Engineering tissue barrier models on hydrogel microfluidic platforms

Daniel Vera[1,2], María García-Díaz[2], Núria Torras[2], Mar Álvarez[1], Rosa Villa[1,3] and Elena Martinez[2,3,4*]*

[1] Institut de Microelectrònica de Barcelona, IMB-CNM (CSIC), Bellaterra, Barcelona, Spain

[2] Biomimetic Systems for Cell Engineering, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Barcelona, Spain

[3] Centro de Investigación Biomédica en Red (CIBER), Madrid, Spain

[4] Department of Electronics and Biomedical Engineering, University of Barcelona (UB), Barcelona, Spain

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ABSTRACT

Tissue barriers play a crucial role in human physiology by establishing tissue compartmentalization and regulating organ homeostasis. At the interface between the extracellular matrix (ECM) and flowing fluids, epithelial and endothelial barriers are responsible for solute and gas exchange. In the last decade, microfluidic technologies and organ-on-chip devices became popular as *in vitro* models able to recapitulate these biological barriers. However, in conventional microfluidic devices, cell barriers are primarily grown on hard polymeric membranes within polydimethylsiloxane (PDMS) channels that do not mimic the cell-ECM interactions nor allow the incorporation of other cellular compartments such as stromal tissue or vascular structures. To develop models that accurately account for the different cellular and acellular compartments of tissue barriers, hydrogels have been integrated into microfluidic setups for tissue barrier-on-chips, either as cell substrates inside the chip, or as self-contained devices. These biomaterials provide the soft mechanical properties of tissue barriers and allow the embedding of stromal cells. Combining hydrogels with microfluidics technology provides unique opportunities to better recreate *in vitro* the tissue barrier models including the cellular components and the functionality of the *in vivo* tissues. Such platforms have the potential of greatly improving the predictive capacities of the *in vitro* systems in applications such as drug development, or disease modelling. Nevertheless, their development is not without challenges in their microfabrication. In this review, we will discuss the recent advances driving the fabrication of hydrogel microfluidic platforms and their applications in multiple tissue barrier models.

INTRODUCTION

Tissue barriers have a major role in regulating the homeostasis of the human body [1]. Located at the boundaries between tissues and their external environment, they are both the first line of defence against external pathogens and the exchange site where nutrients and other important molecules pass through. Endothelial and epithelial cells ensure tissue compartmentalization by sealing their intercellular spaces through the so-called tight junctions and forming highly selective barriers. Endothelial cells are the building units of the circulatory vasculature. Interfacing blood flow and tissues in vessels and capillaries, they ensure haemostasis, transcellular transport, and neutrophil recruitment during immune response. For instance, in the blood-brain barrier, endothelial cells regulate the solute arriving to the central nervous system. On the other hand, epithelial barriers can be found in the kidney, where solutes are filtered, the liver, where the blood is filtered, and lining the surface of the gastrointestinal tract, where nutrients are absorbed. To accomplish their functions, endothelial and epithelial cells forming these barriers are assisted by stromal compartments formed by specific extracellular matrices that contain a variety of cell types. In addition, the luminal side of the barrier is usually exposed to fluid flow, creating a highly dynamic environment (Figure 1, left column). All these components are essential for the proper functionality of the barriers.

While realistic *in vitro* models of biological barriers are essential tools to better understand tissue functioning and perform preclinical drug screenings, in practice they are usually oversimplified in static monolayer cell cultures, often resulting in poor predictive capacities [2]. Within this landscape, the coming of organ-on-chips, which combine microfluidics with cell culture, represented a breakthrough in the field [3]. These devices allowed the co-culture of endothelial or epithelial cells with other cell types and their exposure to dynamic flow

conditions, shear stress and hydrostatic pressure, thus approaching the real structure and functioning of tissue barriers *in vivo*. By employing this approach, numerous organ-on-chip devices mimicking the physiological functions of the blood-brain barrier (BBB), renal tubules, liver and small intestine, among others, have been reported (Figure 1, central column) [4].

However, despite being undoubtedly useful, conventional polydimethylsiloxane (PDMS)-based organ-on-a-chip devices employ hard, non-cell permeable materials that reduce their relevance as *in vitro* models of tissue barriers. Specifically, with this approach cells lack the proper cell-extracellular matrix (ECM) contacts, the basement membrane and the stromal compartment [5,6]. The ECM, which is formed of proteins and polysaccharides produced by the cells, acts both as a physical support and a key regulator of cell function through biochemical and physical cues, playing an active role in tissue remodelling [7]. In tissue barriers, cells seed on top of the basement membrane, a specific ECM that dictates cell polarization (separation between their apical and basolateral compartments) and the sealing of the intercellular spaces [8]. Below the basement membrane, the stromal compartment is formed by an ECM embedding many different cell types such as immune cells, macrophages, adipocytes, pericytes, fibroblasts and other mesenchymal cells, along with blood vessels [9]. While PDMS does not constitute a good ECM-like matrix, hydrogels have been intensively studied as excellent candidates to act as ECM surrogates in tissue engineering for *in vitro* and *in vivo* applications [10,11]. Hydrogels are three-dimensional (3D) networks of polymer chains able to absorb large amounts of fluids. They possess highly tuneable mechanical and chemical properties that can be adjusted to match those of soft tissues [12]. In addition, their porous nature enables the embedding of cells, as oxygen and nutrients are able to diffuse through their structure and provide the proper environment for cell culture [13]. Considering these benefits, the integration of hydrogels into microfluidic devices

represents an excellent opportunity to push the physiological relevance of tissue barrier-on-chips (Figure 1, right column) [14,15]. Both as matrices embedded within the microfluidic chips, and as self-contained perfusable devices, hydrogel microfluidic platforms are the ideal candidates to include the cellular and acellular components of tissue barriers arranged spatially as *in vivo*, while providing fluid flow. Natural, synthetic and combinations of both polymer types have been used to produce hydrogels for this purpose. Collagen [16–18], gelatin [19–21], fibrin [22–24], alginate, hyaluronic acid and agarose [25–27], poly(ethylene glycol)-derivatives such as poly(ethylene glycol) diacrylate (PEGDA) [28–30] and combinations of them have been the most employed hydrogels in microfluidic-tissue barrier devices [31–34]. This approach, however, is still technologically challenging from the fabrication point of view. On the one hand, the hydrogel stiffness need to be soft to match that of the ECM found *in vivo* [12] and that can be limiting factor to achieve self-standing hydrogel structures such as microchannels. On the other hand, other factors such as hydrogel swelling or shrinkage due to dehydration must be considered when designing the microfluidic devices. To overcome these limitations, microfabrication techniques have evolved or have been adapted to the requirements imposed by the hydrogel nature. Therefore, in the first part of this review we address the novel technological strategies developed to produce hydrogel microfluidic devices, highlighting their advantages and drawbacks. Then, in the last sections, we spotlight examples of tissue barriers created employing hydrogels in microfluidic platforms and discuss the opportunities, challenges, and perspectives in this fast-paced research field.

