incorporated into the MeHA-based hydrogel system to facilitate the formation of a more stable and effective IPN structure.



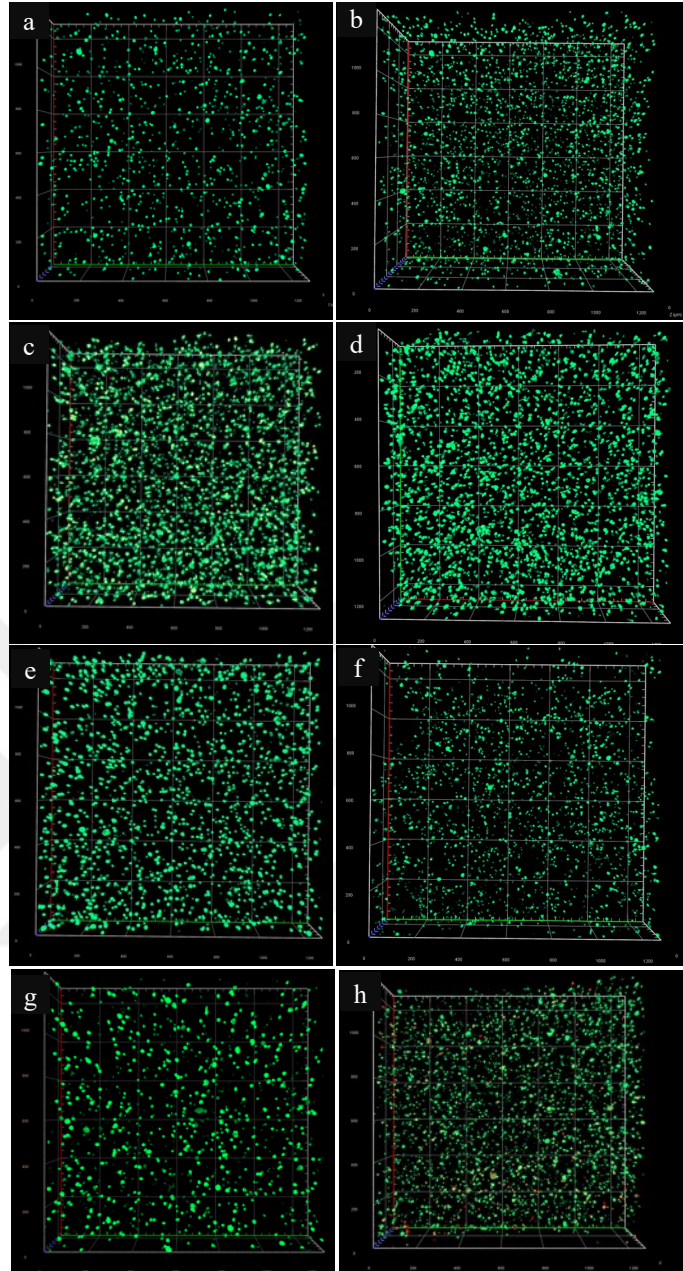


Figure 27. LSCM images of pericyte-like cells in (a, b) 0.5 % MeHA- 1.25% Fnc, (c, d) 0.5 % MeHA- 2.5%, (e, f) 0.5 % MeHA- 5% Fnc, and (g, h) 0.5 % MeHA- 10% Fnc hydrogels after Live/Dead assay at (a, c, e, g) 3rd and (b, d, f, h) 14th day of culture. (green: viable cells, red: dead cells).

The literature survey also revealed that the matrix stiffness and the type of adhesive molecules significantly influenced cell behavior in hydrogels, and cells spread more on stiff hydrogels compared to soft hydrogels (83). Therefore, the MeHA concentration was increased to 0.75% and then to 1% to increase the stiffness of hydrogel, and only collagen-based hydrogel used as a control to evaluate cell

morphology in hydrogel. Pericyte-like cells showed high viability and exhibited typical fibroblastic morphology by spreading on day 3 in the Col-based hydrogel (Figure 28a). Cell viability in the 0.5% MeHA-2% Col hydrogel was moderate and cell spreading was less pronounced compared to the cells in the Col-based hydrogel. However, in this hydrogel pericyte-like cells were slightly expanded and displayed spindle like morphology compared to the other MeHA containing hydrogels (Figure 28b). Cell viability was further reduced in the 0.75% MeHA-1.5% Col hydrogel (Figure 28c). The cells in 1% MeHA: 1% Col hydrogel showed the lowest cell viability, with a high number of dead cells and minimal cell spreading (Figure 28d). These results indicate that elevating the MeHA content at the expense of collagen did not enhance cell morphology, as a higher amount of collagen in the hydrogel solution provided a more favorable environment for pericyte-like cells. Therefore, it was decided to use different ratios of high MeHA and high collagen concentrations in the subsequent studies.

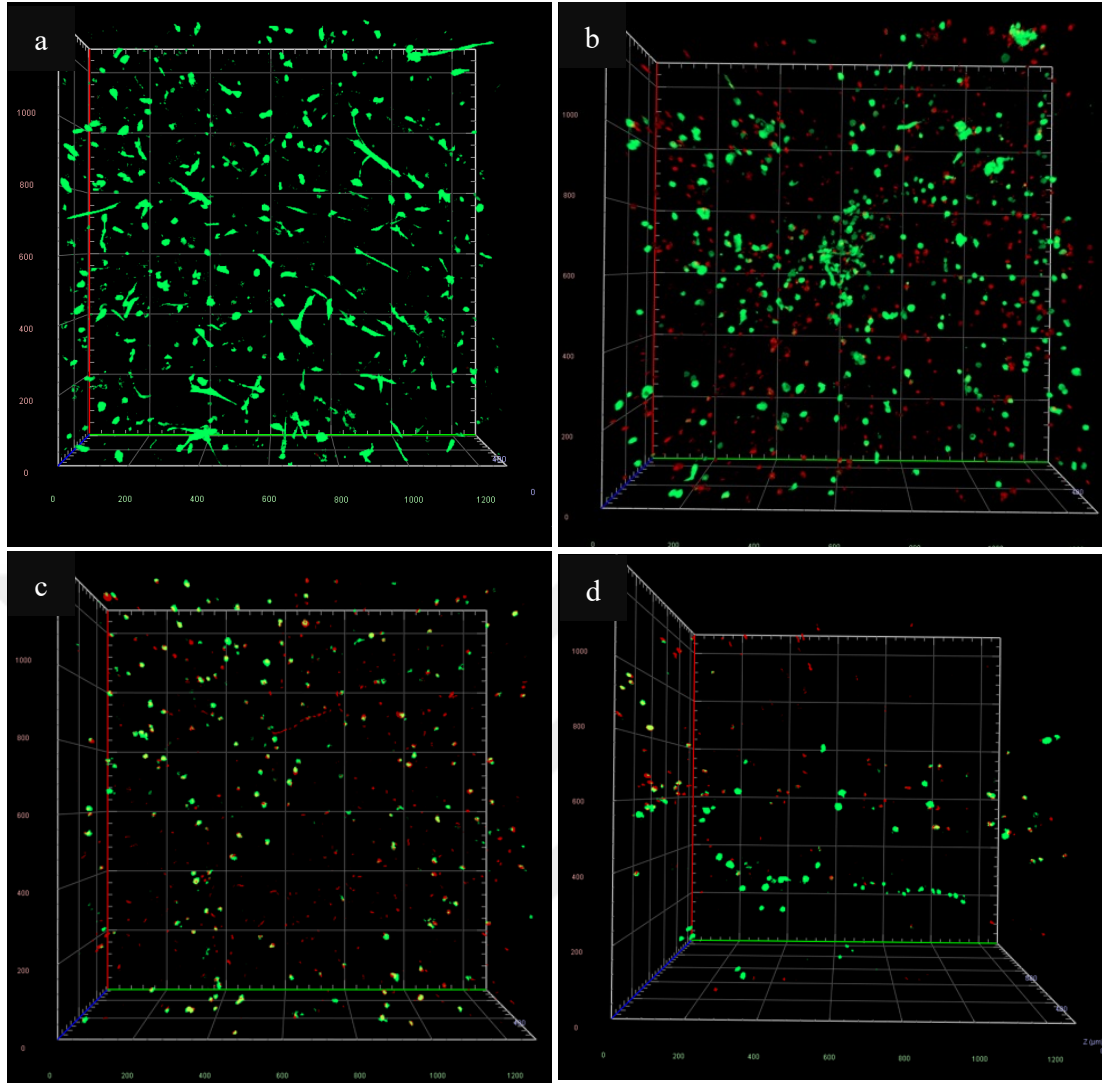


Figure 28. LSCM images of pericyte-like cells in (a) 3% Col, (b) 0.5% MeHA - 2% Col, (c) 0.57% MeHA - 1.5% Col, and (d) 1% MeHA - 1% Col IPN hydrogels after Live/Dead assay on 3rd day of culture (green: viable cells, red: dead cells).

Besides increasing both MeHA and collagen concentrations of the hydrogels, the cell loading density was also increased from 2×10^6 cells/mL to 5×10^6 cells/mL. Consistent with previous findings, cells maintained high viability and exhibited extensive spreading while preserving their characteristic fibroblastic morphology in Col-based hydrogels at both 3% and 6% concentrations (Figure 29a,d). In the 1.25% MeHA–1.5% Col hydrogel, cell viability was moderate, with less pronounced spreading compared to the Col-based hydrogels; however, the cells exhibited some fibroblastic morphology relative to the previous MeHA-Col hydrogels (Figure 29b). In the 0.75% MeHA - 2% Col hydrogel, cell viability and morphology improved

compared to 1.25% MeHA-1.5% Col hydrogel, with more cells displaying spindle-like morphology (Figure 29c). Afterwards, further MeHA and collagen compositions were tested to enhance cell morphology. The hydrogels of 1.5 % MeHA - 1.5% Col and 1% MeHA - 2% Col MeHA did not provide improvement in terms of cell morphology (Figure 29e,f) compared to the 0.75% MeHA - 2% Col hydrogel (Figure 29c). These results suggested that exceeding a MeHA concentration of 0.75% might not effectively enhance cell-matrix interactions. Therefore, it was decided to keep the MeHA concentration constant at 0.75% and increase the collagen concentration to improve cell morphology within the hydrogel. Notably, pericyte-like cells exhibited an elongated morphology in the 0.75% MeHA - 2% Col hydrogel (Figure 29c). As the collagen concentration increased to 4% and 4.5%, while keeping the MeHA concentration at 0.75%, both the number of elongated cells and overall cell viability improved further (Figure 29g,h). These results indicate that collagen concentration positively influences cell viability and morphology, while increasing MeHA concentration beyond a certain threshold may have detrimental effects. Therefore, the optimal hydrogel composition for further studies was determined to be 0.75% MeHA-4.5% Col.

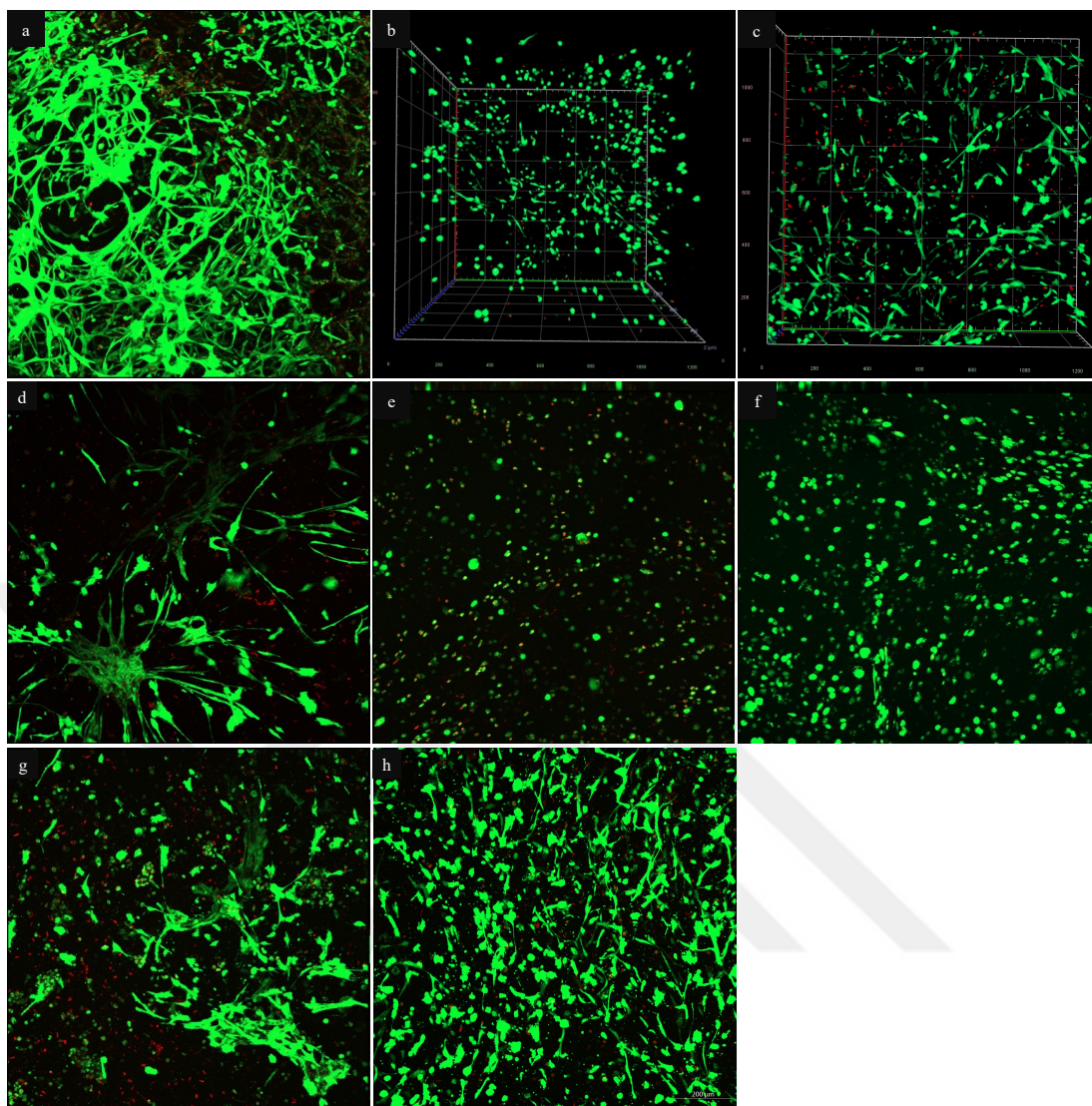


Figure 29. LSCM images of pericyte-like cells in (a) 3% Col, (b) 1.25% MeHA - 1.5% Col, (c) 0.75% MeHA - 2% Col, (d) %6 Col, (e) 1.5 % MeHA - 1.5% Col, (f) 1% MeHA - 2% Col, (g) 0.75% MeHA - 4% Col, (h) 0.75% MeHA - 4.5% Col IPN hydrogels after Live/Dead assay on 3th day of culture (green: viable cells, red: dead cells).

4.2.2 Characterization of the MeHA-Col hydrogel

In this study, a hyaluronic acid-based interpenetrating polymer network (IPN) hydrogel was successfully developed from MeHA and collagen to serve as the brain-side component of the proposed model. Tissue substitutes used in neural tissue engineering meet critical structural and functional requirements, which can be classified based on physical properties such as stiffness and degradation rate, as well as biological characteristics such as cell viability (27). The hydrogel composition was

optimized based on the spreading behavior of pericyte-like cells within the hydrogel, resulting in a final formulation of 0.75% MeHA-4.5% Col (w/v). This formulation was then further characterized in terms of morphology, chemical composition, degradation profile, mechanical properties, swelling degree, and the viability of three cell types.

4.2.2.1 Morphology of the hydrogel

Morphological characterization of the hydrogel was conducted using a scanning electron microscope (SEM). The images showed that the hydrogel exhibited an interconnected porous structure with an average pore size of $73.60 \pm 22.54 \mu\text{m}$, allowing cell penetration and growth, as this size is larger than that of most cell types ($5\text{--}15 \mu\text{m}$) (Figure 30). This structural feature aligns with previously reported porous hydrogels in the literature. For instance, Shi et al. (2022) developed an interconnected GelMA hydrogel with an average pore size of $168 \pm 71 \mu\text{m}$, which supported neural stem cell migration and differentiation (84). In another study, Shahidi et al. (2021) demonstrated that GelMA hydrogels with a median pore size of $10.3 \mu\text{m}$ and a maximum pore size of $1482.3 \mu\text{m}$ enhanced Schwann cell myelination (85). The hydrogel structure developed in this study may enhance its ability to support neuronal cultures by promoting nutrient diffusion, and maintaining cell viability and cell proliferation over extended culture periods.

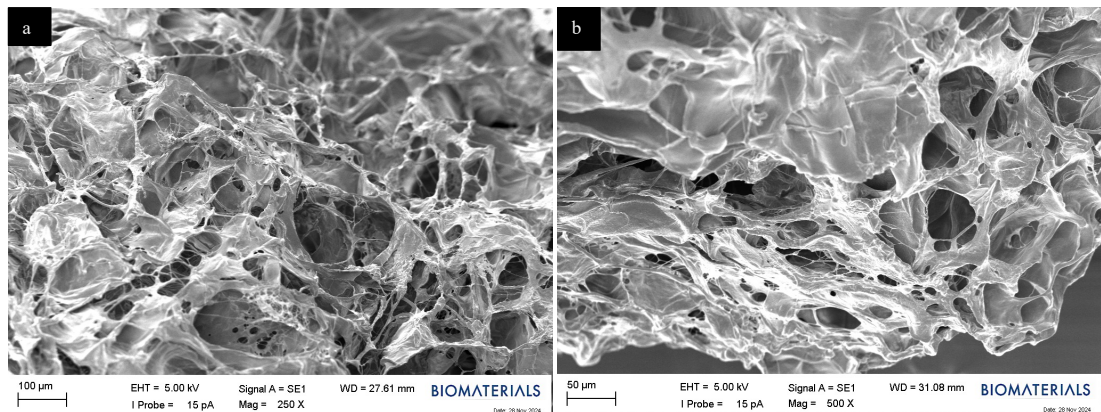


Figure 30. SEM images of the MeHA-Col-based hydrogel at (a) 250X and (b) 500X magnifications.