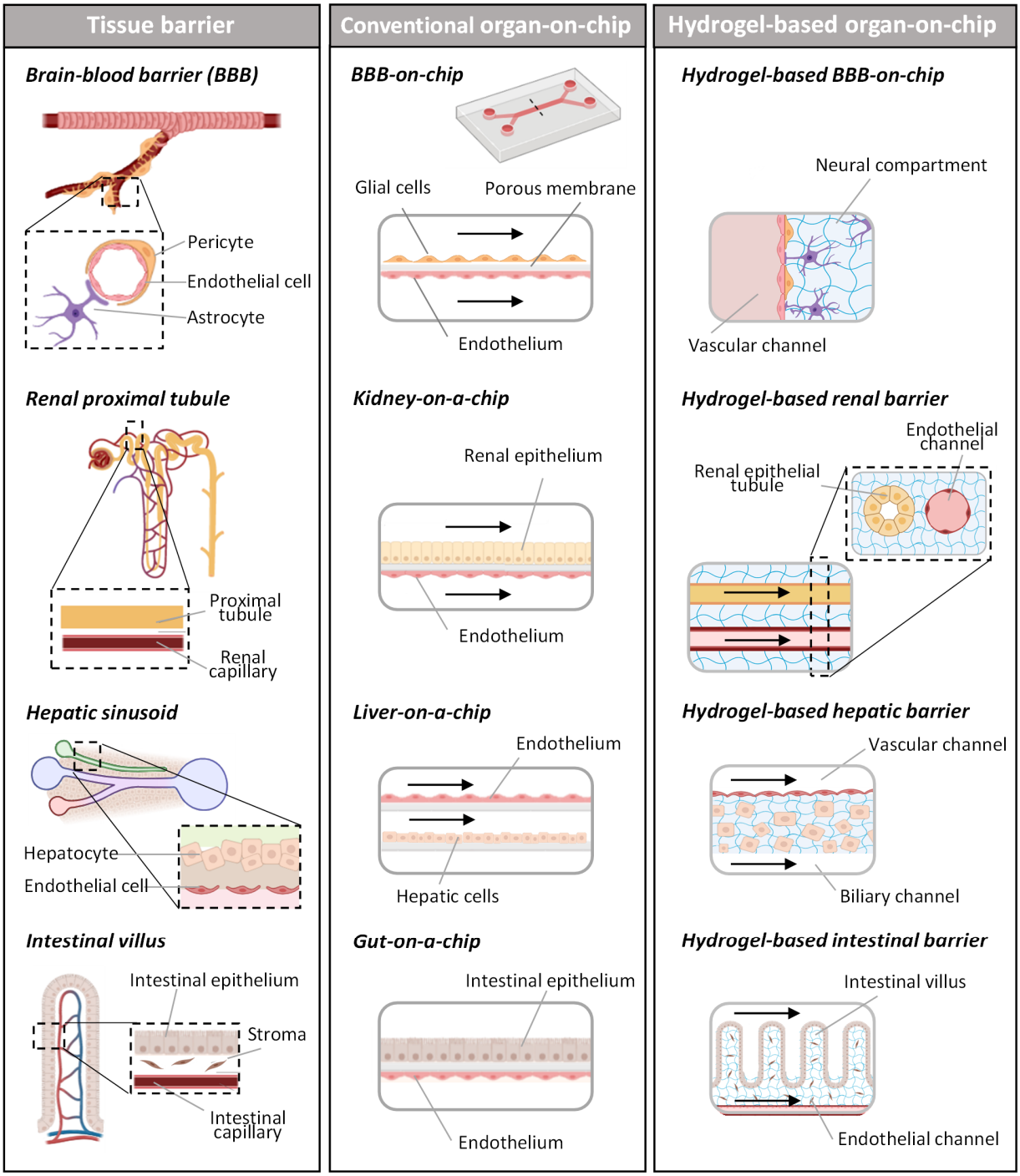


Figure 1. Schematic comparison of the structure of some tissue barriers *in vivo*, conventional PDMS-based organ-on-chip devices and hydrogel-based organ-on-chip platforms aiming to model tissue barrier functions *in vitro* (created with BioRender.com).

MICROFABRICATION TECHNIQUES FOR HYDROGEL MICROFLUIDIC PLATFORMS

Initially adapted from the microfabrication of silicon-based materials, microfabrication tools have been adopted by the bioengineering community to produce tissue barrier models in organ-on-chips [35]. Pushing this concept further, recent advances have enabled the introduction of hydrogels as ECM analogs to potentially revolutionize its impact in *in vitro* studies. The main microfabrication methods used to obtain hydrogel microfluidic platforms for tissue barrier-on-chip applications are briefly addressed in this section, summarized in **Table 1**.

Microfabrication method (Resolution)	**Advantages**	**Drawbacks**	**Hydrogel**	**Hydrogel microfluidic barrier models**	**Refs.**
Soft lithography (100 μm)	Simplicity Compatible with many hydrogels	Only simple shapes Alignment issues Low resolution	Collagen pHEMA GelMA PEG Agarose Fibrin Alginate Collagen/Matrigel	Endothelial barrier Blood-brain barrier Intestinal barrier	16, 29, 30, 36–39
Extrusion-based bioprinting (100 μm)	Fine spatial control of cell-laden hydrogels Reduced number of precursors	High cell shear stress Nozzle-imposed geometrical constraints Poor structural stability	dECM/gelatin GelMA/PEG Gelbrin ECM Gelatin/fibrinogen Collagen Gelatin MeAlg MeHA GelMA	Liver-on-chip Renal proximal tubule model Vessel-on-chip	31, 33, 34, 40–42
Light-based 3D bioprinting (10 μm)	3D complex structures Automated procedure High resolution	Only photocrosslinkable polymers UV/photoinitiator-induced cytotoxicity	PEGDA	Vascular networks	28, 43
Laser-based photopatterning (10 μm)	High resolution *In situ* patterning in cell-laden	Complex setup Expensive equipment	Photodegradable PEG PEG	Vascular networks	44, 45

	hydrogels	Laser-induced cytotoxicity			

Table 1. Summary of the main microfabrication techniques employed to engineer hydrogel microfluidic platforms. Abbreviations: poly(2-hydroxyethyl methacrylate) (pHEMA); gelatin methacryloyl (GelMA); poly (ethylene glycol) (PEG); decellularized extracellular matrix (dECM); gelatin-fibrin ECM (gelbrin ECM); methacrylated alginate (MeAlg); methacrylated hyaluronic acid (MeHA); poly(ethylene glycol) diacrylate (PEGDA).

Soft lithography. Soft lithography, conventionally used to produce PDMS replicas, can be used to produce hydrogel microstructures in a variety of technological approaches [46], thanks to their high flexibility, reproducibility and, in general, their compatibility with a wide range of hydrogels and cell culture requirements These techniques are relatively easy to perform and do not need cleanroom environment or expensive equipment, thus becoming soft lithography-based approaches a popular choice for hydrogel microfluidic platforms. Among them, micromolding is one of the preferred options. In micromolding, a pre-polymer solution is casted onto a usually PDMS mold, which is then removed after polymer gelation. This technique has been used to produce microfluidic channels made from enzymatically crosslinked gelatin [47], thermally crosslinked collagen [48], agarose [49] and photocrosslinkable PEGDA [50], among other materials (Figure 2A). Wires [51], needles [52] and helical springs [53] have also been used as molds. However, the complexity of the hydrogel structures fabricated by micromolding is limited by the geometrical constraints imposed by the mold removal, often requiring multi-step approaches to obtain perfusable constructs. As alternative, sacrificial templating can be considered for demolding, creating molds that can be dissolved after the hydrogel precursor is casted, usually by chemical solvents, water or culture medium [30], combined with high temperatures [54]. Sacrificial

molds are commonly made of carbohydrate glass [30], sodium alginate [20,25], polyvinyl alcohol (PVA) [36], poly(N-isopropylacrylamide) or (PNIPAM) [54], and can be generated by micromolding, electrospinning or 3D printing. This technique has been used to produce complex hollow microchannels, mimicking *in vivo*-like architectures such as vascular networks. For example, PVA sacrificial templating was successfully employed to fabricate hydrogel-embedded branched networks from chemically crosslinked poly(2-hydroxyethyl methacrylate) (pHEMA), thermally crosslinked agarose and photocrosslinked gelatin methacryloyl (GelMA) (Figure 2B) [36]. The resulting fluidic channels, perfused with a bioreactor, supported the culture of human endothelial cells that formed confluent barriers after seven days, while preventing the formation of necrotic cores in cell-laden hydrogels. The limitations of this strategy come from the fact that sacrificial structures need to be mechanically stiff to ensure faithful replicas. In addition, the molds are single use and the mold dissolution process along with the resulting dissolved products must be biocompatible with cell-laden hydrogels to avoid cytotoxic effects. Aside of being used to produce hydrogel-based microfluidic channels, soft lithography approaches have also been exploited to integrate hydrogels within conventional PDMS microfluidic channels. A simple strategy is the fabrication of localized supporting gels by surface tension-assisted patterning [55]. There, a PDMS chip is designed with microposts lining a microchannel where the hydrogel precursor is loaded. This way, the precursor volume is spatially constrained by surface tension, allowing its localized gelation. Typically, the central hydrogel-loaded channel has two parallel outer channels, where culture medium can be perfused. Endothelial or epithelial cells can form functional barriers at the hydrogel-liquid interface and interact with hydrogel-embedded cells. Kamm's group has extensively used this configuration with cell-laden fibrin and collagen hydrogels to recreate BBB models (Figure 2C) [23,37,56]. A similar approach is used in the

commercially available OrganoPlate® system. By capillary force, a collagen solution is filled into the microchannel where chip-integrated bottom stripes spatially confine the hydrogels by meniscus pinning [57]. This technology has been applied to vascular [58] and intestinal studies [38]. Another approach based on surface tension is the so-called viscous finger patterning, developed by Beebe's group, to line the interior of PDMS channels with a layer of hydrogel materials [39]. In this technique, circular hollow lumens are obtained by passive pumping of culture media that displaces the central portion of the hydrogel precursor due to a viscosity gradient between the two fluids (Figure 2D) [59]. After polymerization, cells can adhere and line the inner part of the channel. BBB models where brain endothelial cells were co-cultured with hydrogel-embedded astrocytes and pericytes to study neurovascular inflammation and drug screening have been developed by employing this procedure [60,61]. While the method does not require intricate setups, it is necessary to precisely optimize the process to avoid the complete removal of the precursor or the formation of incomplete structures.

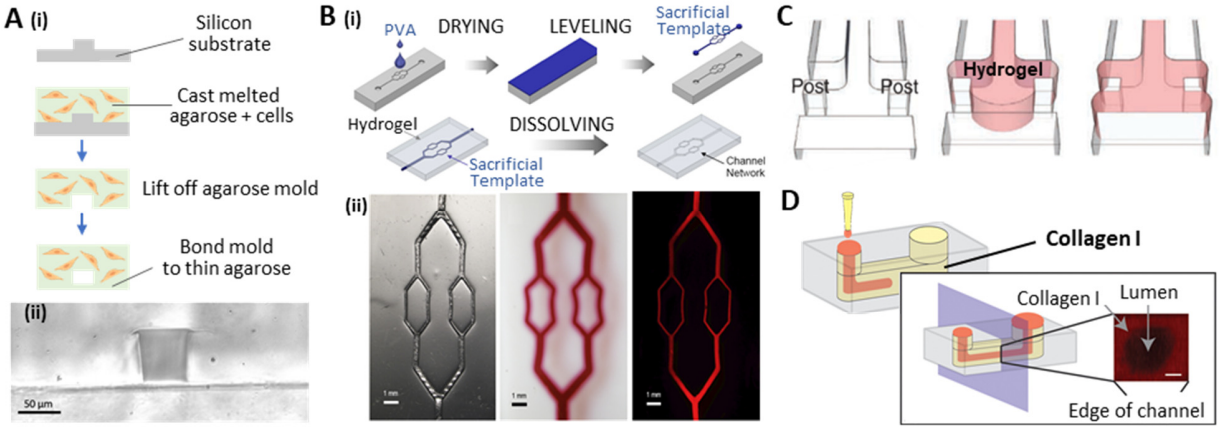


Figure 2. Fabrication of hydrogel-based microfluidics by soft lithography. (A) Replica molding of perfusable hydrogels. **(i)** Mold-based fabrication of cell-laden agarose microchannels **(ii)** Cross-sectional view of hollow channels. Reprinted with permission from ref [49]. Copyright 2007 The Royal Society of Chemistry. **(B)** Sacrificial templating of vascular networks. **(i)**

Schematic of the fabrication process to obtain perfusable hydrogels based on PVA molds. **(ii)** Images of the mold and the microchannels perfused with fluorescent solutions. Reprinted with permission from ref [36]. Copyright 2015 Elsevier. **(C)** Phase-guided patterning of collagen-based hydrogels within a chip channel. The collagen prepolymer solution is inserted into the PDMS channel where it is confined by surface tension through the microposts. Reprinted with permission from ref [55]. Copyright 2012 Springer Nature. **(D)** Schematic process of fabrication of hollow collagen-based channels via viscous finger patterning. Reprinted with permission from ref [59]. Copyright 2012 Sage Publishing.

3D (bio)printing. During the last decade, 3D bioprinting has been consolidated as a popular strategy to produce constructs for tissue engineering employing natural, synthetic or hybrid hydrogels [62,63]. In this review, we will provide a basic description of 3D bioprinting approaches, focusing on the most relevant ones to interface hydrogels with microfluidics for tissue barrier-on-chips.

Extrusion-based 3D bioprinting. In this technique, a cell-laden hydrogel precursor or bioink is loaded into a syringe and extruded through the nozzle by continuous pressure while the nozzle moves along the printing bed, thus creating stacked layers of the extruded filaments. A critical step is the choice of bioinks. Hydrogel precursors must possess the optimal viscosity and good structural stability. Gelatin [64], GelMA [42], methacrylated hyaluronic acid (MeHA) and methacrylated alginate (MeAlg) [41] are considered as appropriate bioinks. They are often copolymerized with PEG derivatives to increase the mechanical stability of the constructs and produce perfusable hydrogel structures [31]. However, relatively high gelation times can still be a problem in terms of mechanical integrity and resolution for the bioprinted structures. On top of that, shear stress caused by the nozzle extrusion can produce cell damage. To overcome these

drawbacks, two main approaches have been proposed: the use of sacrificial inks and the coaxial extrusion of bioinks. The use of sacrificial inks in 3D bioprinting has been proven suitable to create stable hollow structures that can mimic *in vivo* tissue lumens [33,34]. These inks are printed to act as mechanical supports and then are removed once the bioink is crosslinked. Usually, materials with temperature-based gelation properties such as tri-block copolymers of poly(ethylene oxide) poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), trademarked as Pluronic®, are used [42]. For example, 3D renal proximal tubules were formed on cell-laden gelatin-fibrin hydrogels casted on top of 3D Pluronic bioprinted filament (Figure 3A) [34]. Once the sacrificial ink was evacuated by thermal cooling, the resulting hollow network was epithelialized with proximal tubule epithelial cells that formed a polarized epithelium with improved phenotypic and functional properties. On the other hand, coaxial extrusion of bioinks has been recently investigated to generate perfusable multilayer hydrogel structures [31,65,66]. In this approach, the nozzle can extrude several bioink layers simultaneously in a coaxial configuration through concentrically assembled needles. It can combine several hydrogels and crosslinking methods to spatially control the number of layers and shape of the extruded tubules along the process, offering high versatility on the design. For instance, a PEG-derivative polymer (PEGOA) loaded with urothelial smooth muscle cells and GelMA/alginate loaded with urothelial cells were simultaneously extruded. The two-step crosslinking strategy, with $CaCl_2$ and UV light, resulted in cell-embedded tubular structures mimicking the epithelium of the urinary tract (Figure 3B) [31].