4.2.2.2 Chemical characterization of the hydrogel

Chemical composition of the hydrogel was shown with FTIR spectroscopy, confirming the effective integration of MeHA and collagen within the hydrogel matrix (Figure 31). The MeHA-Col based hydrogel exhibited a characteristic peak at a 1020 cm^{-1} , corresponding to the saccharide unit (86). Due to collagen does not carry saccharide units in its chemical structure, this peak specifically attributed to the MeHA. However, the Col-based hydrogel showed bands at 1632 cm^{-1} (amide I), 1547 cm^{-1} (amide II), 1236 cm^{-1} (amide III), 1449 cm^{-1} (pyrrolidine ring), which are characteristic peaks indicative of the collagen structure (87). In the FTIR spectrum of the MeHA-Col hydrogel showed that the amide I, amide II, and amide III bands from collagen along with the saccharide-related peak from MeHA, were present in the hybrid hydrogel, confirming the successful integration of MeHA and collagen.

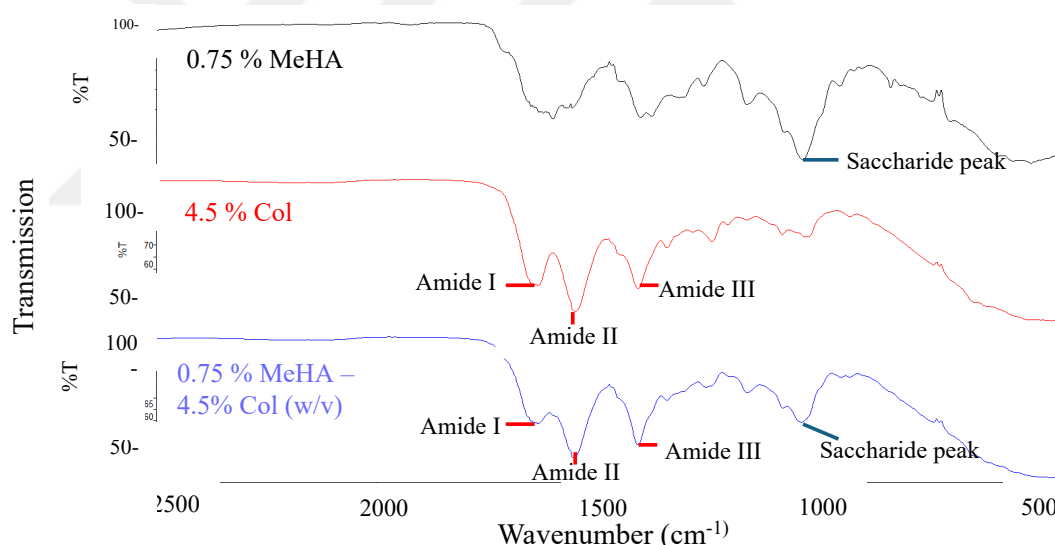


Figure 31. FTIR spectra of the MeHA-Col-based hydrogel.

4.2.2.3 Degradation profile of the hydrogel

Materials used for CNS *in vitro* modeling should possess a long-term degradation profile to permit remodeling in accordance with cell growth and morphogenesis. Lancaster et al. (2013) reported that human induced pluripotent stem cells require approximately 20-30 days to differentiate and self-organize into cerebral organoids (88). Therefore, materials used in CNS modeling must exhibit prolonged stability for

at least approximately 30 days. Designing a 3D microenvironment that supports long-term cell culture while maintaining mechanical integrity is crucial. The degradation profile of the MeHA-Col hydrogel showed that it lost approximately 20% of its initial weight within the first five days, after which the remaining weight remained largely stable throughout the experimental period (Figure 32). Similar to the degradation profile of collagen-integrated fibrous membranes (Figure 18), this behavior is most likely attributed to the rapid degradation of collagen in the hydrogel, followed by the slower, prolonged degradation of MeHA.

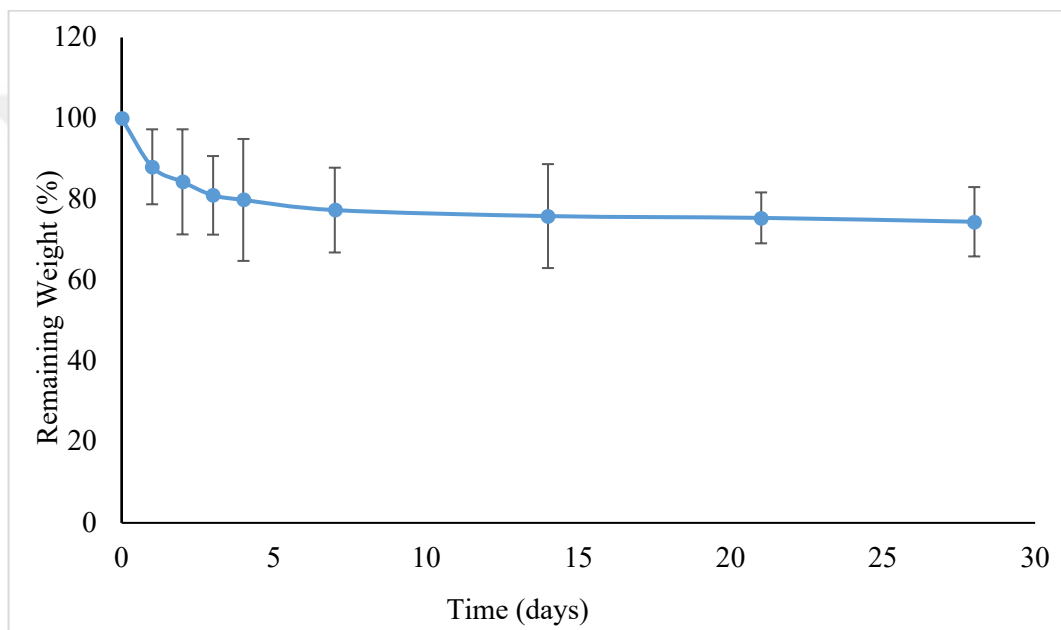


Figure 32. Degradation profile of the MeHA-Col-based hydrogel over time.

4.2.2.4 Mechanical properties of the hydrogel

In addition to the long-term degradation profile, the mechanical properties of the hydrogel are crucial to achieve CNS characteristics *in vitro*. A matrix with a Young's modulus of below 80 kPa is considered the most suitable candidate for neuronal culture (89). However, matrices based on natural materials undergo rapid degradation in an aqueous environment. Therefore, achieving both mechanical stability and controlled degradation remains a key challenge in CNS modeling. The Young's modulus of the MeHA-Col hydrogel was measured as 2.02 ± 0.24 kPa (Figure 33). These findings, together with the hydrogel's degradation profile, suggest that the MeHA-Col based

IPN hydrogel has the potential to address these challenges in CNS modeling and serve as a promising platform for drug toxicity assessment and *in vitro* 3D CNS disease modeling.

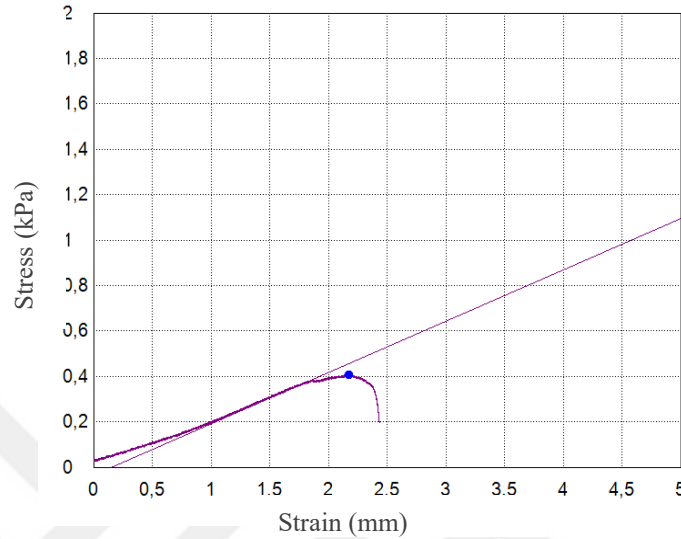


Figure 33. Stress-strain curve of the MeHA-Col based IPN hydrogel

4.2.2.5 Equilibrium swelling behavior of the hydrogel

The water-retention ability of hydrogels is an important parameter, as it directly affects their mechanical properties, cell behavior, and nutrient diffusion. Hydrogels with excessively high water-retention (>90%) can become too soft, leading to a loss of mechanical stability and structural integrity (90). Hydrogels with approximately 80% water-retention have been shown to support cell adhesion and proliferation while allowing sufficient oxygen and nutrient diffusion (91). To evaluate the water-retention ability of the hydrogels, the equilibrium swelling degrees of MeHA, MeHA-Col, and Col-based hydrogels were determined to be $100.14 \pm 12.12\%$, $83.53 \pm 11.34\%$, and $77.95 \pm 6.16\%$, respectively (Figure 34). The addition of collagen into the MeHA solution significantly reduced the swelling degree of the hydrogel ($p < 0.05$). This reduction in water retention may be attributed to the formation of an interpenetrating network hydrogel composed of MeHA and collagen. Such an organization enhances the mechanical stability of the hydrogel while maintaining a hydrated microenvironment conducive to cell culture. These findings suggest that the MeHA-Col-based hydrogel exhibits a favorable balance between swelling capacity and

structural integrity, making it a promising candidate for *in vitro* 3D CNS disease modeling applications.

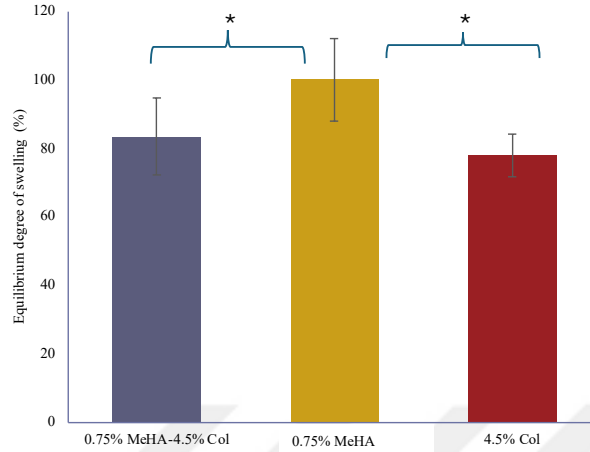


Figure 34. Equilibrium swelling degree (%) of MeHA-Col, MeHA, and Col-based hydrogels (* $p < 0.05$).

4.3 *In Vitro* Studies: Cells And Their Incorporation Into the Fibrous Membrane And Hydrogel

4.3.1. Characterization of endothelial cells

Endothelial cell-cell adhesion plays a crucial role in maintaining BBB integrity by enhancing monolayer stability and regulating selective permeability. The BBB's highly restrictive barrier function relies on a continuous endothelial layer with strong intercellular connections. CD31 mediates homophilic interactions between adjacent endothelial cells, improving monolayer integrity and supporting endothelial barrier function (92). In this study, CD31 expression was investigated to evaluate endothelial cell adhesion and monolayer formation. Immunocytochemistry results confirmed that HUVECs expressed CD31 at cell borders and formed a confluent endothelial monolayer on the tissue culture plate (Figure 35). This monolayer formation confirms the suitability of HUVECs as an endothelial cell source for barrier modeling.

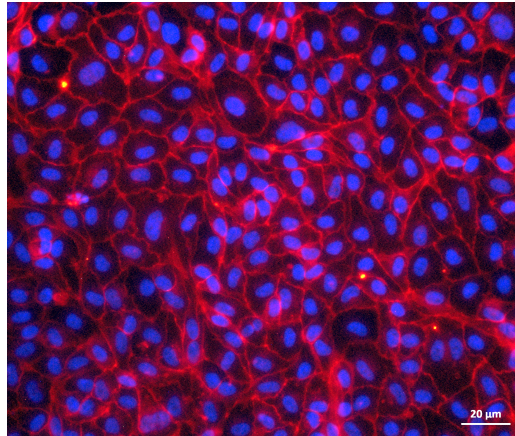


Figure 35. LSCM images of HUVECs on the tissue culture plate stained against CD31 (red) and counterstained with DAPI (blue) for nucleus.

4.3.2. Characterization of pericyte-like cells

Pericytes are the multifunctional, vascular mural cells embedded in basement membranes of small blood vessels, the capillaries (24). Pericytes at the blood–brain barrier (BBB) exhibit a characteristic “bump-on-a-log” morphology, defined by a prominent cell body with long, thin processes that extend along vessels to help regulate barrier permeability (93). They express α -SMA, which contributes to their contractile properties and distinctive morphology. LSCM images showed that pericyte-like WJ MSCs exhibited a spindle-like actin cytoskeletal organization with elongated cytoplasmic processes (Figure 36a,b). Immunocytochemistry results confirmed the expression of α -SMA, reinforcing their structural and functional resemblance to pericytes (Figure 36c,d).

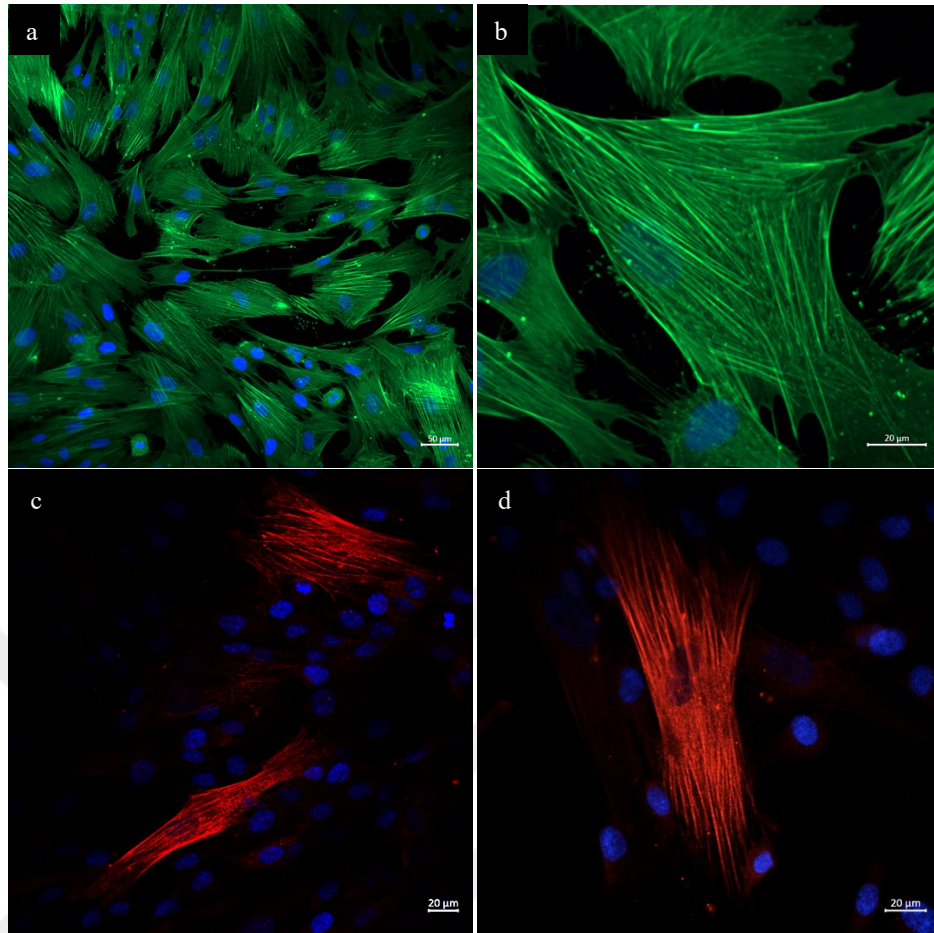


Figure 36. LSCM images of pericyte-like cells on tissue culture plate stained with (a,b) Phalloidin (green) for actin filaments, immunostained against (c,d) α -SMA (red) and counterstained with DAPI (blue) for nucleus.

4.3.3. Characterization of astrocytes

Astrocytes are the predominant glial cell type in the neurovascular unit (NVU). They simultaneously interact with both neurons and capillaries, serving as a cellular bridge to neuronal tissue and enabling versatile connections (17, 25). They have star-shaped morphology, which is characterized by multiple branched processes extending from their cell body. GFAP is an intermediate filament protein commonly used as an astrocyte marker (94). Its expression is generally increased in activated astrocytes, such as those cultured in 2D environments. In contrast, astrocytes cultured in biomimetic environments exhibit a highly branched morphology and lower GFAP expression, indicative of a quiescent state (95). Aquaporin-4 (AQP4) is highly expressed in astrocyte end-feet and plays a crucial role in endothelial cell-astrocyte

signaling. Its high expression serves as a functional marker of astrocyte-incorporated BBB models.