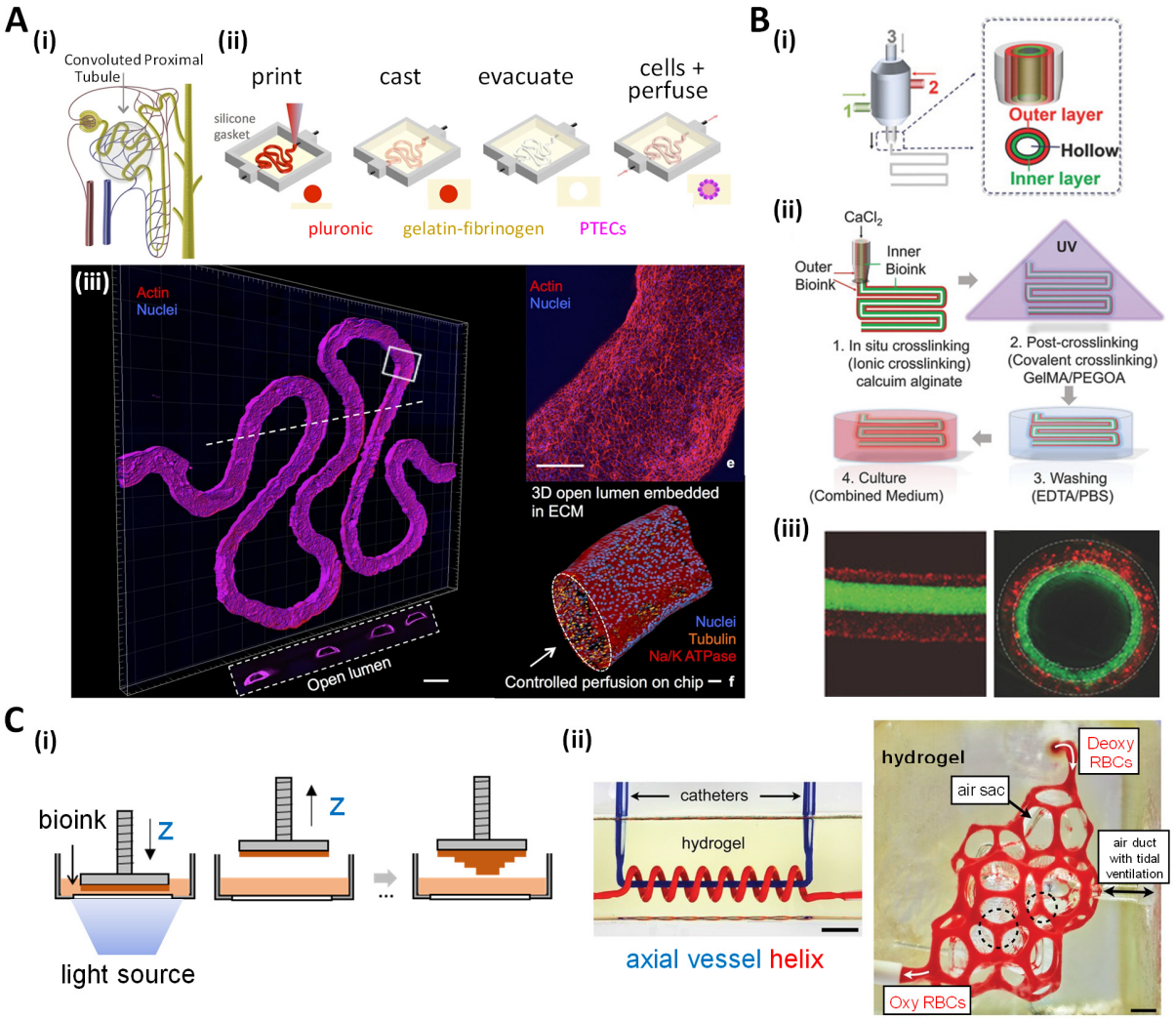


Figure 3. Fabrication of hydrogel-based microfluidics by bioprinting. (A) Sacrificial ink-based bioprinting of perfusable networks. **(i)** Schematic illustration of the renal proximal tubule. **(ii)** Schematic illustration of the fabrication process. The vascular ink that contains Pluronic is printed, and the ink with gelatin and fibrinogen is casted on the perfusion chip. Finally, the fugitive ink is evacuated creating the renal proximal tube (PTECs: primary tubular epithelial cells). **(iii)** 3D rendered confocal images of the printed convoluted proximal tubule (blue: nuclei, red: actin or NaK ATPase, orange: tubulin). Reprinted with permission from ref [34]. Copyright Springer Nature2016. **(B)** Coaxial extrusion-based bioprinting of hollow tubules. **(i)** Schematic

illustration of the coaxial nozzle to generate multilayer structures. **(ii)** Fabrication process of the PEGOA-GelMA-alginate multilayered hollow tubes. **(iii)** Fluorescent images of longitudinal and cross-sectional views of double-layered hollow fibers. Reprinted with permission from ref [31]. Copyright 2018 John Wiley and Sons. **(C)** Fabrication of hydrogel-based microfluidics by light-based bioprinting. **(i)** Schematic illustration of the working principle of digital light projection (DLP) stereolithography to print hydrogel structures based on a layer-by-layer procedure. **(ii)** DLP printing of entangle vessel topologies within PEGDA-based hydrogels to study (left) red blood cell oxygenation and **(**right) airway sac ventilation. Reprinted with permission from ref [28]. Copyright 2019 AAAS.

Light-based 3D bioprinting. Often referred as stereolithography (SLA), light-based 3D bioprinting can produce high-resolution hydrogel structures of photocrosslinkable polymers [67]. It represents a straightforward approach to create self-contained, mechanically robust, and localized hydrogel microstructures for microfluidic platforms by layer-by-layer photopolymerization. Two main strategies are defined depending on the light source employed: laser-assisted stereolithography (laser-assisted SLA) and digital light projection stereolithography (DLP - SLA). In laser-assisted SLA, a focused laser beam raster scans a layer of the hydrogel precursor to induce spatially localized photocrosslinking [68,69]. The laser energy dosage is one of the most important parameters to be considered in this process, as an excess of laser light can induce photoablation of the hydrogel matrix. Moreover, as the laser has to scan all the surface of each layer, printing times can last up to several hours, thus compromising the viability of the embedded cells. To overcome this speed limitation, DLP-SLA has gained increasing attention, since it allows for a full x-y plane photopolymerization per exposure, speeding up the printing process and allowing for an increase in size of the printed designs

(Figure 3C) [43]. Once a layer is photopolymerized, either a vat containing the hydrogel precursor or the supporting stage moves vertically for the next layer printing step. For the resolution, it is important to carefully select the concentration of photoinitiators and the optimal light exposure conditions (the power density of the light and the exposure time applied per printed layer) to avoid cytotoxic effects. In addition, photoabsorbers might be added to the hydrogel-photoinitiator mixture to limit the polymerization process to the desired layer position and thickness by absorbing light excess. Grigoryan *et al.* found that food dyes such as tartrazine and curcumin are effective photoabsorbers to print intravascular networks within PEGDA hydrogels [28]. Using a custom DLP-SLA setup, they achieved remarkable complex structures, such as 3D static mixers, bicuspid valves and entangled helical networks, which were applied in studies of red blood cells oxygenation and blood flow changes during ventilation in vascularized alveolar models (Figure 3C) [28].

Laser-based photopatterning. Photopatterning relies on the photodegradation of small focal volumes of polymer by laser focusing due to multiphoton absorption [70]. By adjusting the laser frequency and the pulse time, the continuous degradation of hydrogel voxels by nano to femtosecond laser pulses allows for precise formation of hollow microchannels without compromising the overall structure [71,72]. Lutolf´s group has used this approach to generate vascular channels and intestinal models on PEG-based, collagen-I and Matrigel hydrogels [44,73]. In one of the studies, new vessel branches could be microfabricated *in situ* from existing ones without damaging the embedded cells, allowing spatiotemporal control over the vascular pattern (Figure 4A) [44]. Hydrogels with photolabile groups have also been developed to induce controlled photoscission of polymeric chains. Arakawa *et al.* employed a cytocompatible laser-based

strategy to create vascular networks within a photosensitive hydrogel [45]. The pre-polymer composition was a mixture of a PEG derivative covalently linked with a synthetic peptide containing a photodegradable moiety, cell adhesion and cleavable motifs. Photopatterned vessel sizes were in the physiological range, with diameters as small as 10 µm and supported endothelial cell attachment and proliferation and the co-culture of stromal cells (Figure 4B) [45]. Despite the progress, the high cost and complexity of the equipment, along with the long fabrication times limit the use of this approach in the bioengineering field.