LSCM images showed that astrocytes exhibited filamentous actin network, forming extensive cell-cell interactions (Figure 37a). Individual astrocytes displayed a characteristic star-shaped morphology with multiple branched processes radiating from the cell body (Figure 37b). Immunocytochemistry results demonstrated that astrocytes expressed GFAP, which is an indicator of their active state when cultured in a 2D environment (Figure 37c). In addition, astrocytes exhibited a punctate distribution of AQP4 in their cytoplasm and along their cellular processes. This punctate distribution, observed as discrete spots, correlates with the known tendency of AQP4 to form clusters rather than being uniformly distributed (Figure 37d).

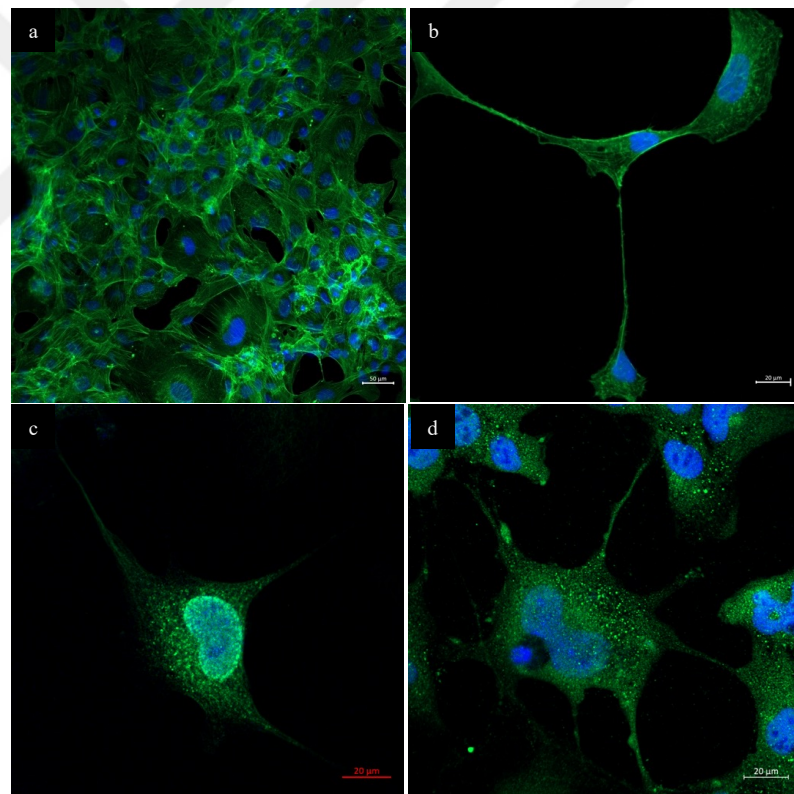


Figure 37. LSCM images of astrocytes on tissue culture plate stained with (a,b) Phalloidin (green) for actin filaments, immunostained against (c) GFAP (green) and (d) AQP-4 (green), and counterstained with DAPI (blue) for nucleus.

4.3.4. Characterization of neurons

Neurons are referred to as 'the pacemakers of the NVU' because they play a crucial role in homeostatic mechanisms through the dynamic nature of their neural activity (18, 25). In NVU models, the incorporation of neurons is essential for investigating neurovascular coupling, synaptic communication, and homeostatic mechanisms between the brain and blood sides. Neurons are highly specialized cells with a polarized morphology, characterized by small cell bodies and distinct neurites. β -Tubulin is a core component of microtubules and plays a crucial role in neuronal structure and neurite outgrowth. Specifically, β -Tubulin III is widely used as a neuronal marker, particularly in early stages of differentiation and neurite extension. Microtubule-associated proteins (MAPs), as their name suggests, stabilize microtubules in neurons.

LSCM results demonstrated that neurons formed an extensive interconnected network, establishing cell-cell interactions (Figure 38a,b). Immunocytochemistry results showed that cells expressed β -Tubulin III in their cytoplasm, confirming the early neuronal differentiation phase of SH-SY5Y cells (Figure 38c). Additionally, neurons expressed mature neuronal marker MAP-2, which was mainly localized in the cytoplasm and extended along neurite-like projections (Figure 38d).

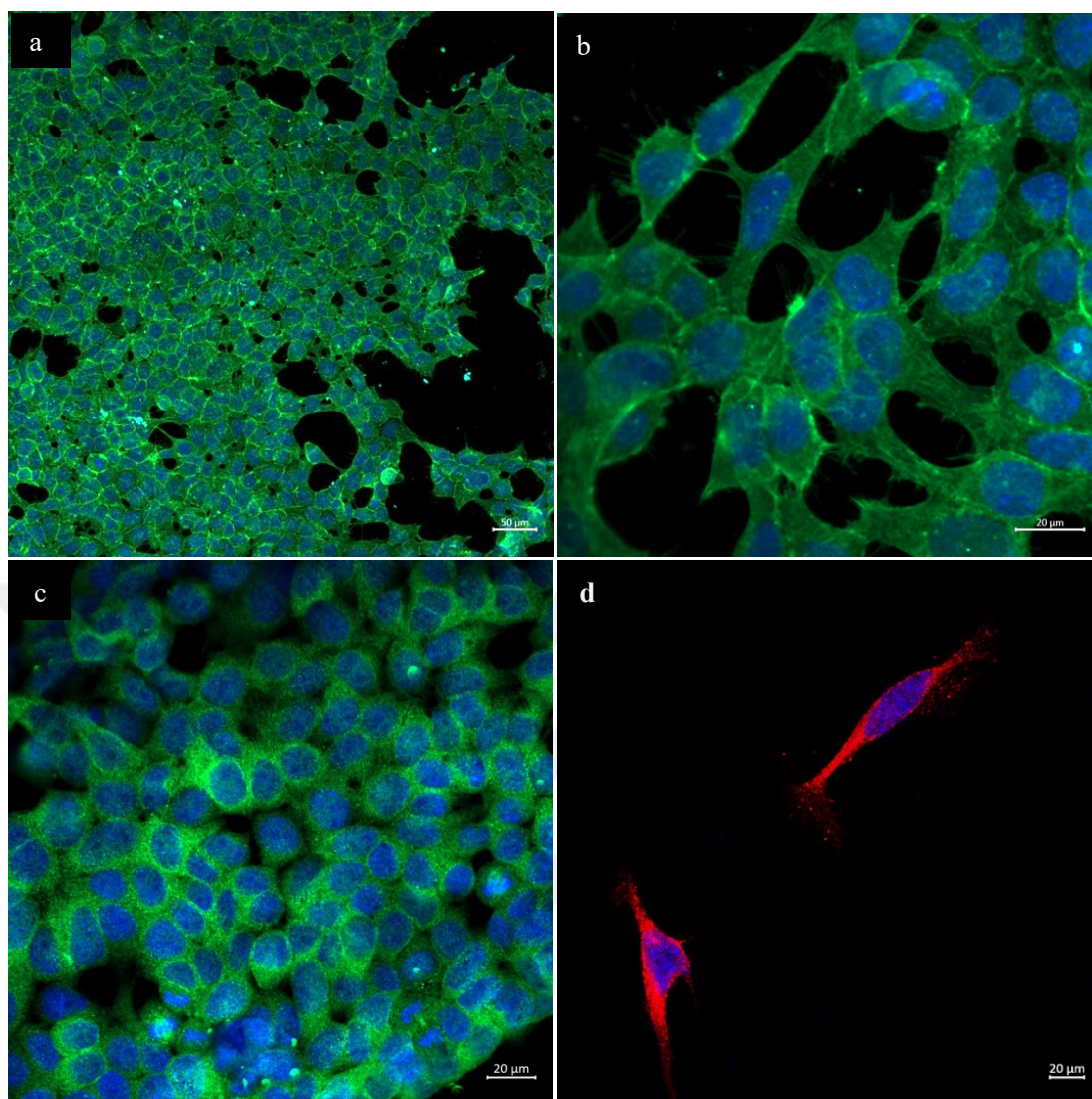


Figure 38. LSCM images of neurons on tissue culture plate stained with (a,b) Phalloidin (green) for actin filaments, immunostained against (c) β -Tubulin III (green) and (d) MAP-2 (red) , and counterstained with DAPI (blue) for nucleus.

4.3.5. Cell behaviour on the fibrous membrane and in the hydrogel

4.3.5.1. Cell viability and confluency on the fibrous membrane

The fibrous membranes were coated with fibronectin, gelatin and a mixture of fibronectin and gelatin, and then ECs' viability and distribution on these membranes were assessed using a Live/Dead assay. The results showed that the cells on the fibronectin coated PCL and PCL-Col membranes were dense and form uniform cell layer, covering the surface of the membrane (Fig 39a). The highest cell confluency was achieved on the fibronectin-coated PCL membranes at $78.13 \pm 10.72\%$, whereas

the fibronectin-coated PCL-Col membranes showed a confluency of $40.54 \pm 2.99\%$ (Fig 39a,b,g). In contrast, cell confluency on gelatin-coated PCL and PCL-Col membranes were 10.60 ± 2.52 and $3.18 \pm 0.78 \%$, respectively (Figure 39 c,d,g). It was observed that the cells exhibited low levels of viability and attachment and could not cover the surface of the gelatin-coated membrane, comparing to the cells on the membranes coated with fibronectin or with the fibronectin-gelatin mixture. On the fibronectin-gelatin mixture coated membranes, cell confluency was moderate at $40.54 \pm 2.99 \%$ on PCL membrane, and was low on PCL-Col based membranes as $6.36 \pm 0.90\%$ (Figure 39e,f,g). This could be due to the gelatin coating, which may further reduce surface hydrophobicity, making the membrane less suitable for cell attachment. These results indicated that gelatin did not support cell attachment as effectively as fibronectin on either membrane type, and cell confluency was lower on collagen-incorporated membranes under all coating conditions. Consequently, it was decided to proceed with fibronectin-coated PCL membranes for the further studies.

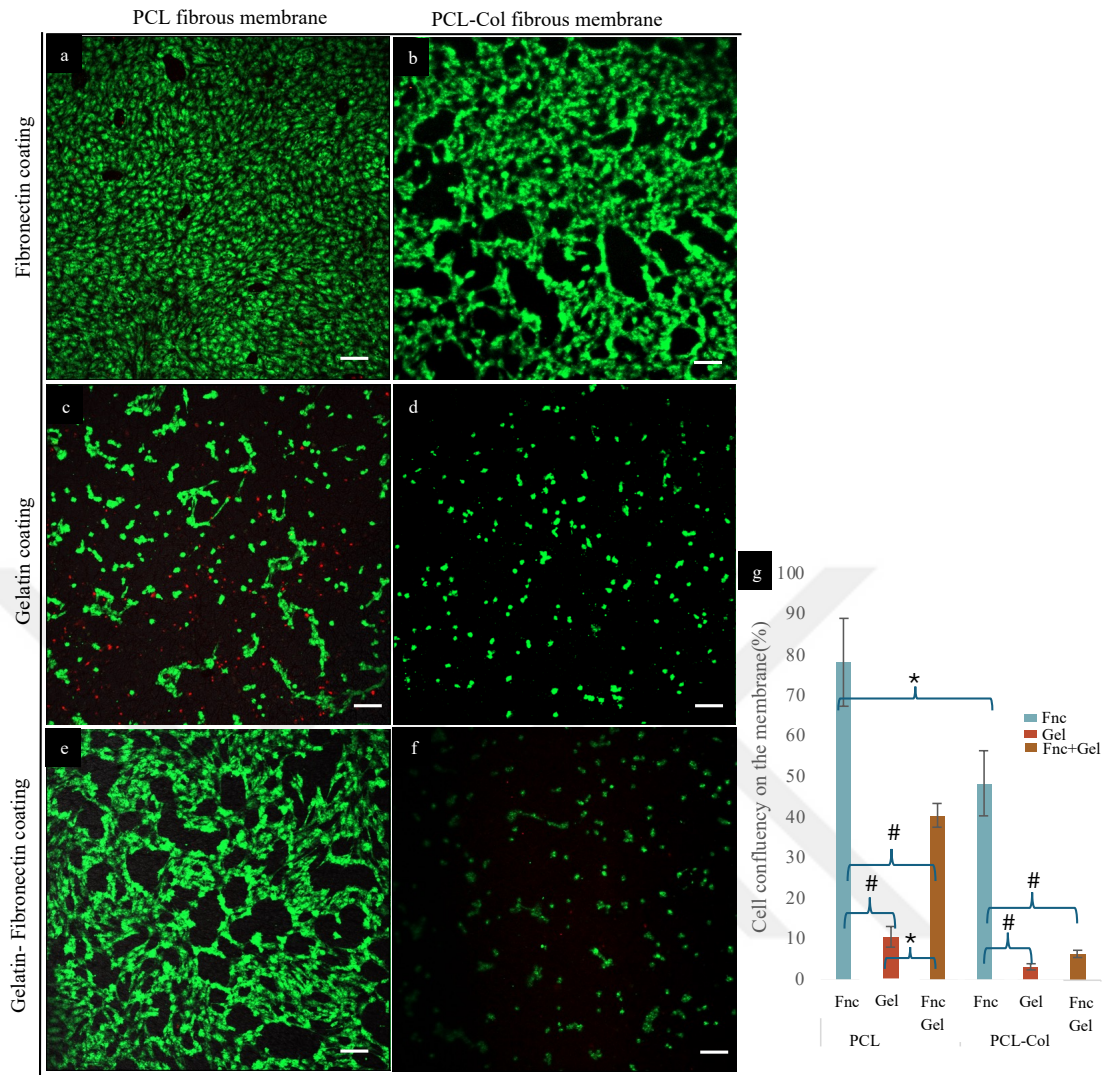


Figure 39. LSCM images of ECs on (a,c,e) PCL and (b,d,f) PCL-Col fibrous membranes coated with (a,b) fibronectin, (c,d) gelatin and (e,f) a mixture of fibronectin-gelatin after Live/Dead assay (viable cells: green, dead cells: red) (scale bar: 100 μ m). (g) Quantification of cell confluency (area coverage) on the membranes (* $p < 0.05$, # $p < 0.001$).

4.3.5.2. Cell viability and distribution in the hydrogel

The *in vitro* characterization of the optimized hydrogel composition (0.75%–4.5% MeHA-Col, w/w) was performed using a Live/Dead assay to assess cell viability (Figure 40). CLSM images demonstrated the successful incorporation of cells into the hydrogel matrix (Figure 40 a,c,e). On day 3 of culture, cell viability was calculated as 84.40%, 90.27%, and 97.27% for pericyte-like cells, astrocytes, and neurons, respectively, with observable spreading of pericyte-like cells and astrocytes (Figure

40g). By day 7, enhanced spreading was evident across all cell types, indicating improved cellular interaction, adaptation, and compatibility with the hydrogel environment (Figure 40 b,d,f). Moreover, cell viability increased to 96.57%, 92.97%, and 97.13% for pericyte-like cells, astrocytes, and neurons, respectively (Figure 40g).

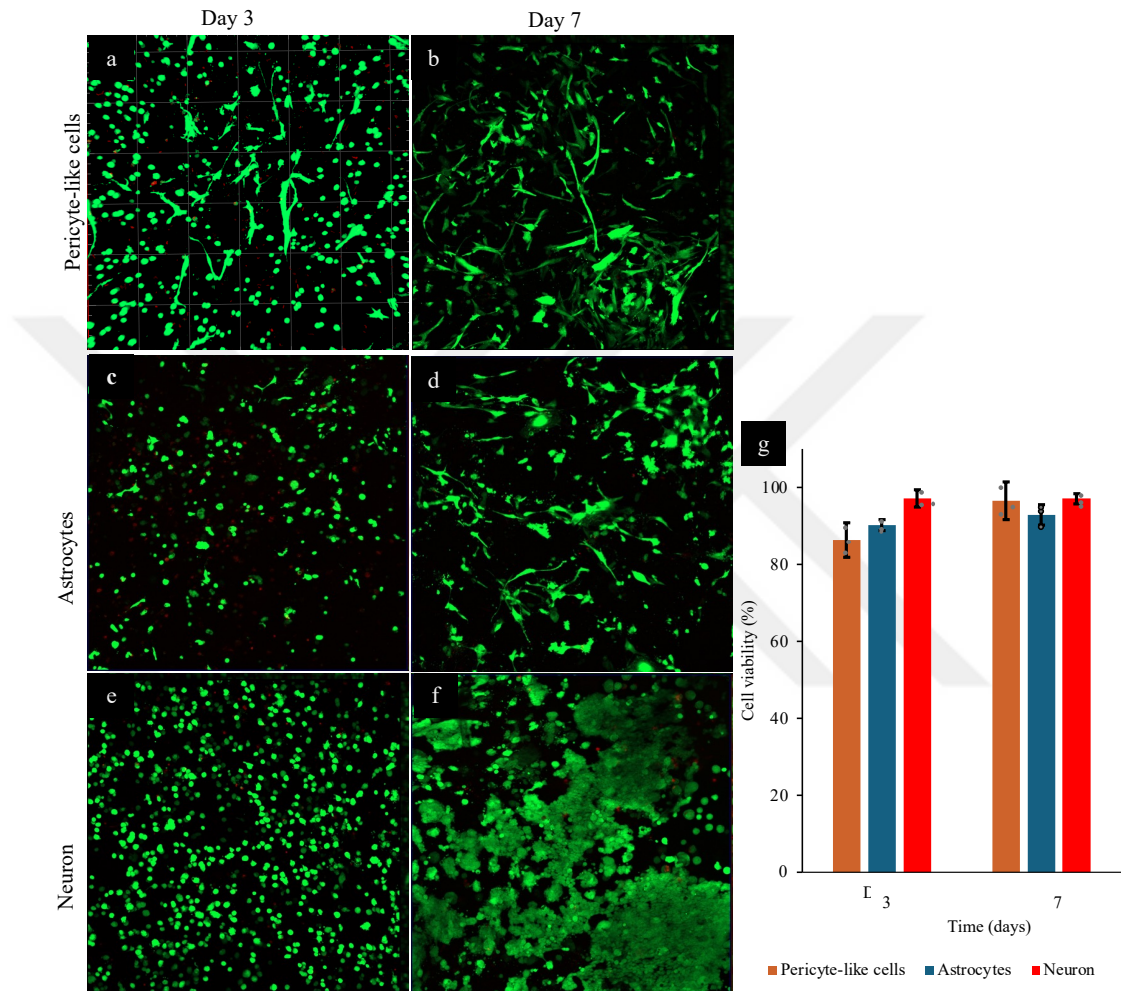


Figure 40. LSCM images of (a,b) pericyte-like-cells, (c,d) astrocytes and (e,f) neurons (viable cells: green, dead cells: red) (scale bar: 100 μm). (g) Quantification of cell viability (%) in the hydrogel.