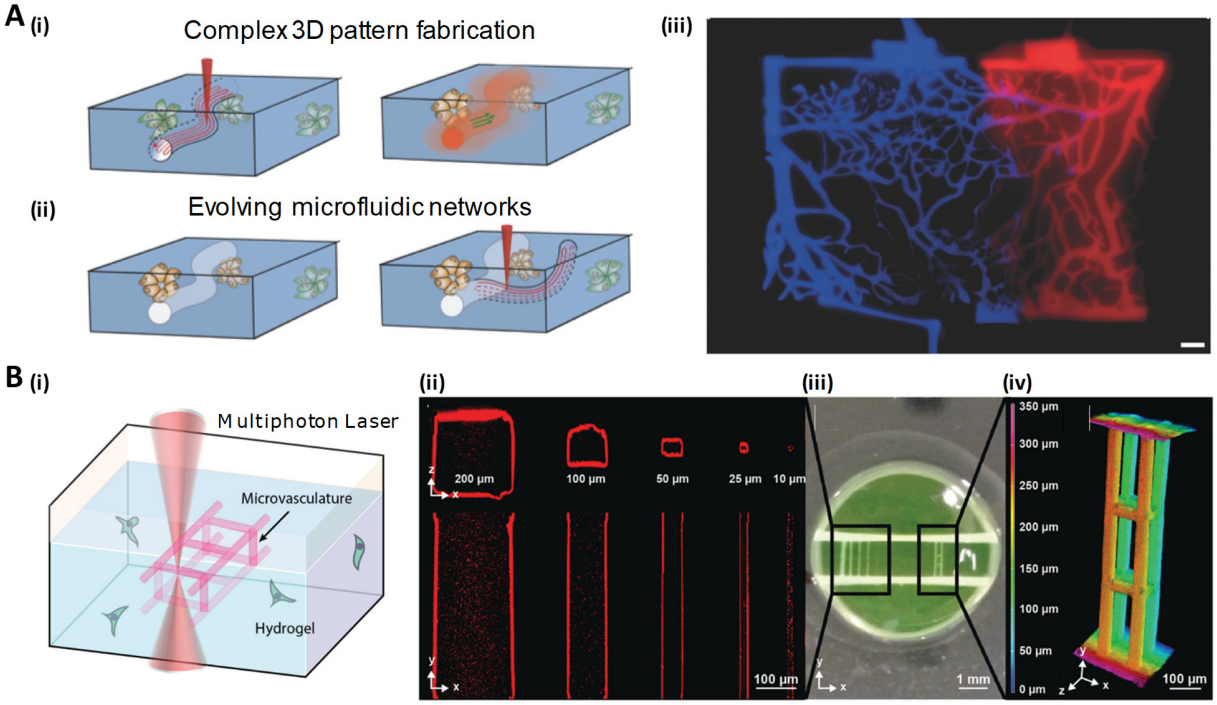


Figure 4. Fabrication of hydrogel-based microfluidics by laser-based photopatterning. (A) Photopatterning of complex microfluidic networks. **(i)** Schematic illustration of the microfabrication of channels in cell-laden hydrogels by laser photodegradation. **(ii)** Spatiotemporal control over the microchannel structure. **(iii)** Photograph of micropatterned capillary network perfused with dyes mimicking arteriovenous circulation. Reprinted with

permission from ref [44]. Copyright 2016 John Wiley and Sons. **(B)** Laser photoscission of synthetic photolabile hydrogels. **(i)** Schematic illustration of the fabrication of microchannels in the presence of encapsulated stromal cells by multiphoton excitation that induces a localized photocleavage of the hydrogel. **(ii)** Cross-sectional view of perfused hydrogel with red fluorescent beads to visualize different diameters of the photocleaved microchannels. **(iii)** Photograph of photopatterned hydrogel with parallel channels (left) and 3D multi-layered channels (right). **(iv)** Colour-mapped 3D representation of fabricated interconnected channels. Reprinted with permission from ref [45]. Copyright 2017 John Wiley and Sons.

MODELS OF TISSUE BARRIERS IN HYDROGEL MICROFLUIDIC PLATFORMS

For tissue barriers, the combination of hydrogels and microfluidics has allowed to overcome some of the limitations of conventional tissue barrier-on-chips based on cell culture membranes [74]. Targeted tissue barriers for organ-on-chip applications have been primarily those used in pharmaceutical research for absorption, distribution, metabolism, and excretion (ADME) studies. The intestinal barrier is responsible for the absorption of oral-delivered drugs, while the vascular system ensures their distribution to other compartments where other barriers such as the BBB can selectively exclude their uptake. Metabolism of the compounds mostly relies on the liver, while excretion of waste metabolites is ensured by renal filtration systems. Advanced hydrogel microfluidic platforms can provide physiological and pathological models of these tissues. On the one hand, this might improve the drug development process, but it might also provide a mechanistic insight of these physiological compartments that can lead to a better understanding of diseases and therapeutic target predictions. In the following sections, we discuss

representative examples of hydrogel microfluidic platforms which have demonstrated to successfully recapitulate key functions of these organ-specific barriers.

Endothelial barrier models. Vascular networks are organized in complex 3D geometries to ensure nutrient and oxygen supply to organ tissues. Blood vessels are lined by endothelial cells that form tight barriers and interact with the surrounding connective tissues to modulate the state of the barrier. Under blood flow, endothelial barriers are exposed to mechanical forces such as lateral blood pressure, which can range from 1 kPa to 15 kPa [75]. The size of blood vessels, which extends from a few micrometers for capillaries to 25 mm for the aorta, and the elasticity of the vascular ECM also affect the endothelial barrier microenvironment. The structural and mechanical tunability of hydrogels allows engineering intricated and complex structures with different dimensions and mechanical properties, mimicking the ones found *in vivo* [76,77]. Two main strategies have been adopted to fabricate vessel-on-chip systems. One relies on the pre-design of vascular channel networks within hydrogels, based on one or several of the microfabrication techniques previously explained, that later on will be seeded with endothelial cells to create functional barriers [78–80]. The main advantage of using microfabrication techniques is the precise tailoring of the geometry and the size of the channels, thus controlling key dynamic parameters such as fluid flow and solute gradients. For example, micromolding was applied to create collagen-based microvessel networks to form endothelial barriers. Under dynamic conditions, it was shown that fluid shear stress and vessel geometry modulate the formation and morphology of cell-secreted von Willebrand factor bundles and fibres (Figure 5A) [78]. The second strategy pursued to fabricate vessel-on-chip devices relies on the intrinsic properties of endothelial cells, which can spatially self-assemble to form vascular networks when seeded within 3D matrices [81–83]. These matrices are formed from natural polymers such as fibrin [24], that

can be remodelled by the cells while being mechanically stable to avoid their collapse during cell culture. The integration of hydrogels within microfluidic devices has been exploited to study the multi-step process of vascular formation that happens *in vivo.* To that end, a microvascular model-on-chip combined the capillary network formation and engineered vessels to better recapitulate vasculogenesis [82,84]. A PDMS chip was designed and micromolded to obtain multiple central chambers for hydrogel loading where the capillary network self-assembled, lined by two outer laminin-coated microchannels mimicking the artery/vein. Endothelial cells together with perivascular fibroblasts formed a lumenized network within the fibrin gels that was tightly interconnected to the engineered artery/vein channel and had *in vivo*-like barrier properties (Figure 5B) [84]. This microvascular chip represents a model to study the transport across the endothelial barrier in a more physiologically relevant microenvironment than the traditional Transwell assays. Furthermore, the hydrogel channel allows the incorporation of perivascular cells found in the surrounding tissues such as fibroblasts, pericytes or smooth muscle cells that dramatically enhances the potential of hydrogel-based microfluidics as *in vitro* models of endothelial barriers [17]. These complex microvascular networks can also serve as platforms for disease modelling [32,42]. For example, Zhang *et al.* developed a thrombosis-on-chip model by means of sacrificial bioprinting [42]. Pluronic was used to generate GelMA hollow channels where endothelial cells formed a confluent monolayer. Perfusion of whole blood supplemented with calcium chloride, induced both endothelial damage and formation of blood clots and thrombi, which were cleared from the lumen by a thrombolytic agent. By using fibroblasts loaded within the GelMA channels, this chip also modelled fibrotic thrombosis. Fibroblasts migrated within the hydrogel towards the blood clots, releasing ECM proteins and forming fibrotic microtissues within the vessel lumen (Figure 5C) [42]. Furthermore, employing hydrogel microfluidic devices

allows for the visualization of the endothelial barrier permeability in pathological situations such as haematological disorders or infectious diseases like malaria [32]. Microvessels made of agarose-gelatin via micromolding, were exposed to patient-derived sickle red blood cells. By using fluorescence dyes and hydrogel porosity, increased barrier permeability and vessel obstruction were observed in the channels (Figure 5D) [32].