4.4. Establishment Of The Multicellular NVU Model

4.4.1. Assembling the NVU model

The NVU model was established using the custom-designed 3D-printed system to evaluate the integration of its components prior to *in vitro* studies. Additionally, hydrogels of varying thicknesses were integrated onto the opposite side of the endothelial cell-seeded membrane to assess their influence on the EC monolayer formation, a critical factor for the barrier function of the model. The custom-designed 3D-printed system was composed of two parts: the main body part that holds fibrous membrane, and a ring to secure the membrane (Figure 41a). While establishing the NVU model, fibrous membrane was first placed and fixed into the system, followed by addition of the MeHA-Col based hydrogel solution over the fibrous membrane on the ring side of the system. Gelation of the hydrogel solution was then achieved through sequential crosslinking: UV and temperature crosslinking for MeHA and collagen, respectively (Figure 41b). Stereomicroscope images showed that the hydrogel formed over the fibrous membrane on the ring side of the system and remained in place even when inverted, which is the position during EC seeding and cell culture (Figure 41c). SEM image demonstrated the successful integration of the hydrogel solution, forming a continuous hydrogel layer on the basolateral side of the membrane while preventing penetration into the apical side (Figure 41d). This effective integration was attributed to the nanoscale fibrous nature of the membrane, which facilitated hydrogel adhesion. Moreover, both components retained their structural integrity within the custom-designed 3D system, preserving their distinct morphologies, the fibrous architecture of the membrane and the porous network of the hydrogel.

Mechanical properties of the surface affect the mechanical tension generated at the focal adhesions (FA) of endothelial cells, which spreads through actin cytoskeletal organization and influences cell-cell interactions (2). For example, ECs on the softer surface (in the kPa range) showed increased cadherin expression compared to those on stiffer surfaces (in the GPa range) (74). This tension extends to adherens junctions, helping to maintain endothelial monolayer integrity and thereby supporting barrier function. Considering the potential effect of hydrogel thickness on the mechanical

properties of the membrane, hydrogels of varying thicknesses were applied to assess their influence on endothelial cell monolayer formation over the membrane. The hydrogel thicknesses were adjusted by modifying their volumes and were set to 2.5 mm, 5 mm, and 10 mm. As the hydrogel thickness increased, it made direct contact with the bottom of the well plate, increasing pressure on the fibrous membrane (Figure 41f-i). CD31 immunostaining showed that hydrogel thickness, by inducing mechanical forces, influenced endothelial monolayer formation. ECs on the membranes without hydrogel integration adhered well and formed a continuous monolayer on the fibrous membrane by day 3 (Figure 41f). Similarly, ECs on the 2.5 mm hydrogel-incorporated membrane, where the hydrogel did not induce mechanical compression, adhered well and formed a continuous monolayer on the membrane (Figure 41g). However, on the 5 mm hydrogel-incorporated membrane, where mechanical stress might arise, monolayer disruption was observed (Figure 41h). On the 10 mm hydrogel-incorporated membrane, which might be under the highest mechanical stress, EC monolayer integrity was further compromised, and cell detachments from the membrane were observed (Figure 41i).

These preliminary results highlighted the potential applications of the model and emphasized that hydrogel thickness, by inducing a potential mechanical compression, negatively impacted endothelial cell adhesion and disrupted or prevented continuous monolayer formation, a key benchmark of *in vitro* barrier models. Therefore, this factor should be carefully considered in the design of biomimetic models.

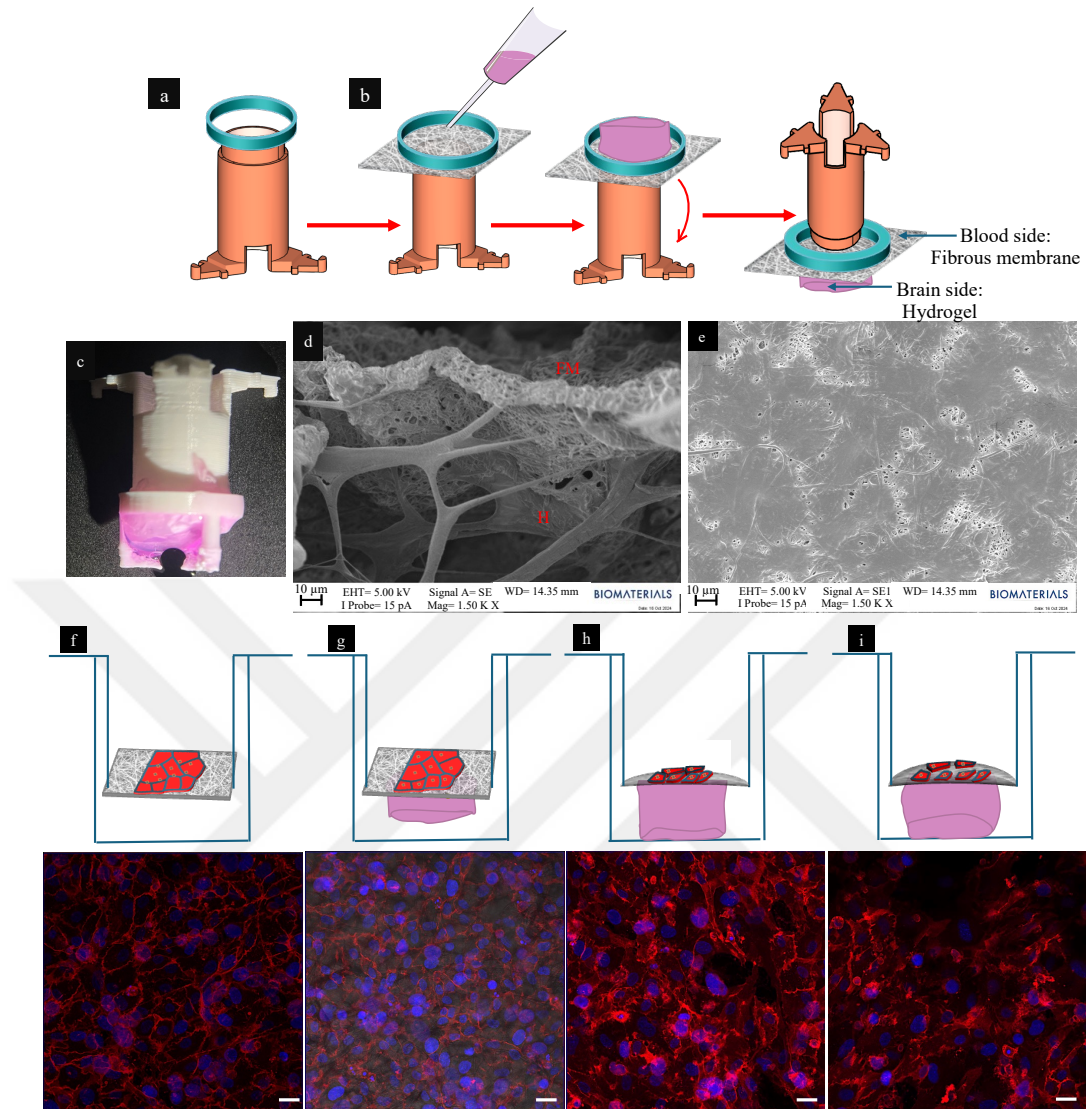


Figure 41. Establishment of the BBB model. (a) Schematic presentation of the custom-designed 3D-printed system, including the main body part and the securing ring. (b) Stepwise establishment of the BBB model. (c) Stereomicrograph of the model. SEM images of (d) the BBB model composed of the fibrous membrane (FM) and hydrogel (H), and (e) ECs on the fibrous membrane. (f-i) CD31 expression (red) of ECs on the membrane: (f) without hydrogel integration, integrated with a (g) 2.5 mm, (h) 5 mm, and (i) 10 mm hydrogel. (H; hydrogel, FM; fibrous membrane) (scale bar: 20 μm)

4.4.2. Cell behaviour in the multicellular NVU model

An initial hybrid multicellular NVU model was successfully constructed using the custom-designed 3D-printed system by sequential addition of two distinct cell-loaded hydrogel solutions onto the fibrous membrane, one containing astrocytes and pericyte-like cells, and the other containing neurons, and hydrogel was formed to mimic the brain side of the BBB (Figure 42a,b). This configuration enabled the spatial organization of neural and vascular cell types within a simplified 3D architecture, allowing the assessment of cell viability, morphology, and early cell-cell interactions. ECs were subsequently seeded on the opposite side of the fibrous membrane to constitute the apical side of the model. (Figure 42c). Endothelial cell-cell adhesion is a key feature of BBB integrity, contributing to monolayer stability and highly selective permeability. The critical barrier function of the BBB is characterized by the formation of a continuous endothelial monolayer with strong intercellular interactions. CD31 mediates homophilic interactions between adjacent endothelial cells, improving monolayer integrity and supporting endothelial barrier function (92). In this study, CD31 expression was examined to evaluate endothelial cell adhesion and monolayer formation, providing complementary insight into barrier function. LSCM imaging with Z-stack confirmed that ECs formed a continuous monolayer on the nanofibrous membrane surface in both monoculture and co-culture conditions (Figure 42d). These results suggest that the nanoscale fibrous architecture of the membrane, with mechanical properties in the kPa range, may provide a biomimetic microenvironment conducive to endothelial adhesion and continuous monolayer formation. This finding is consistent with previous reports emphasizing the role of nanoscale topography and substrate stiffness in regulating endothelial behavior (74, 96). LSCM results showed that ECs successfully adhered to the nanofibrous membrane and formed a monolayer by day 7 in both monoculture and multicellular models (Figure 42e-i). However, on day 7, the immunostained CD31 fluorescence intensity per cell was significantly higher in the multicellular group than in the monoculture group (Figure 42e,i,h) ($p < 0.05$), suggesting enhanced endothelial cell-cell adhesion in the presence of supporting cells. Similarly, on day 14, CD31 expression intensity was higher in the multicellular group compared to the monoculture group. Moreover, morphological differences were observed among the groups. In the monoculture model, ECs

maintained a monolayer structure, showing a significant increase in CD31 expression relative to day 7 ($p < 0.05$) (Figure 42 e,f,h). In the multicellular model, ECs exhibited a denser and more compact arrangement by day 14, in the multicellular model, ECs exhibited a denser and more compact arrangement by day 14 (Figure 42j), which was accompanied by a significant increase in CD31 signal intensity per cell compared to day 7 ($p < 0.05$) (Figure 42 j,h). The elevated CD31 expression in the multicellular model suggests enhanced endothelial cell-cell adhesion, which may contribute to stronger intercellular junctions and improved monolayer integrity.

SEM analysis demonstrated distinct variations in surface coverage across the groups. In the monoculture model, ECs displayed a well-defined arrangement of cells with visible gaps on the fibrous membrane (Figure 42g). However, in the multicellular model, a more continuous endothelial monolayer was formed with fewer exposed areas on the membrane at day 14 (Figure 42k). These findings suggest that the presence of additional related cell types within the biomimetic hydrogel in the multicellular model may enhance EC adhesion and monolayer stability.

The brain side of the multicellular NVU model consisted of astrocytes, pericyte-like cells, and neurons loaded in a MeHA-Col hydrogel. LSCM imaging confirmed that the cells successfully integrated into the 3D microenvironment of the model, exhibiting a star-shaped morphology characteristic of astrocyte and a fibroblastic morphology for pericyte-like cell (Figure 42k). The images further demonstrated that cells maintained their immunophenotypic identity within the hydrogel, even after co-culture with multiple cell types, as confirmed by AQP-4 expression in astrocytes and α -SMA expression in pericyte-like cells (Figure 42l-o).

AQP-4 expression is associated with EC-astrocyte signaling and is a key hallmark of astrocyte-integrated models (94). The expression of AQP-4 in astrocytes within our hydrogel might indicate that indirect EC-astrocyte interactions were established in the model (Figure 42l-o). Similarly, α -SMA is a well-known marker of pericytes and is associated with vascular stability. The expression of α -SMA in pericyte-like cells further supports the physiological relevance of the model. These results suggest that the hydrogel-based microenvironment not only supports cellular viability and phenotypic maintenance but also facilitates critical cell-cell interactions necessary for

barrier functionality. LSCM imaging demonstrated that the hybrid model successfully enabled multicellular culture of brain-side cells with communication, while the fibrous membrane maintained a physical separation between ECs and the brain-side compartment, allowing for indirect signaling between these two regions. Notably, imaging findings indicated that CD31 expression in ECs was enhanced in the multicellular model compared to the monoculture model. This improvement may be attributed to the presence of a native-like brain-side environment, where paracrine signals from cells within the hydrogel contribute to endothelial function and barrier integrity. The hybrid model's structural organization demonstrates its promise as a physiologically relevant platform for investigating neurovascular interactions, BBB development, and disease-associated changes, reinforcing its value for translational research.

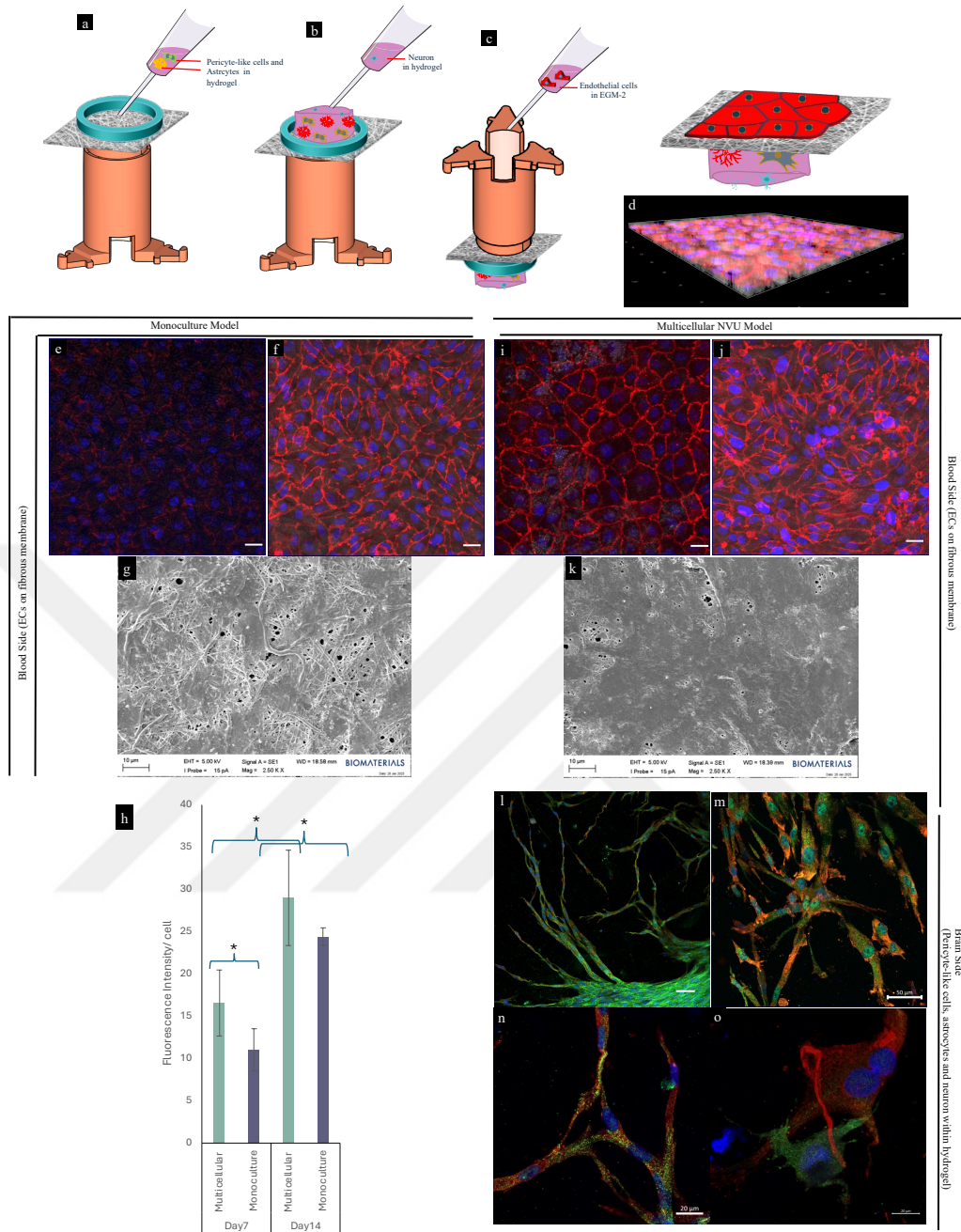


Figure 42. Schematic presentation of sequential integration of (a) astrocytes and pericyte-like cell loaded hydrogel and (b) neuron loaded MeHA-Col hydrogels on the fibrous membrane, (c) seeding of ECs on the fibrous membrane. C31 expression (red) of ECs on the apical side of the the monoculture and multicellular models at (e,i) day 7 and (f,j) day 14 SEM images of ECs on the fibrous membrane of the (g) monoculture and (k) multicellular model on day 14. (h) Quantitative analysis of CD31 intensity/cell ($* < 0.05$). (l-o) Immunostaining of astrocytes against AQP-4 (green), pericyte-like cell against α -SMA (red), and neurons (green) in MeHA-Col hydrogel at day 14 (green).

4.4.3. Functionality of blood brain barrier in the tissue engineered NVU model

Besides the expression of specific endothelial cell markers, the barrier function of a model is also associated with transendothelial electrical resistance (TEER) and restricted permeability (19). TEER measurements were performed over time to evaluate barrier functionality in the monoculture and the multicellular models. Both culture models exhibited a similar trend, with TEER values rising during the first seven days and reaching a peak at day 7. The maximum TEER values were $166.86 \pm 5.75 \Omega \cdot \text{cm}^2$ and $121.70 \pm 13.58 \Omega \cdot \text{cm}^2$ for the multicellular and the monoculture models, respectively (Figure 43a). However, TEER values were significantly higher in the multicellular model than in the monoculture model at all time points, except on day 7 ($p < 0.05$). Additionally, whereas the monoculture model showed a sharp decline in TEER after day 10, the multicellular model maintained a more stable TEER profile, indicating enhanced barrier stability over time.

Permeability to 0.4 kDa fluorescein and 20 kDa dextran was measured on days 14 and 21 as an indicator of barrier integrity (Figure 43b). The permeability coefficient of the multicellular model for 20 kDa dextran was $8.14 \pm 6.18 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ and $8.85 \pm 6.25 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on days 14 and 21, respectively. In contrast, the permeability coefficient of the monoculture model for 20 kDa dextran was significantly higher as $69.80 \pm 12.40 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ and $118.66 \pm 69.80 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on days 14 and 21, respectively. Similarly, the permeability coefficient for 0.4 kDa fluorescein in the multicellular model was $301.64 \pm 95.68 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on day 14 and $251.87 \pm 103.83 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on day 21. The monoculture model exhibited significantly higher permeability values of $588.49 \pm 147.93 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ and $671.67 \pm 276.88 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on days 14 and 21, respectively ($p < 0.05$). The multicellular model demonstrated significantly lower permeability to both tracers compared to the monoculture model at both time points, which was consistent with the imaging results (Figure 43b). Moreover, no significant change in permeability was observed between day 14 and day 21, suggesting that the barrier function was fully established by day 14 and remained stable thereafter. This sustained integrity is likely supported by the long-term stability of the fibrous membrane and hydrogel components, which provided a consistent microenvironment for both the endothelial monolayer and brain-side cells.

The development of physiologically relevant *in vitro* models with high translational predictability is crucial, especially for drug screening studies, as they enable better prediction of human responses, reduce costs and reliance on animal studies, and accelerate drug development (97). Therefore, the clinical relevance of our tissue-engineered, physiologically relevant multicellular NVU model was validated by evaluating the permeability of ceftriaxone and amikacin. Ceftriaxone is an antibiotic that exhibits limited BBB penetration under normal physiological conditions but can cross the barrier more effectively in pathological states such as meningitis, where increased permeability allows for higher drug accumulation (98). In contrast, the penetration of amikacin into the brain is highly restricted. The permeability of the multicellular model at day 14 to ceftriaxone and amikacin was investigated and measured as $42.7 \pm 2.61 \times 10^{-8}$ and $1.41 \pm 1.55 \times 10^{-8} \text{ cm}\cdot\text{s}^{-1}$, respectively (Figure 43c). The permeability of ceftriaxone was significantly higher than that of amikacin, reflecting a trend that is observed under normal physiological conditions. These findings demonstrate the potential of our physiologically representative model for pharmacological research, particularly for studying CNS drug transport and therapeutic applications.

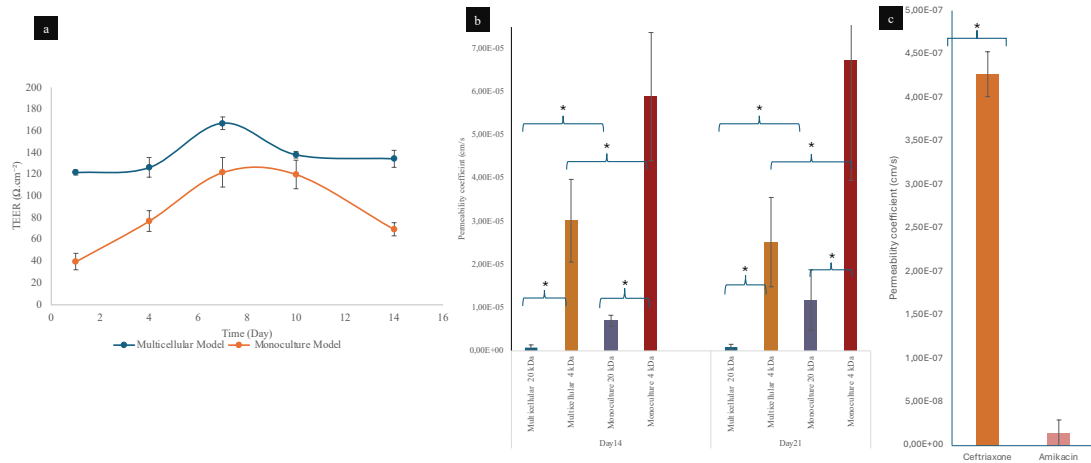


Figure 43. Evaluation of barrier function in the NVU model. (a) TEER value vs Time graph and (b) permeability coefficients to 0.4 kDa fluorescein and 20 kDa dextran in the monoculture and multicellular models. (c) Permeability coefficients to ceftriaxone (Mw 554.6 Da) and amikacin (Mw 585.6 Da) in the multicellular model on day 14 of culture

4.5. Establishment Of The NVU-On-A-Chip Model

4.5.1. Morphological analysis of the NVU-on-a-chip model

Two distinct microfluidic NVU-on-chip models, a basic model and a complex branched model, integrated with the hybrid multicellular NVU model developed on the custom-designed 3D-printed platform, were successfully fabricated to allow dynamic perfusion and precise compartmentalization (Figure 44 and 45). This advanced configuration aimed to more closely mimic the tubular and branched geometry of capillaries while enhancing barrier integrity and neurovascular interactions under controlled flow conditions. ECs were seeded on the luminal side of the branched, tubular fibrous membrane, while two distinct hydrogel solutions, one containing neurons and the other containing astrocytes and pericytes, were sequentially introduced around the membrane within the chamber to reconstruct the multicellular neurovascular microenvironment cultured for 14 days. (Figure 44a and Figure 45a). SEM images showed that the hydrogels were successfully integrated around the outer surface of the tubular, fibrous membrane without obstructing the lumen (Figure 44b,c and 45b,c). Both the membrane and surrounding hydrogel structures remained intact over the 14-day culture period, preserving the fibrous morphology of the membrane and the porous architecture of the hydrogel, thereby demonstrating structural stability under long-term perfusion conditions (Figure 44d, 45b). A hydrogel network with numerous interconnected, thin, filamentous structures was observed under SEM at high magnifications (Figure 44d,e,f and 45d,e,f).

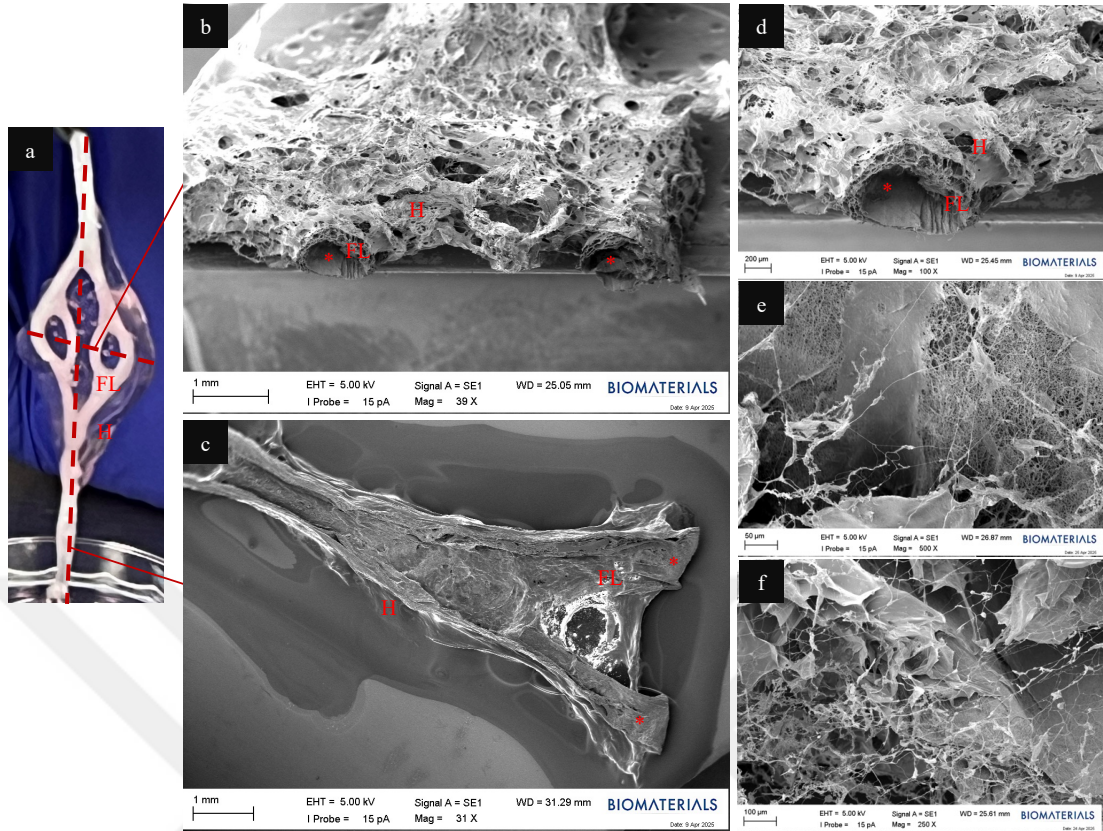


Figure 44. Basic NVU-on-a-chip model. (a) Photograph of the fabricated model. SEM images showing (b) vertical and (c) horizontal cross-sectional views of the chip. (d,e,f) Magnified images of the hydrogel matrix including the fibrous tubular membrane (FL; fibrous membrane, H; hydrogel, * lumen of the tubular fibrous membrane).

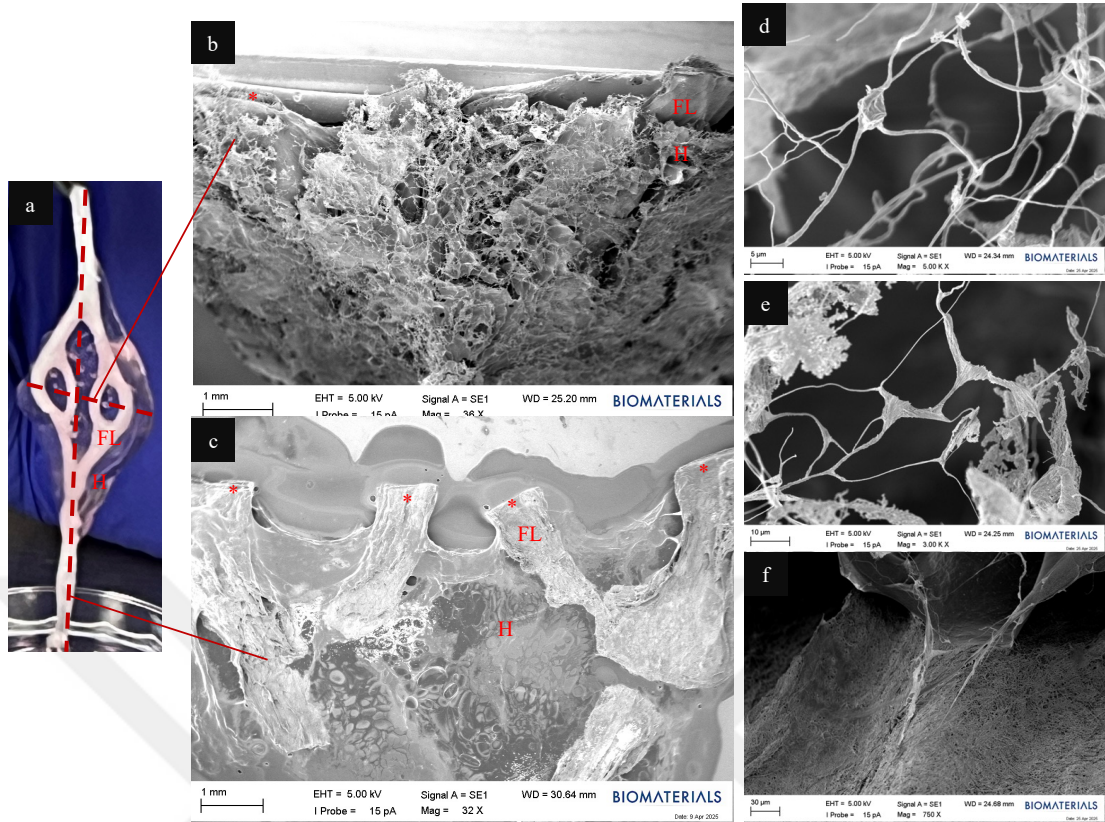


Figure 45. Complex NVU-on-chip model. (a) Photograph of the fabricated model. SEM images showing (b) vertical and (c) horizontal cross-sectional views of the chip. (d,e,f) Magnified images of the hydrogel matrix showing its fibrous network and membrane–hydrogel interface. (FL; fibrous membrane, H; hydrogel)

4.5.2. Cell behavior in the multicellular NVU-on-a-chip model

The viability and behavior of cells within the NVU-on-a-chip model were evaluated using live/dead assay and immunostaining methods, following establishment of a multicellular culture under dynamic perfusion conditions. Live/dead assay results demonstrated that cells were viable and widely distributed throughout the hydrogel compartments of the NVU-on-a-chip model (Figure 46). Viable cells were present in both the basic and complex NVU-on-a-chip models on day 3 and day 7, demonstrating sustained cell survival throughout the culture period (Figure 46a–d)

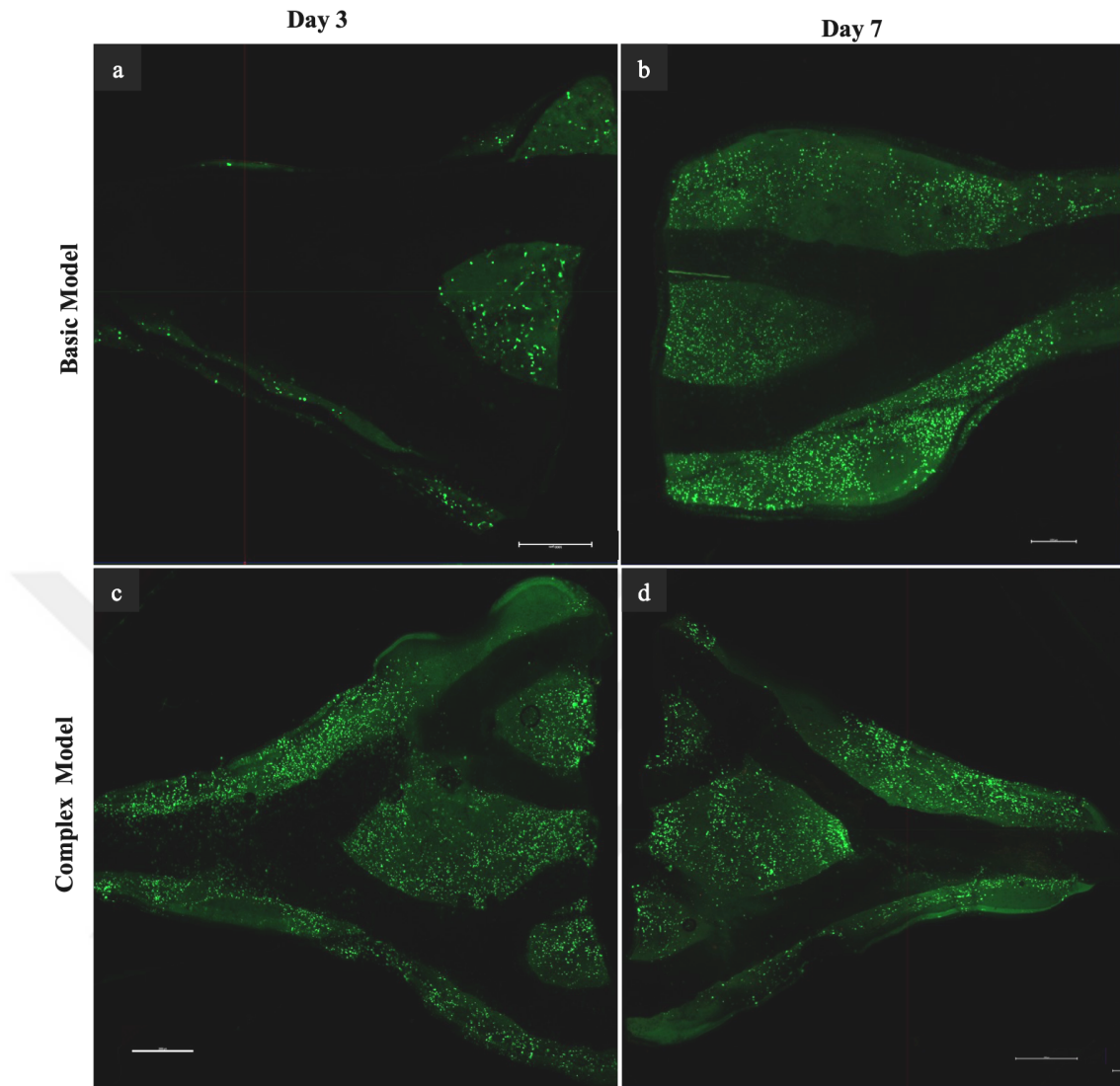


Figure 46. LSCM images showing the results of the Live/Dead assay.. Pericytes-like cells, astrocytes and neurons loaded within the hydrogel surrounding the tubular, fibrous membrane of the (a, b) basic and (c, d) complex NVU-on-a-chip models. (Scale bar: 1000 μm)