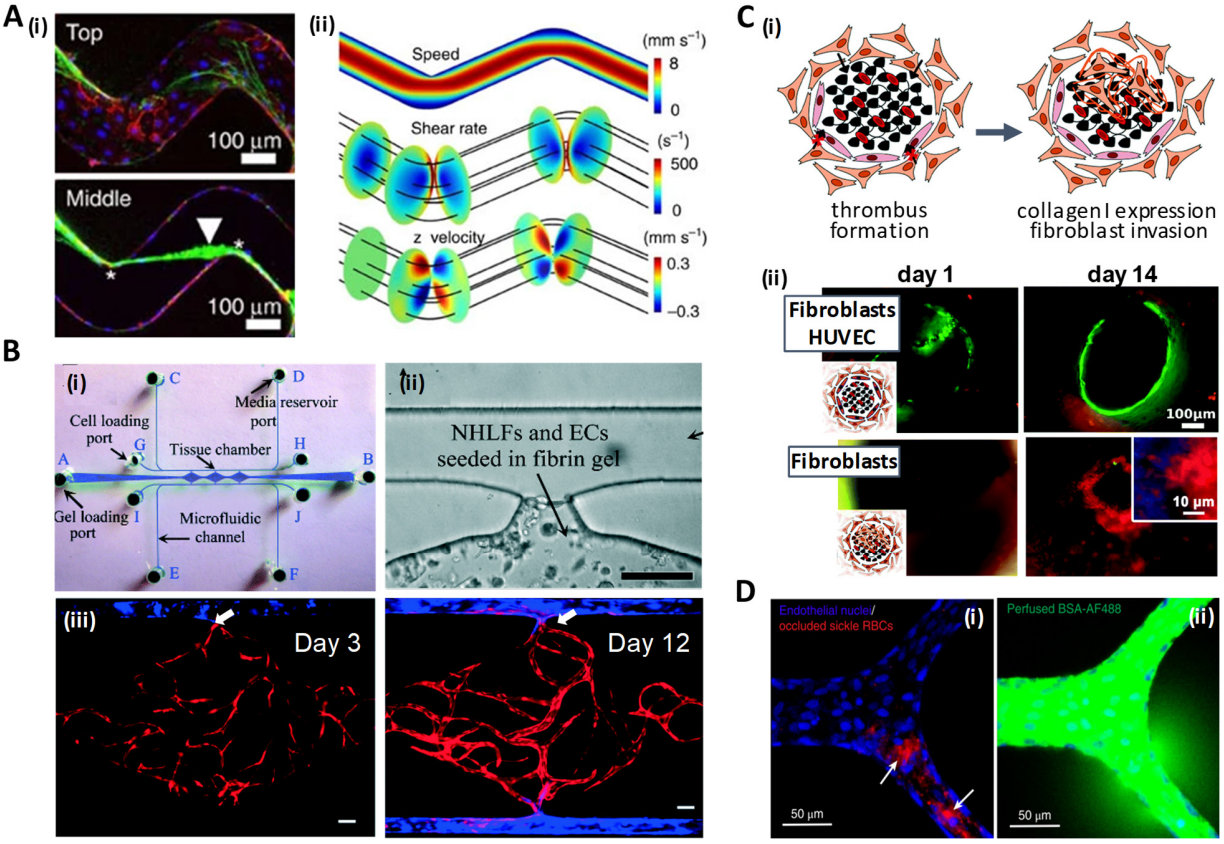


Figure 5. Endothelial barrier models. (A) Micromolded perfusable collagen-I channels to study endothelial cell secretion of von Willebrand factor (VWF) proteins. **(i)** Confocal images at different z levels to visualize VWF fiber formation (green staining) within a tortuous channel covered by endothelial cells (blue and red staining). **(ii)** Computational simulation of fluid flow within the vessels to correlate channel geometry and shear stress with VWF strand morphology

and location. Reprinted with permission from ref [78].Copyright 2015 Springer Nature. **(B)** Self-assembled vascular channels. **(i)** Top view photograph of the chip with the three channels for gel and cell loading, and media perfusion. **(ii)** Fibrin hydrogel loaded in the microfluidic chamber with endothelial cells (ECs) and fibroblasts (NHLFs). **(iii)** Self-formation of a vascular network in a multi-step manner (white arrows indicate interconnection between the blue-stained endothelial cells located at the outer channels and the red-stained vascular network embedded in the hydrogel). Reprinted with permission from ref [84]. Copyright 2016 The Royal Society of Chemistry. **(C)** Vascular thrombosis-on-chip. **(i)** Schematic representation of thrombus formation in a vessel lumen. **(ii)** Confocal images of collagen-I deposition (red) by hydrogel embedded fibroblasts with and without endothelial cell barrier (green) to model the early stage of thrombus formation and the formation of a fibrotic clot over 14 days. Reprinted with permission from ref [42]. Copyright 2016 The Royal Society of Chemistry. **(D)** Study of sickle red blood cell disease with hydrogel microfluidic chips. **(i)** Confocal images of endothelialized channels (DAPI staining) occluded with sickle red blood cells (red staining) **(ii)** and co-localized leakage of perfused fluorescent protein (BSA-AF488). Reprinted with permission from ref [32]. Copyright 2018 Springer Nature.

Brain-blood barrier (BBB) models. The central nervous system is a challenging target for therapeutic drugs. The BBB protects the neural tissues from toxic compounds in a very efficient manner by selectively restricting the uptake of small molecules and drugs. This BBB barrier is formed by endothelial cells lining the capillary walls, astrocytes ensheathing the walls, and pericytes embedded in the basement membrane. To develop efficient therapeutic strategies that selectively cross the BBB, a better understanding of this multicellular and complex barrier is required. Conventional neurovascular studies rely on *in vivo* animal models and *in vitro* static

cell culture platforms. Both approaches show limitations, either in terms of low throughput and ethics concerns, or in the lack of mimicking the cell microenvironment, respectively. To mitigate these limitations, conventional microfluidic systems have been used. They consist on PDMS chips where a semi-permeable membrane supports endothelial cells and neural cells on each side while been perfused [85]. Even though this configuration recreates the fluid flow the BBB is exposed to, it does not allow the formation of 3D architectures where different neural cell types can interact with each other [86]. Thus, there is a growing interest in including ECM analogues within these models to obtain more biomimetic systems. Novel hydrogel microfluidic platforms have shown to support the co-culture of different types of neural cells under flow. Surface-tension patterning is the preferred microfabrication technique to shape cell-laden hydrogels in BBB models because of its simplicity. It has been employed to establish an *in vitro* neurovascular model where endothelial cells, derived from induced pluripotent stem cells, pericytes and astrocytes were embedded in a fibrin matrix (Figure 6A) [23]. Endothelial cells formed self-assembled perfusable microvessels with low permeability and strong tight junctions. Direct interactions of the microvessels with astrocytes and pericytes improved cell barrier maturation and function, compared to endothelial cell-only models, as shown in other studies [37,61]. These improved properties were recently exploited to study PEG-coated nanoparticle transport across the barrier [56]. Using time-dependant image analysis of nanoparticle distribution inside and outside the microvasculature, the impact of size and functionalization of the nanoparticles on their permeability could be assessed, proving the suitability of this *in vitro* model for pre-clinical drug screening evaluations. In addition to its relevancy in drug delivery, the BBB is involved in pathological processes such as tumour metastasis. By including hydrogels in the microfluidic device, tumour cell extravasation in the central nervous system could be

studied [87]. In this work, replica molding was employed to fabricate a multiplexed PDMS microfluidic chip consisting of a vascular channel and another one in which a cell-laden collagen hydrogel was loaded. The extravasation of lung and breast cancer cells across the formed BBB was observed, reproducing similar results of brain metastasis seen *in vivo* (Figure 6B). This chip was used for testing chemotherapeutic drugs approved for brain cancer treatment as a pre-clinical screening tool. By targeting hydrogel-embedded glioma cells, different efficacy results were obtained for each drug in the presence of the BBB.