LSCM with tile-scanned immunofluorescence imaging allowed visualization of the full microfluidic geometry, and magnified images facilitated detailed evaluation of compartment-specific organization and matrix–cell interactions (Figure 47). The wide-field views showed the large-scale spatial distribution of cells within the MeHA-Col hydrogel compartments in both basic and complex NVU-on-a-chip models (Figure 47a,b). To better visualize the branched tubular fibrous membrane, a set of samples was cryosectioned. CD31 expression indicated that ECs successfully adhered to the luminal side of the fibrous membrane and formed a monolayer under perfusion-

integrated co-culture conditions in the NVU-on-a-chip models, particularly in the basic model (Figure 47c). Additionally, the successful integration and immunotypic maintenance of astrocytes, pericyte-like cells, and neurons were seen within the both basic and complex NVU-on-chip models (Figure 47d,e,f). The expressions of AQP-4 by astrocytes, α -SMA by pericyte-like cells, and MAP-2 by neurons showed that the cells preserved their immunophenotypic identity in the NVU-on-chip models under perfusion and co-culture conditions. Moreover, independently stained samples were employed to visualize clearly the marker expression and morphology of each cell type following the co-culture. It was observed that astrocytes expressing AQP-4 exhibited their characteristic star-shaped morphology (Figure 47g), pericyte-like cells expressed α -SMA (figure 47h) and displayed a fibroblastic appearance, and neurons expressing MAP-2 extended neurite-like processes from their cell bodies (Figure 47 i).

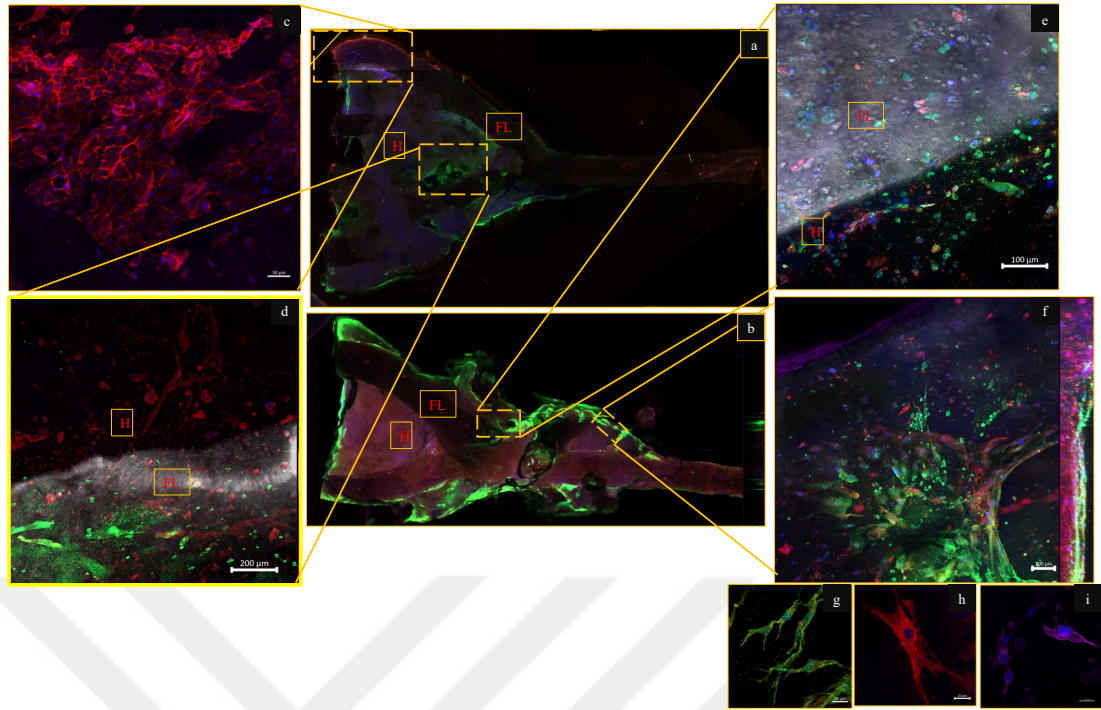


Figure 47. LSCM images of the basic and complex NVU-on-a-chip models after immunocytochemistry. (a,b) Tile-scanned images of the cells, (c) CD31 expression (red) of ECs on the lumen side of the model, (d,e,f) AQP-4 expression (green) of astrocytes, α -SMA expression (red) of pericyte-like cells, and MAP-2 expression (magenta) of neurons (grey region). Representative images of (g) AQP-4 expression (green) of astrocytes, (h) α -SMA expression (red) of pericyte-like cells (red) and (i) MAP-2 expression (magenta) of neurons from the samples stained individually ((FL; fibrous lumen, H; hydrogel)).

4.5.3. Barrier function of the NVU-on-a-chip model

The barrier function of both the basic and complex NVU-on-a-chip models, with specific tubular designs and integrated perfusion system, was evaluated with permeability studies and compared to a static, hybrid NVU model established on a custom-designed 3D-printed system, in which ECs were cultured on a flat fibrous membrane. The permeability coefficient of the basic and complex models was calculated as $3.18 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ and $0.48 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$, respectively. The lower permeability value of the complex model can be partly attributed to its larger effective surface area, which directly influences the permeability calculation. The branched architecture offers increased curvature and additional surface features that may support tighter endothelial organization and improved barrier integrity. However, both the basic and complex chip models exhibited lower permeability to 0.4 kDa fluorescein

compared to the NVU model established on the 3D-printed system. These results may indicate that the incorporation of a tubular design and perfusion integration enhances the barrier function of the NVU-on-a-chip models.



5 DISCUSSION

This study describes the establishment of a tissue-engineered, physiologically relevant multicellular NVU model that integrates key characteristics of both the blood-side and brain-side of the barrier. By reconstructing essential structural components of the native BBB, this model aimed to achieve an enhanced barrier function and a better recapitulation of its physiological properties. Traditionally, blood-brain barrier (BBB) and neurovascular unit (NVU) studies have relied on animal models, which are often considered as the gold standard due to their ability to provide the native microenvironment of the BBB and incorporate immune system interactions. However, approximately 80% of drugs that demonstrate efficacy in animal models fail in human trials, primarily due to differences in cellular and molecular composition, as well as genetic variations between species. To overcome these limitations, there is a critical need for an accurately replicated BBB/NVU models that can be employed in various applications, including CNS disease modeling, high-throughput drug screening, and neurotoxicity testing. Considering this, we developed a 3D *in vitro* multicellular NVU model designed to more effectively recapitulate the heterogeneous tissue architecture of both the blood and brain sides of the native barrier.

The blood side of the model consists of a branched, tubular fibrous membrane fabricated via electrospinning to mimic the intrinsic complexity of the vascular basement membrane. The nanoscale fibrous architecture of the membrane was designed to mimic continuous, non-fenestrated capillaries, enabling nutrient transport and indirect communication between endothelial cells and the other cell type, while preventing cell migration. Fibrous membranes have been previously employed in BBB models instead of PET membranes due to their mechanical properties and morphological resemblance to the native basement membrane (35). To the best of our knowledge, this is the first study to incorporate a fibrous membrane with a branched, tubular architecture into a BBB model and to investigate its influence on barrier function—highlighting the potential of topographically guided membrane geometries in enhancing *in vitro* BBB modeling. Mechanical properties of the surface affect the mechanical tension generated at the focal adhesions (FA) of endothelial cells, which spreads through cytoskeletal elements and influences cell-cell interactions (2). For

instance, endothelial cells on the softer surface (in the kPa range) showed increased cadherin expression compared to those on stiffer surfaces (in the GPa range) (74). This tension extends to adherens junctions, helping to maintain endothelial monolayer integrity and thereby supporting barrier function. In our model, the Young's modulus of PCL and PCL-Col membranes was measured as 0.75 ± 0.10 MPa and 0.74 ± 0.15 MPa, respectively. The Young's modulus in kPa-range of the membranes was achieved by incorporating acetic acid into the electrospinning setup. Beyond mechanical stiffness, surface morphology and composition also play a critical role in endothelial attachment and monolayer formation. The interplay between topographical guidance and mechanical compliance is likely to influence both the maturation and the functional integrity of the endothelial barrier within such 3D architectures.

There should be a balance between hydrophilicity and hydrophobicity on the surface for cell adhesion and growth (99). Therefore, it seems that the presence of both collagen and fibronectin in our membranes reduces cell growth due to excessive hydrophilicity, which can lead to an overly hydrated surface that prevents cells from making proper contact (100). Based on these findings, PCL-based fibrous membranes were selected for the establishment of the multicellular NVU model to ensure a suitable balance between mechanical and surface properties.

The brain side of the model was composed of a hydrogel designed to mimic the native brain extracellular matrix (ECM). The brain ECM is primarily composed of hyaluronic acid (HA), tenascin, and lectins, which contribute to its distinct biochemical and mechanical properties. HA plays a crucial role in maintaining the brain's soft mechanical environment, and hydrogels with a stiffness below 80 kPa are considered optimal for neuronal culture. Material selection for brain ECM mimetic remains one of the key challenges in CNS studies. Unlike other tissues, the brain lacks fibronectin and collagen, but most BBB models incorporate these proteins either on the basolateral side in 2D cultures or within passive scaffold matrices that provide only structural support without actively influencing BBB function. However, human astrocytes cultured in hydrogels composed of collagen, HA, and Matrigel exhibited a morphology resembling their *in vivo* phenotype, along with a low GFAP expression and high AQP-4 expression, indicating a more physiologically relevant astrocytic state (95). For a

brain ECM-mimicking hydrogel to be effective, essential requirements include a slow degradation rate to ensure stability in culture for at least 21 days, softness to replicate the mechanical properties of the native brain ECM, and biological activity to support cell adhesion and function (27). However, achieving these requirements remains challenging, as the soft, natural polymer matrices often results in rapid hydrolysis within the aqueous environment of cell culture media. Therefore, designing a model that supports the long-term cell culture while maintaining mechanical integrity is crucial.

The brain side of our model was designed as a hyaluronic acid-based hydrogel to imitate chemical composition and the soft mechanical properties of brain tissue. To this end, MeHA was synthesized and employed as the hydrogel matrix. However, unmodified HA does not support integrin-mediated cell adhesion, which prevents cells loaded in MeHA-based hydrogels from expressing their tissue-specific morphology (83). To address this limitation, we developed a MeHA and collagen-based hydrogel to enhance structural integrity, extend the degradation profile due to MeHA, and provide cell attachment sites through collagen. In the MeHA-collagen hybrid hydrogel, MeHA provides structural and mechanical support, which in turn regulates the formation of the collagen network that serves cell adhesion sites. At lower MeHA concentrations (below 0.75%), insufficient crosslinking may lead to inadequate structural support for collagen fibrillogenesis, resulting in a weaker and incomplete collagen network. In contrast, excessively high MeHA concentrations (above 1%) may lead to elevated polymer density, which could limit collagen fibril organization by reducing hydrogel porosity. Compared to previously reported MeHA-based hydrogels, our imaging results demonstrated that pericyte-like cells and astrocytes exhibited enhanced cellular expansion and extended their projections within the hydrogel (101-103). Moreover, astrocytes expressed high levels of AQP-4, a key benchmark for assessing the physiological relevance of hydrogel-based BBB models. MeHA hydrogels are used as bio-inks for osteogenic substitutes to provide passive structural support, where cells exhibit limited expansion (101). Alternatively, they are employed to enhance cell spreading and investigate stem cell fate through the addition of cell-adhesive motifs or MMP-sensitive domains (102, 103). However, we optimized the MeHA hydrogel composition by testing different proteins and their concentrations to

provide a biomimetic microenvironment that facilitates cellular interactions (Fig. 2). The optimized MeHA-collagen hydrogel was then well-characterized to identify key features of a microenvironment that supports the culture of three distinct cell types and their co-culture (Fig 3). This improvement can be attributed to the presence of integrin-binding sites in the crosslinked collagen, which enhanced astrocytic attachment, morphology, and functional marker expression. To the best of our knowledge, this is the first model to consider the brain side of the native barrier and incorporate a hydrogel that mimics the native brain ECM and evaluates its effect on barrier formation.

Our tissue-engineered multicellular model was initially established by integrating a fibrous membrane in flat form as the blood side and a MeHA-collagen-based hydrogel as the brain side within a custom-designed, 3D-printed system. Before the co-culture studies, we investigated the mechanical effect of hydrogel compression on the fibrous membrane by assessing CD31 expression in the monoculture model. CD31 is known to mediate mechanotransduction processes by regulating endothelial responses to biomechanical stimuli (104). Therefore, its expression serves as an indicator for evaluating whether mechanical alterations in the underlying substrate influence monolayer formation and barrier integrity. Current *in vitro* and *in vivo* BBB models primarily focus on biochemical cues within the basement membrane and brain ECM (105-108). However, the mechanical properties of the brain ECM are known to change with aging, neurodegenerative diseases, and neuroinflammatory conditions (109, 110). Despite this, the influence of ECM mechanics on endothelial integrity remains underexplored. In a preliminary experimental setup, we investigated how mechanical compression affects CD31 expression and monolayer integrity. We showed that hydrogel thickness-induced mechanical compression negatively impacts endothelial cell adhesion, disrupting continuous monolayer formation which suggests a potential role for ECM mechanics in BBB regulation. While these initial findings highlight the relevance of mechanical forces, further investigations, including TEER measurements and permeability assays, are needed to determine how pathological conditions—such as neurodegenerative diseases, glioblastoma-induced ECM stiffening, or ischemic conditions—modulate BBB integrity. Nevertheless, our model could serve as a useful platform for studying BBB dysfunction in mechanically altered

microenvironments and for evaluating therapeutic strategies targeting ECM-mediated barrier disruption.

In the evolutionary development of *in vitro* BBB models, various approaches have been explored to achieve more accurate cellular responses and restrictive barrier properties. These models range from traditional Transwell systems to microfluidic chip designs incorporating different strategies, such as sandwich design, parallel-design chips, 3D tubular design, and vasculogenesis-based designs (47, 111-114). Critical design requirements of BBB can be listed as 3D vessel like structure, cell-cell interaction, perfusion incorporation, and promising recapitulation of the basement membrane. While the 3D vessel-like structure, cell-cell communication, and perfusion integration have been successfully achieved in existing models, the accurate mimicry of the basement membrane remains a challenge. Therefore, our initial NVU model was evolved into two microfluidic platforms—a basic and a complex NVU-on-chip design that incorporate tubular electrospun fibrous membranes embedded within PDMS housings. These designs enabled precise compartmentalization, controlled shear stress application, and improved spatial organization, offering a more physiologically relevant platform to investigate barrier function under dynamic conditions. The surrounding hydrogel, mimicking the brain-side matrix, was integrated around the membrane to reconstruct the neural compartment. With this design approach, we achieved simultaneous integration of key features—perfusion, three-dimensional flow, and a geometrically complex, fibrous basement membrane—meeting the critical design criteria for constructing an ideal *in vitro* BBB/NVU model.

Previous studies have demonstrated that dynamic flow conditions and 3D vascular structures improve endothelial cell polarization, enhance tight junction formation, and ultimately result in higher TEER values and lower permeability (111, 112). TEER values reported in perfusion-integrated tri-culture sandwich-design microfluidic chips were $150 \Omega \cdot \text{cm}^2$ using the HUVECs, while tri-culture perfusion-integrated Transwell systems reached $500 \Omega \cdot \text{cm}^2$. In comparison, our initial 2D multicellular co-culture model—established on a Transwell-like system—achieved a TEER value of $166.86 \pm 5.75 \Omega \cdot \text{cm}^2$, suggesting that the inclusion of a fibrous basement membrane and a biomimetic brain-side environment contributed to improved barrier function.

However, TEER measurements could not be performed for the NVU-on-a-chip models due to the incompatibility between the branched tubular membrane's lumen geometry and the commercially available TEER electrode configuration. Therefore, barrier function in the NVU-on-a-chip system was evaluated based on the permeability coefficient using 0.4 kDa fluorescein.

ECs are one of the main constituents of *in vitro* BBB models, forming the barrier between the blood and the brain. Therefore, the selection of the EC source is a critical consideration. In a 3D tubular design microfluidic chip model, iPSC-derived brain microvascular endothelial cells (BMECs) were employed, achieving TEER values of $2,000 \Omega \cdot \text{cm}^2$ for seven days (113). However, the differentiation and maintenance of iPSC-derived ECs are time-consuming, expensive, and require specialized expertise. In another study, BMECs were seeded on gelatin based fibrous membrane and TEER values were in the range of $20\text{-}30 \Omega \cdot \text{cm}^2$ (35). Another ECs source is HUVECs, which may not fully replicate BBB-specific properties (115). However, they are widely used in *in vitro* studies due to their ease of culture and rapid proliferation (111, 112). In this study, HUVECs were employed as the endothelial cells and seeded onto the fibrous membrane. Given that the primary objective of this study was to investigate the role of the basement membrane and the brain-side microenvironment in BBB function, HUVECs provided a practical and reproducible alternative. Although the use of brain-specific endothelial cells may further enhance the physiological relevance of the model, our findings suggest that the key barrier properties were successfully achieved in the current setup. An important benchmark of the BBB is the continuous expression of tight junction proteins such as zonula occludens-1 (ZO-1), claudins, and occludin. In this study, CD31 staining was used to assess cell-cell adhesion, focusing on adherent junction formation. However, ZO-1 staining was not performed, which could have provided additional validation of tight junction assembly and monolayer integrity. Despite this limitation, barrier function was evaluated using TEER measurements and permeability assays, which serve as functional indicators of tight junction integrity. LSCM images demonstrated that CD31 expression was localized at the cell borders, confirming monolayer integrity on the membrane. Additionally, CD31 expression was disrupted under mechanical changes, further supporting its potential as an indicator of monolayer integrity and junction stability.

The permeability of the models—including the initial co-culture model established on a custom-designed 3D-printed system, as well as the basic and complex NVU-on-a-chip platforms—was investigated using conventional tracers, 0.4 kDa fluorescein and 20 kDa dextran. Additionally, permeability was assessed using ceftriaxone and amikacin to evaluate the translational potential of the model. The permeability coefficient for 15 kDa dextran in a tri-culture BBB model was reported as $2.5 \times 10^{-7} \text{ cm s}^{-1}$ (111). The permeability coefficient for 20 kDa dextran in a co-culture model established on a fibrous membrane was $18.2 \pm 9.3 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ at day 12, and $5.6 \pm 2.3 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ on day 21 (35). In comparison, the permeability coefficient of our model for 20 kDa dextran was $8.14 \pm 6.18 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ at day 14 and $8.85 \pm 6.25 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ at day 21. The higher permeability of our model for 20 kDa dextran compared to the 15 kDa dextran in the tri-culture model from the literature could be attributed to the fact that, although the permeability assay was performed under static conditions, the reference model was established under perfusion conditions. Additionally, their TEER values were also higher than those in our model, which correlates with the permeability results. The permeability of our multicellular model was higher than that of the co-culture model established on a fibrous membrane at day 14. However, at day 21, their permeability values were slightly lower than ours, suggesting that their model's barrier function improved until day 21, whereas our model maintained its barrier function for at least one week. Under physiological conditions, molecules larger than 0.4 kDa cannot cross the BBB via passive diffusion. However, some molecules larger than 0.7 kDa can cross through active transport mechanisms mediated by transport proteins or receptor-mediated transcytosis. The permeability to 0.4 kDa Lucifer Yellow in a previously reported model was $3.38 \pm 2.65 \times 10^{-7} \text{ cm s}^{-1}$ (113). In comparison, the permeability of our model to 0.4 kDa Fluorescein disodium salt was $301.64 \pm 95.68 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$ at day 14. Their permeability result correlated with the TEER values which was higher than the $2,000 \Omega \cdot \text{cm}^2$ for seven days. This significant difference in permeability and TEER values can be attributed to their model's use of a tubular design approach, where iPSC-derived ECs were cultured under perfusion, contributing to enhanced barrier function. However, permeability to 0.4 kDa Fluorescein of basic NVU-on-a-chip model and complex NVU-on-a-chip model were calculated as 0.04×10^{-7} and $0.48 \times 10^{-7} \text{ cm} \cdot \text{s}^{-1}$

at day 14. These values suggest that both chip-based models exhibit lower permeability, which may reflect enhanced barrier integrity.

Although ceftriaxone (554.6 Da) and amikacin (585.6 Da) have similar molecular weights, ceftriaxone can cross the BBB to a limited extent due to its moderate hydrophilicity or transporter-mediated uptake. In contrast, amikacin is a highly hydrophilic aminoglycoside that lacks a specific transport mechanism, preventing its entry into the brain. The permeability to ceftriaxone and amikacin of our model was calculated as $42.7 \pm 2.61 \times 10^{-8}$ and $1.41 \pm 1.55 \times 10^{-8} \text{ cm} \cdot \text{s}^{-1}$, respectively at day 14. The observed differences in permeability between 0.4 kDa fluorescein disodium salt and ceftriaxone may partially stem from differences in their detection methods. While fluorescein disodium salt permeability was assessed using fluorescence intensity measurements, ceftriaxone permeability was quantified based on absorbance at a specific wavelength.

Permeability to ceftriaxone and amikacin of our model—both measured using the same detection method— showed that amikacin exhibits significantly lower permeability, whereas ceftriaxone demonstrates relatively higher permeability. This finding aligns with the known pharmacokinetics of these compounds: amikacin, a highly hydrophilic aminoglycoside, is largely excluded from the brain, whereas ceftriaxone, despite its limited passive diffusion, can partially penetrate the BBB through carrier-mediated mechanisms. Thus, while our preliminary results suggest a correlation between ceftriaxone permeability and the barrier function of our BBB model, further investigations incorporating additional compounds with diverse physicochemical properties are required to confirm our initial findings and account for potential methodological influences. This approach could serve as a promising strategy to evaluate the translational potential of *in vitro* BBB /NVU models.

The NVU-on-a-chip system developed in this study—featuring distinct blood- and brain-side compartments, integrated perfusion capability, and a three-dimensional architecture—provides a robust platform for investigating bidirectional interactions between vascular and neural components. In the literature, BBB/NVU-on-a-chip models have evolved through various design strategies, with many early approaches relying on sandwich-based configurations in which two compartments are separated

by a flat porous membrane(43). Reported transendothelial electrical resistance (TEER) values for non-perfused sandwich models are typically around $25 \Omega \cdot \text{cm}^2$, whereas perfusion-integrated versions can reach values of up to $\sim 300 \Omega \cdot \text{cm}^2$. In these co-culture models, permeability to a 4 kDa tracer is commonly reported to be approximately $5 \times 10^{-5} \text{ cm/s}$.

In a parallel chip design employing two horizontally arranged chambers separated by an array of PDMS microchannels, a co-cultured neonatal BBB model exhibited high barrier integrity, with a transendothelial electrical resistance of approximately $250 \text{ k}\Omega$ and a permeability to a 40 kDa tracer of $\sim 1.1 \times 10^{-6} \text{ cm/s}$, indicative of a highly restrictive barrier phenotype(50). Similarly, a 3D tubular BBB model formed by casting hydrogel around a cylindrical mold and subsequent endothelialization demonstrated very high barrier integrity, with a TEER of $\sim 4,100 \Omega \cdot \text{cm}^2$ and a permeability to a 0.4 kDa tracer of $\sim 3\text{--}5 \times 10^{-7} \text{ cm/s}$ (113).

In the present study, direct TEER measurements could not be performed in the NVU-on-a-chip due to the branched fibrous architecture, which precluded proper electrode placement. However, TEER was successfully measured in a corresponding transwell co-culture configuration, where the multicellular model achieved a high TEER value of $166.86 \pm 5.75 \Omega \cdot \text{cm}^2$. Permeability measurements further confirmed strong barrier function, with permeability coefficients of $3.18 \times 10^{-7} \text{ cm/s}$ and $0.48 \times 10^{-7} \text{ cm/s}$ for the basic and complex models, respectively.

The establishment of this multicellular, three-dimensional, branched NVU-on-a-chip model provides a strong foundation for physiologically relevant *in vitro* studies through the integration of a biomimetic basement membrane and a brain-side extracellular matrix. Future efforts will focus on enhancing the biological fidelity and predictive power of the platform by incorporating human iPSC-derived brain microvascular endothelial cells, which are expected to yield higher TEER values and more physiologically relevant transporter expression compared to HUVECs. The neural compartment can also be refined by including mature neurons and microglia to enable investigation of complex neurovascular signaling and inflammatory responses. From a translational perspective, expanding drug testing beyond ceftriaxone and amikacin to include compounds with diverse physicochemical properties will further

strengthen the platform's translational relevance. Collectively, these advancements will position the NVU-on-a-chip as a valuable tool for personalized medicine, drug screening, and mechanistic studies of central nervous system disorders.



6 CONCLUSION

This study presented the development of NVU/BBB models with distinct blood-side and brain-side compartments. The initial co-culture model was constructed on a custom-designed 3D-printed platform, followed by the fabrication of more advanced NVU-on-a-chip systems incorporating tubular and branched fibrous membrane configurations. The blood-side compartment was formed using electrospun fibrous membranes to mimic the structural and mechanical properties of the native basement membrane, while the brain-side compartment was composed of a hyaluronic acid-based hydrogel designed to imitate the biochemical composition of brain ECM. Both components were optimized and characterized to ensure structural and functional relevance for neurovascular modeling. The cellular components included ECs on the blood-side compartment, and astrocytes, pericyte-like cells, and neurons on the brain-side compartment. Cellular morphology and marker expression were evaluated using LSCM and immunocytochemistry, confirming the phenotypic characteristics of each cell type within the model.

The models were constructed by seeding the ECs on fibrous membrane, and sequential integration of neuron loaded hydrogel and astrocytes- and the pericyte-like cell loaded hydrogel. The model was evaluated through Live/Dead staining, immunofluorescence analysis, TEER measurement (in 2D platform), and permeability assays using conventional tracers and drug molecules with known BBB profiles. Results demonstrated that the cells remained viable, maintained their immunophenotype, and exhibited improved barrier function under co-culture conditions, along with reduced permeability across the developed barrier.

Taken together, the development of multiple NVU models—from a simple 3D-printed co-culture system to a more complex microfluidic chip platform—was achieved in this study. To the best of our knowledge, this is the first BBB model to incorporate a geometrically complex branched fibrous membrane, offering a topography-guided approach for mimicking the vascular basement membrane. In parallel, the brain-side compartment was designed using a hydrogel that replicates the biochemical composition of the brain ECM. By recapitulating the key structural and

functional features of the NVU, the resulting system provides a valuable platform for future studies involving disease modeling, compound permeability screening under physiologically relevant conditions, and contributing to the reduction of animal testing.



7 REFERENCES

1. Barichello T, Collodel A, Hasbun R, Morales R. An Overview of the Blood-Brain Barrier. In: Barichello T, editor. *Blood-Brain Barrier*. New York, NY: Springer New York; 2019. p. 1-8.
2. Ferro MP, Heilshorn SC, Owens RM. Materials for blood brain barrier modeling in vitro. *Materials Science and Engineering: R: Reports*. 2020;140:100522.
3. Oddo A, Peng B, Tong Z, Wei Y, Tong WY, Thissen H, et al. Advances in Microfluidic Blood-Brain Barrier (BBB) Models. *Trends Biotechnol*. 2019;37(12):1295-314.
4. Aazmi A, Zhou H, Lv W, Yu M, Xu X, Yang H, et al. Vascularizing the brain in vitro. *iScience*. 2022;25(4):104110.
5. Brain Basics: Know Your Brain 2022 [cited 2022 16.06.2022]. Available from: <https://www.ninds.nih.gov/health-information/patient-caregiver-education/brain-basics-know-your-brain>.
6. Pawlina. MHRaW. Nerve Tissue. *Histology: A Text and Atlas: with Correlated Cell and Molecular Biology*. 6th ed: Lippincott Williams & Wilkins; 2011. p. 352-400.
7. Paul Ehrlich – Biographical NobelPrize.org. [cited 2023 11.11.2023]. Available from: <https://www.nobelprize.org/prizes/medicine/1908/ehrlich/biographical/>.
8. Goldmann EE. Vitalfärbung am Zentralnervensystem: Beitrag zur Physio-Pathologie des Plexus chorioideus und der Hirnhäute: Königl. Akademie der Wissenschaften; 1913.
9. Goldmann EE. Die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der ‘vitalen Färbung. *Beitr Klin Chir*. 1909;64:192-265.
10. Ribatti D, Nico B, Crivellato E, Artico M. Development of the blood-brain barrier: A historical point of view. *The Anatomical Record Part B: The New Anatomist*. 2006;289B(1):3-8.
11. Stern L, Gautier R. Recherches Sur Le Liquide Céphalo-Rachidien: I.–Les Rapports Entre Le Liquide Céphalo-Rachidien et la Circulation Sanguine. *Archives Internationales de Physiologie*. 1921;17(2):138-92.
12. Saunders NR, Dreifuss JJ, Dziegielewska KM, Johansson PA, Habgood MD, Møllgård K, et al. The rights and wrongs of blood-brain barrier permeability studies: a walk through 100 years of history. *Front Neurosci*. 2014;8:404.
13. Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J Cell Biol*. 1967;34(1):207-17.
14. Crone C, Olesen SP. Electrical resistance of brain microvascular endothelium. *Brain Res*. 1982;241(1):49-55.
15. Iadecola C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron*. 2017;96(1):17-42.
16. Daneman R, Prat A. The blood-brain barrier. *Cold Spring Harb Perspect Biol*. 2015;7(1):a020412.
17. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci*. 2006;7(1):41-53.
18. Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev*. 2005;57(2):173-85.
19. Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J Physiol*. 1990;429:47-62.
20. Stamatovic SM, Johnson AM, Keep RF, Andjelkovic AV. Junctional proteins of the blood-brain barrier: New insights into function and dysfunction. *Tissue Barriers*. 2016;4(1):e1154641.
21. Lochhead JJ, Yang J, Ronaldson PT, Davis TP. Structure, Function, and Regulation of the Blood-Brain Barrier Tight Junction in Central Nervous System Disorders. *Frontiers in Physiology*. 2020;11.
22. Ahn C, Shin DH, Lee D, Kang SM, Seok JH, Kang HY, et al. Expression of claudins, occludin, junction adhesion molecule A and zona occludens 1 in canine organs. *Mol Med Rep*. 2016;14(4):3697-703.
23. Mandell KJ, Parkos CA. The JAM family of proteins. *Advanced Drug Delivery Reviews*. 2005;57(6):857-67.
24. Sweeney MD, Ayyadurai S, Zlokovic BV. Pericytes of the neurovascular unit: key functions and signaling pathways. *Nat Neurosci*. 2016;19(6):771-83.
25. Muoio V, Persson PB, Sendeski MM. The neurovascular unit - concept review. *Acta Physiol (Oxf)*. 2014;210(4):790-8.

26. Potjewyd G, Moxon S, Wang T, Domingos M, Hooper NM. Tissue Engineering 3D Neurovascular Units: A Biomaterials and Bioprinting Perspective. *Trends Biotechnol.* 2018;36(4):457-72.
27. Hopkins AM, DeSimone E, Chwalek K, Kaplan DL. 3D in vitro modeling of the central nervous system. *Prog Neurobiol.* 2015;125:1-25.
28. Lam D, Enright HA, Cadena J, Peters SKG, Sales AP, Osburn JJ, et al. Tissue-specific extracellular matrix accelerates the formation of neural networks and communities in a neuron-glia co-culture on a multi-electrode array. *Sci Rep.* 2019;9(1):4159.
29. Elkin BS, Azeloglu EU, Costa KD, Morrison B, 3rd. Mechanical heterogeneity of the rat hippocampus measured by atomic force microscope indentation. *J Neurotrauma.* 2007;24(5):812-22.
30. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006;126(4):677-89.
31. Whitesides GM. The origins and the future of microfluidics. *Nature.* 2006;442(7101):368-73.
32. Zhao Y, Wang EY, Lai FBL, Cheung K, Radisic M. Organs-on-a-chip: a union of tissue engineering and microfabrication. *Trends Biotechnol.* 2023;41(3):410-24.
33. Williams-Medina A, Deblock M, Janigro D. In vitro Models of the Blood-Brain Barrier: Tools in Translational Medicine. *Frontiers in Medical Technology.* 2021;2.
34. Hatherell K, Couraud PO, Romero IA, Weksler B, Pilkington GJ. Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. *J Neurosci Methods.* 2011;199(2):223-9.
35. Bischel LL, Coneski PN, Lundin JG, Wu PK, Giller CB, Wynne J, et al. Electrospun gelatin biopapers as substrate for in vitro bilayer models of blood-brain barrier tissue. *Journal of Biomedical Materials Research Part A.* 2016;104(4):901-9.
36. Qi D, Wu S, Lin H, Kuss MA, Lei Y, Krasnoslobodtsev A, et al. Establishment of a Human iPSC- and Nanofiber-Based Microphysiological Blood-Brain Barrier System. *ACS Appl Mater Interfaces.* 2018;10(26):21825-35.
37. Hoheisel D, Nitz T, Franke H, Wegener J, Hakvoort A, Tilling T, et al. Hydrocortisone Reinforces the Blood-Brain Barrier Properties in a Serum Free Cell Culture System. *Biochemical and Biophysical Research Communications.* 1998;244(1):312-6.
38. Schrot S, Weidenfeller C, Schäffer TE, Robenek H, Galla HJ. Influence of hydrocortisone on the mechanical properties of the cerebral endothelium in vitro. *Biophys J.* 2005;89(6):3904-10.
39. Hatherell K, Couraud P-O, Romero IA, Weksler B, Pilkington GJ. Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. *Journal of Neuroscience Methods.* 2011;199(2):223-9.
40. Wuest DM, Wing AM, Lee KH. Membrane configuration optimization for a murine in vitro blood-brain barrier model. *Journal of Neuroscience Methods.* 2013;212(2):211-21.
41. Mondrinos MJ, Yi Y-S, Wu N-K, Ding X, Huh D. Native extracellular matrix-derived semipermeable, optically transparent, and inexpensive membrane inserts for microfluidic cell culture. *Lab on a Chip.* 2017;17(18):3146-58.
42. Katt ME, Linville RM, Mayo LN, Xu ZS, Searson PC. Functional brain-specific microvessels from iPSC-derived human brain microvascular endothelial cells: the role of matrix composition on monolayer formation. *Fluids and Barriers of the CNS.* 2018;15(1):7.
43. Booth R, Kim H. Characterization of a microfluidic in vitro model of the blood-brain barrier (μ BBB). *Lab on a Chip.* 2012;12(10):1784-92.
44. Griep LM, Wolbers F, de Wagenaar B, ter Braak PM, Weksler BB, Romero IA, et al. BBB ON CHIP: microfluidic platform to mechanically and biochemically modulate blood-brain barrier function. *Biomedical Microdevices.* 2013;15(1):145-50.
45. Achyuta AK, Conway AJ, Crouse RB, Bannister EC, Lee RN, Katnik CP, et al. A modular approach to create a neurovascular unit-on-a-chip. *Lab Chip.* 2013;13(4):542-53.
46. Brown JA, Pensabene V, Markov DA, Allwardt V, Neely MD, Shi M, et al. Recreating blood-brain barrier physiology and structure on chip: A novel neurovascular microfluidic bioreactor. *Biomicrofluidics.* 2015;9(5):054124.
47. Maoz BM, Herland A, FitzGerald EA, Grevesse T, Vidoudez C, Pacheco AR, et al. A linked organ-on-chip model of the human neurovascular unit reveals the metabolic coupling of endothelial and neuronal cells. *Nature Biotechnology.* 2018;36(9):865-74.
48. Park T-E, Mustafaoglu N, Herland A, Hasselkus R, Mannix R, FitzGerald EA, et al. Hypoxia-enhanced Blood-Brain Barrier Chip recapitulates human barrier function and shuttling of drugs and antibodies. *Nature Communications.* 2019;10(1):2621.