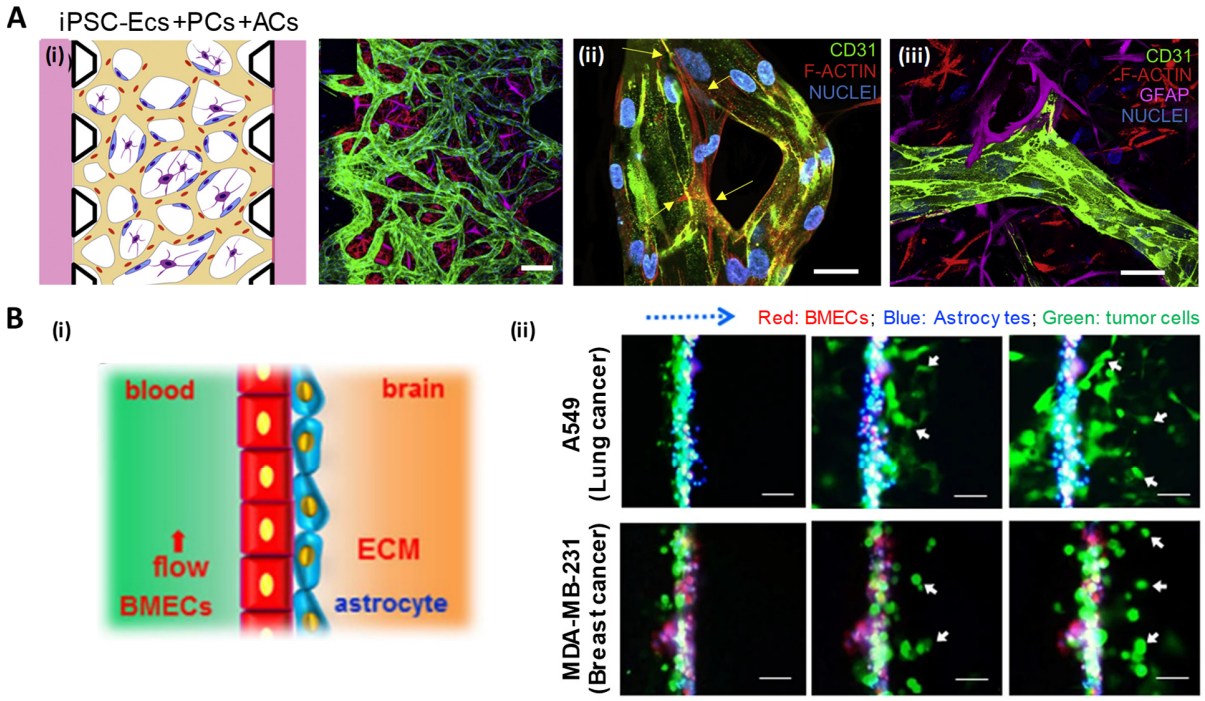
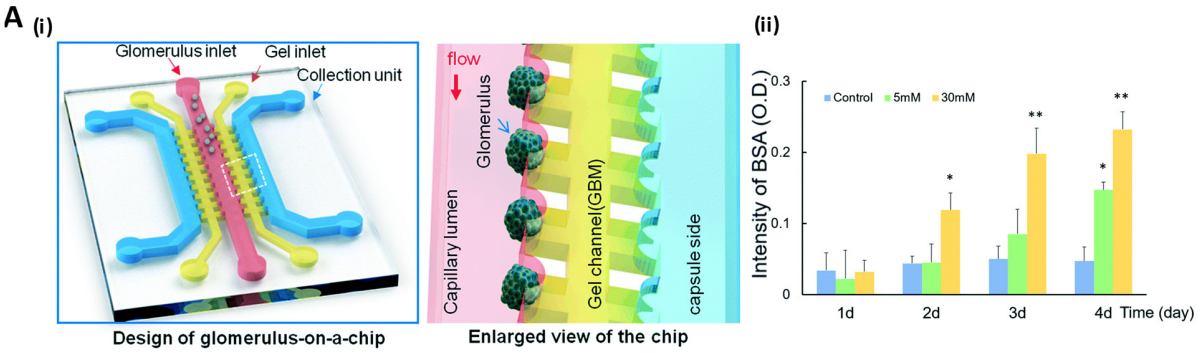


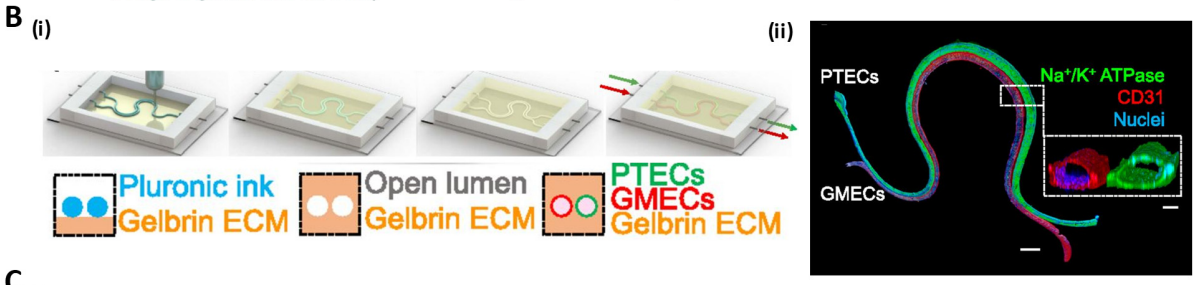
Figure 6. Brain-blood barrier models. (A) Neurovascular brain-blood barrier model. **(i)** Schematic view of the monoculture of induced pluripotent stem cell-derived endothelial cells (iPSC ECs), co-cultured with pericytes (PCs), and tri-cultured with astrocytes (ACs) within a fibrin hydrogel on a chip. **(ii)** Cross-sectional view of hollow microvessels (green) surrounded by pericytes (blue) and **(iii)** by astrocytes (magenta). Reprinted with permission from ref 23. Copyright 2018 Elsevier. **(B)** Brain-blood barrier model to study metastatic brain tumors. **(i)**

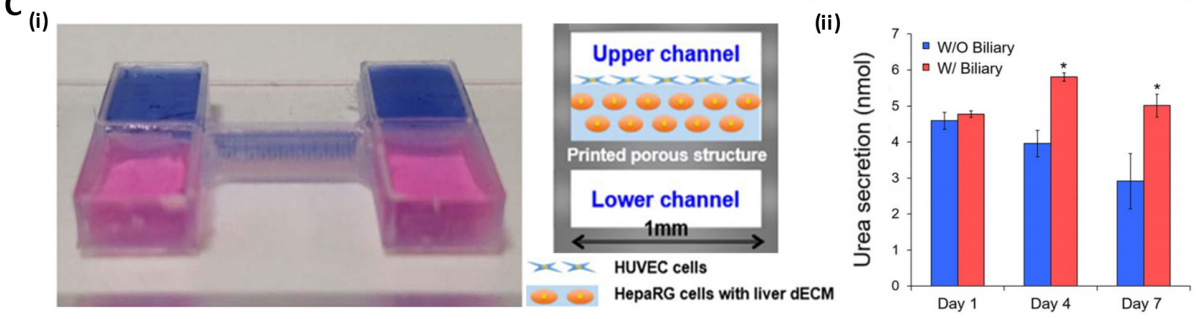
Schematic illustration of the device design to allow the co-culture of brain microvascular endothelial cells (BMECs) and astrocytes. **(ii)** Time-lapse fluorescence images of the migration of breast and lung cancer cells across the BBB model. Reprinted with permission from ref [87]. Copyright 2016 Springer Nature.