49. Prabhakarapandian B, Shen M-C, Nichols JB, Mills IR, Sidoryk-Wegrzynowicz M, Aschner M, et al. SyM-BBB: a microfluidic blood brain barrier model. *Lab on a Chip*. 2013;13(6):1093-101.
50. Deosarkar SP, Prabhakarapandian B, Wang B, Sheffield JB, Krynska B, Kiani MF. A Novel Dynamic Neonatal Blood-Brain Barrier on a Chip. *PLoS One*. 2015;10(11):e0142725.
51. Terrell-Hall TB, Ammer AG, Griffith JI, Lockman PR. Permeability across a novel microfluidic blood-tumor barrier model. *Fluids Barriers CNS*. 2017;14(1):3.
52. Adriani G, Ma D, Pavesi A, Kamm RD, Goh EL. A 3D neurovascular microfluidic model consisting of neurons, astrocytes and cerebral endothelial cells as a blood-brain barrier. *Lab Chip*. 2017;17(3):448-59.
53. Xu H, Li Z, Yu Y, Sizdahkhani S, Ho WS, Yin F, et al. A dynamic in vivo-like organotypic blood-brain barrier model to probe metastatic brain tumors. *Scientific Reports*. 2016;6(1):36670.
54. van der Helm MW, van der Meer AD, Eijkel JCT, van den Berg A, Segerink LI. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers*. 2016;4(1):e1142493.
55. Chrobak KM, Potter DR, Tien J. Formation of perfused, functional microvascular tubes in vitro. *Microvascular Research*. 2006;71(3):185-96.
56. Herland A, van der Meer AD, FitzGerald EA, Park TE, Sleeboom JJ, Ingber DE. Distinct Contributions of Astrocytes and Pericytes to Neuroinflammation Identified in a 3D Human Blood-Brain Barrier on a Chip. *PLoS One*. 2016;11(3):e0150360.
57. Partyka PP, Godsey GA, Galie JR, Kosciuk MC, Acharya NK, Nagele RG, et al. Mechanical stress regulates transport in a compliant 3D model of the blood-brain barrier. *Biomaterials*. 2017;115:30-9.
58. de Graaf MNS, Cochrane A, van den Hil FE, Buijsman W, van der Meer AD, van den Berg A, et al. Scalable microphysiological system to model three-dimensional blood vessels. *APL Bioengineering*. 2019;3(2).
59. Li H, Cheng F, Li W, Cao X, Wang Z, Wang M, et al. Expanding sacrificially printed microfluidic channel-embedded paper devices for construction of volumetric tissue models in vitro. *Biofabrication*. 2020;12(4):045027.
60. Bertassoni LE, Cecconi M, Manoharan V, Nikkhah M, Hjortnaes J, Cristino AL, et al. Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs. *Lab Chip*. 2014;14(13):2202-11.
61. Hong S, Song Y, Choi J, Hwang C. Bonding of Flexible Membranes for Perfusable Vascularized Networks Patch. *Tissue Eng Regen Med*. 2022;19(2):363-75.
62. Zhang YS, Davoudi F, Walch P, Manbachi A, Luo X, Dell'Erba V, et al. Bioprinted thrombosis-on-a-chip. *Lab on a Chip*. 2016;16(21):4097-105.
63. Li Z, Ribeiro de Souza L, Litina C, Markaki A, Al-Tabbaa A. A novel biomimetic design of a 3D vascular structure for self-healing in cementitious materials using Murray's law. *Materials & Design*. 2020;190:108572.
64. Saramito P. *Complex fluids*. 1 ed: Springer Cham; 2016.
65. Eom S, Park SM, Hong H, Kwon J, Oh S-R, Kim J, et al. Hydrogel-Assisted Electrospinning for Fabrication of a 3D Complex Tailored Nanofiber Macrostructure. *ACS Applied Materials & Interfaces*. 2020;12(46):51212-24.
66. Burdick JA, Chung C, Jia X, Randolph MA, Langer R. Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. *Biomacromolecules*. 2005;6(1):386-91.
67. Kole GE, Hasirci V, Yucel D. Development of a Tri-Layered Vascular Construct and In Vitro Evaluation of Endothelialization. *Macromolecular Bioscience*. 2024;24(5):2300369.
68. Köle GE. *In Vitro Development of Multilayered Construct for Blood Vessel Tissue Engineering [Master Thesis]*. Istanbul: Acıbadem Mehmet Ali Aydınlar University; 2020.
69. Tian X, Brookes O, Battaglia G. Pericytes from Mesenchymal Stem Cells as a model for the blood-brain barrier. *Scientific Reports*. 2017;7(1):39676.
70. Lim D, Kamotani Y, Cho B, Mazumder J, Takayama S. Fabrication of microfluidic mixers and artificial vasculatures using a high-brightness diode-pumped Nd:YAG laser direct write method. *Lab on a chip*. 2003;3:318-23.
71. Murray CD. The Physiological Principle of Minimum Work: I. The Vascular System and the Cost of Blood Volume. *Proc Natl Acad Sci U S A*. 1926;12(3):207-14.
72. Murray Cd. The Physiological Principle Of Minimum Work Applied To The Angle Of Branching Of Arteries. *J Gen Physiol*. 1926;9(6):835-41.
73. Ladoux B, Mège R-M. Mechanobiology of collective cell behaviours. *Nature Reviews Molecular Cell Biology*. 2017;18(12):743-57.

74. Andresen Eguiluz RC, Kaylan KB, Underhill GH, Leckband DE. Substrate stiffness and VE-cadherin mechano-transduction coordinate to regulate endothelial monolayer integrity. *Biomaterials*. 2017;140:45-57.
75. Elamparithi A, Punnoose A, Kuruvilla S, Ravi M, Rao S, Paul S. Electrospun polycaprolactone matrices with tensile properties suitable for soft tissue engineering. *Artificial cells, nanomedicine, and biotechnology*. 2015;44:1-7.
76. Miele D, Catenacci L, Rossi S, Sandri G, Sorrenti M, Terzi A, et al. Collagen/PCL Nanofibers Electrospun in Green Solvent by DOE Assisted Process. An Insight into Collagen Contribution. *Materials*. 2020;13(21):4698.
77. Ågren R, Zeberg H. Low-Resistance silver bromide electrodes for recording fast ion channel kinetics under voltage clamp conditions. *Journal of Neuroscience Methods*. 2020;348:108984.
78. Khasim S. Conductivity and DSC studies of polyethylene glycol and its salt complexes. *Indian Journal of Engineering and Materials Sciences*. 2000;Volume-7:456-8.
79. Candreva A, Nisco GD, Rizzini ML, D'Ascenzo F, Ferrari GMD, Gallo D, et al. Current and Future Applications of Computational Fluid Dynamics in Coronary Artery Disease. *RCM*. 2022;23(11).
80. John E. Hall MEH. Guyton and Hall Textbook of Medical Physiology. 14th Edition ed: Elsevier; 2021.
81. Grinberg L, Anor T, Cheever E, Madsen JR, Karniadakis GE. Simulation of the human intracranial arterial tree. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*. 2009;367(1896):2371-86.
82. Pankov R, Yamada KM. Fibronectin at a glance. *Journal of Cell Science*. 2002;115(20):3861-3.
83. Caliali SR, Burdick JA. A practical guide to hydrogels for cell culture. *Nature Methods*. 2016;13(5):405-14.
84. Shi M, Xu Q, Ding L, Xia Y, Zhang C, Lai H, et al. Cell Infiltrative Inner Connected Porous Hydrogel Improves Neural Stem Cell Migration and Differentiation for Functional Repair of Spinal Cord Injury. *ACS Biomaterials Science & Engineering*. 2022;8(12):5307-18.
85. Shahidi S, Janmaleki M, Riaz S, Sanati Nezhad A, Syed N. A tuned gelatin methacryloyl (GelMA) hydrogel facilitates myelination of dorsal root ganglia neurons in vitro. *Materials Science and Engineering: C*. 2021;126:112131.
86. Zhang N, Liu X, Yu L, Shanks R, Petinaks E, Liu H. Phase composition and interface of starch–gelatin blends studied by synchrotron FTIR micro-spectroscopy. *Carbohydrate Polymers*. 2013;95(2):649-53.
87. Júnior ZSS, Botta SB, Ana PA, França CM, Fernandes KPS, Mesquita-Ferrari RA, et al. Effect of papain-based gel on type I collagen - spectroscopy applied for microstructural analysis. *Scientific Reports*. 2015;5(1):11448.
88. Lancaster MA, Renner M, Martin C-A, Wenzel D, Bicknell LS, Hurles ME, et al. Cerebral organoids model human brain development and microcephaly. *Nature*. 2013;501(7467):373-9.
89. Man AJ, Davis HE, Itoh A, Leach JK, Bannerman P. Neurite Outgrowth in Fibrin Gels Is Regulated by Substrate Stiffness. *Tissue Engineering Part A*. 2011;17(23-24):2931-42.
90. Gong JP, Katsuyama Y, Kurokawa T, Osada Y. Double-Network Hydrogels with Extremely High Mechanical Strength. *Advanced Materials*. 2003;15(14):1155-8.
91. Liu Y, Chan-Park MB. Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering. *Biomaterials*. 2009;30(2):196-207.
92. Wimmer I, Tietz S, Nishihara H, Deutsch U, Sallusto F, Gosselet F, et al. PECAM-1 Stabilizes Blood-Brain Barrier Integrity and Favors Paracellular T-Cell Diapedesis Across the Blood-Brain Barrier During Neuroinflammation. *Frontiers in Immunology*. 2019;10.
93. Pfau SJ, Langen UH, Fisher TM, Prakash I, Nagpurwala F, Lozoya RA, et al. Characteristics of blood–brain barrier heterogeneity between brain regions revealed by profiling vascular and perivascular cells. *Nature Neuroscience*. 2024;27(10):1892-903.
94. DeStefano JG, Jamieson JJ, Linville RM, Searson PC. Benchmarking in vitro tissue-engineered blood-brain barrier models. *Fluids Barriers CNS*. 2018;15(1):32.
95. Placone AL, McGuiggan PM, Bergles DE, Guerrero-Cazares H, Quiñones-Hinojosa A, Searson PC. Human astrocytes develop physiological morphology and remain quiescent in a novel 3D matrix. *Biomaterials*. 2015;42:134-43.
96. Salehi A, Sprejz S, Ruehl H, Olayioye M, Cattaneo G. An imprint-based approach to replicate nano- to microscale roughness on gelatin hydrogel scaffolds: surface characterization and effect on endothelialization. *J Biomater Sci Polym Ed*. 2024;35(8):1214-35.

97. Ingber DE. Is it Time for Reviewer 3 to Request Human Organ Chip Experiments Instead of Animal Validation Studies? *Adv Sci (Weinh)*. 2020;7(22):2002030.
98. Nau R, Sörgel F, Eiffert H. Penetration of drugs through the blood-cerebrospinal fluid/blood-brain barrier for treatment of central nervous system infections. *Clin Microbiol Rev*. 2010;23(4):858-83.
99. Arima Y, Iwata H. Effect of wettability and surface functional groups on protein adsorption and cell adhesion using well-defined mixed self-assembled monolayers. *Biomaterials*. 2007;28(20):3074-82.
100. Nuttelman CR, Mortisen DJ, Henry SM, Anseth KS. Attachment of fibronectin to poly(vinyl alcohol) hydrogels promotes NIH3T3 cell adhesion, proliferation, and migration. *Journal of Biomedical Materials Research*. 2001;57(2):217-23.
101. Poldervaart MT, Goversen B, de Ruijter M, Abbadessa A, Melchels FPW, Öner FC, et al. 3D bioprinting of methacrylated hyaluronic acid (MeHA) hydrogel with intrinsic osteogenicity. *PLOS ONE*. 2017;12(6):e0177628.
102. Khetan S, Burdick JA. Patterning network structure to spatially control cellular remodeling and stem cell fate within 3-dimensional hydrogels. *Biomaterials*. 2010;31(32):8228-34.
103. Khetan S, Guvendiren M, Legant WR, Cohen DM, Chen CS, Burdick JA. Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nature Materials*. 2013;12(5):458-65.
104. Caligiuri G. Mechanotransduction, immunoregulation, and metabolic functions of CD31 in cardiovascular pathophysiology. *Cardiovascular Research*. 2019;115(9):1425-34.
105. Yao Y, Chen Z-L, Norris EH, Strickland S. Astrocytic laminin regulates pericyte differentiation and maintains blood brain barrier integrity. *Nature Communications*. 2014;5(1):3413.
106. Rohde F, Danz K, Jung N, Wagner S, Windbergs M. Electrospun Scaffolds as Cell Culture Substrates for the Cultivation of an In Vitro Blood-Brain Barrier Model Using Human Induced Pluripotent Stem Cells. *Pharmaceutics*. 2022;14(6).
107. Al-Ahmad AJ, Patel R, Palecek SP, Shusta EV. Hyaluronan impairs the barrier integrity of brain microvascular endothelial cells through a CD44-dependent pathway. *J Cereb Blood Flow Metab*. 2019;39(9):1759-75.
108. Ventorp F, Barzilay R, Erhardt S, Samuelsson M, Träskman-Bendz L, Janelidze S, et al. The CD44 ligand hyaluronic acid is elevated in the cerebrospinal fluid of suicide attempters and is associated with increased blood–brain barrier permeability. *Journal of Affective Disorders*. 2016;193:349-54.
109. Ryu Y, Iwashita M, Lee W, Uchimura K, Kosodo Y. A Shift in Tissue Stiffness During Hippocampal Maturation Correlates to the Pattern of Neurogenesis and Composition of the Extracellular Matrix. *Front Aging Neurosci*. 2021;13:709620.
110. Pavuluri K, Huston J, Ehman RL, Manduca A, Vemuri P, Jack CR, et al. Brain mechanical properties predict longitudinal cognitive change in aging and Alzheimer's disease. *Neurobiology of Aging*. 2025;147:203-12.
111. Bolden CT, Skibber MA, Olson SD, Zamorano Rojas M, Milewicz S, Gill BS, et al. Validation and characterization of a novel blood–brain barrier platform for investigating traumatic brain injury. *Scientific Reports*. 2023;13(1):16150.
112. Ahn SI, Sei YJ, Park H-J, Kim J, Ryu Y, Choi JJ, et al. Microengineered human blood–brain barrier platform for understanding nanoparticle transport mechanisms. *Nature Communications*. 2020;11(1):175.
113. Grifno GN, Farrell AM, Linville RM, Arevalo D, Kim JH, Gu L, et al. Tissue-engineered blood-brain barrier models via directed differentiation of human induced pluripotent stem cells. *Scientific Reports*. 2019;9(1):13957.
114. Bang S, Lee S-R, Ko J, Son K, Tahk D, Ahn J, et al. A Low Permeability Microfluidic Blood-Brain Barrier Platform with Direct Contact between Perfusable Vascular Network and Astrocytes. *Scientific Reports*. 2017;7(1):8083.
115. Adriani G, Ma D, Pavesi A, Kamm RD, Goh ELK. A 3D neurovascular microfluidic model consisting of neurons, astrocytes and cerebral endothelial cells as a blood–brain barrier. *Lab on a Chip*. 2017;17(3):448-59.

APPENDIX

8 CURRICULUM VITAE