Renal barrier models. Kidneys are responsible for the filtration and reabsorption of specific solutes in a selective manner according to their size and charge. Their functional units, called nephrons, regulate the exchange of these solutes through specific barriers. Among these, glomeruli are networks of blood vessels which are encapsulated in a cup-like sac and located at the proximal site of the kidney, where the filtrate enters the tubular nephron. Glomeruli have been reproduced on-chip to construct a model of diabetic nephropathy, a vascular pathology induced by high blood glucose [88]. Micromolding was used to produce a PDMS chip, consisting of a capillary channel mimicking the vascular lumen, a hydrogel channel representing the glomerular basement membrane, and a collection channel that acts as the glomerular capsule (Figure 7A). The hydrogel channel was filled with Matrigel to support the growth of primary glomerular microtissues. The collection channel allowed collection of renal filtrates for further characterization. Under high glucose medium perfusion, the glomerular barrier showed higher permeability values and protein leakage was observed, reproducing the *in vivo* pathological responses of the glomeruli to hyperglycaemia (Figure 7A) [88]. Another important part of the nephron is the proximal tubule, which takes an essential role in nutrient transport of the renal filtrate from the nephron to the bloodstream. Different studies with hydrogel microfluidic platforms have model it by generating hollow perfusable structures [89,90]. Convoluted proximal tubules were produced using 3D bioprinting techniques to fabricate their complex shape. For instance, twisted hollow channels within enzymatically crosslinked gelatin/fibrin matrix were

produced by sacrificial templating using Pluronic, allowing epithelial cells to grow and form a functional barrier under flow [34]. Recently, the same approach was employed to mimic the proximal tubule-endothelial barrier through bioprinting of two adjacent microchannels (Figure 7B) [33]. Reabsorption of proteins such as albumin and glucose were confirmed with this model. Furthermore, exposing the renal epithelial barrier to a hyperglycaemic state induced a dysfunction of the endothelial barrier, suggesting a crosstalk between the two barriers.


A
(i)
Glomerulus inlet
Gel inlet
Collection unit
flow
Glomerulus
Capillary lumen
Gel channel(GBM)
capsule side
Design of glomerulus-on-a-chip
Enlarged view of the chip
(ii)
Intensity of BSA (O.D.)
Control
5mM
30mM
1d
2d
3d
4d
Time (day)


B
(i)
(ii)
Pluronic ink
Gelbrin ECM
Open lumen
Gelbrin ECM
PTECs
GMECs
Gelbrin ECM
PTECs
GMECs
Na+/K+ ATPase
CD31
Nuclei


C
(i)
Upper channel
Printed porous structure
Lower channel
1mm
HUVEC cells
HepaRG cells with liver dECM
(ii)
Urea secretion (nmol)
W/O Biliary
W/ Biliary
Day 1
Day 4
Day 7

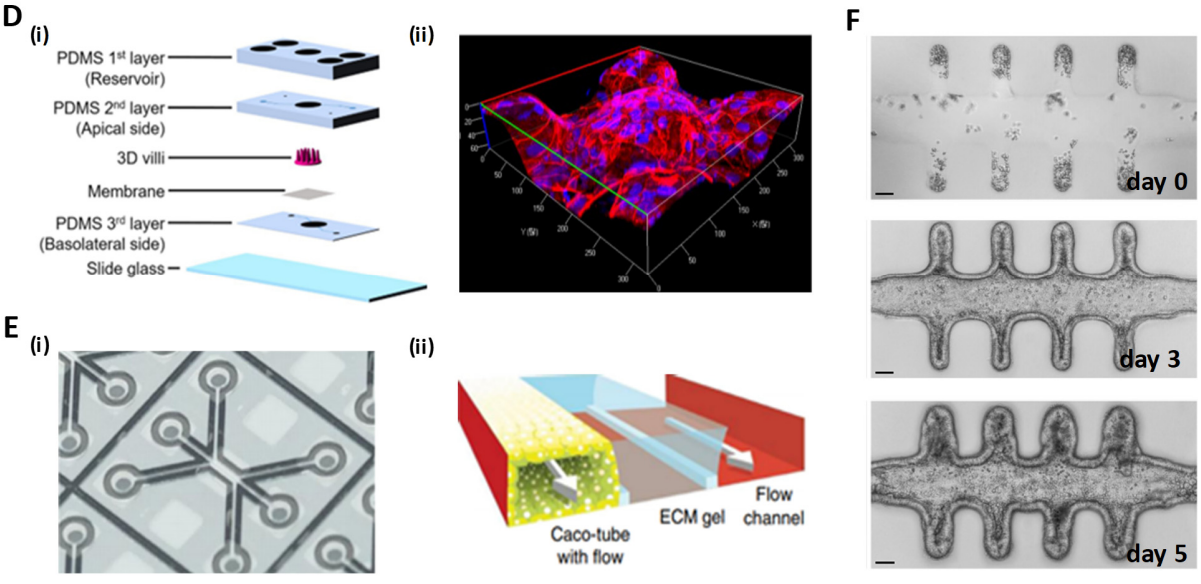

D
(i)
PDMS 1st layer
(Reservoir)
PDMS 2nd layer
(Apical side)
3D villi
Membrane
PDMS 3rd layer
(Basolateral side)
Slide glass
(ii)
E
(i)
(ii)
Caco-tube
with flow
ECM gel
Flow
channel
F
day 0
day 3
day 5

Figure 7. Epithelial barrier models. (A) Renal glomerulus-on-chip. **(i)** Schematic illustration of the microchip device with a capillary channel, a gel loading channel and a collection channel mimicking the compartments of the renal glomerulus. **(ii)** Bovine Serum Albumin (BSA) filtration rate through the glomerular barrier under different glucose concentrations to quantify barrier permeability. Reprinted with permission from ref [88]. Copyright 2017 The Royal Society of Chemistry. **(B)** Vascularized proximal tubule model. **(i)** Schematic view of the bioprinting process of the channels using sacrificial inks. **(ii)** Immunostaining image of the glomerular microvascular endothelial cells (GMECs, red) and proximal tubule epithelial cells (PTECs, green). Reprinted with permission from ref [33]. Copyright 2019 National Academy of Sciences. **(C)** Bioprinted hepatic model. **(i)** Image of the 3D printed device with an upper and lower channel for co-culture of endothelial cells (HUVEC) and hepatic cells (HepaRG). **(ii)** Urea secretion levels with and without lower biliary channel. Reprinted with permission from ref [40]. Copyright 2019 IOP Publishing. **(D)** Microfluidic intestinal model with a 3D villous-like scaffold. **(i)** Detailed view of the gut-on-chip. **(ii)** Confocal image of intestinal enterocytes (Caco-2 cells) cultured on top of the hydrogel. Reprinted with permission from ref [91]. Copyright 2017 Springer Nature. **(E)** Tubular gut-on-chip. **(i)** Photograph of the multiplexed three-lane microfluidic chip OrganoPlate®. **(ii)** Schematic view of collagen-based scaffold to support Caco-2 cell barrier formation within the chip. Reprinted with permission from ref [38]. Copyright 2017 Springer Nature. **(F)** Formation of an intestinal organoid tubule on a 3D crypt-shaped hydrogel of collagen/Matrigel during 5 days under perfusion. Reprinted with permission from ref [73]. Copyright 2020 Springer Nature.

Hepatic barrier models. The liver sustains critical physiological functions within the human body such as detoxification, drug metabolism, bile acid production and protein synthesis.

Exchange of metabolites and oxygen occur at the liver sinusoid, where hepatocytes interact with a defenestrated barrier of endothelial cells. In pre-clinical studies, hepatoxicity tests are a standard procedure to assess the risks of discovered drugs on human health. However, several drug withdrawals due to their hepatoxic effects have shown the limitations of current toxicological models [92]. Due to this, great effort has been put to develop functional liver-on-chips that could be implemented in the pre-clinical testing pipeline. Conventional microfluidics has proven success cases in this field [93]. However, they lack an *in vivo*-like 3D matrix where hepatocytes can develop and interact directly with the endothelial barrier. Thus, hydrogels are well suited to reproduce the spatial architecture of hepatic tissue barriers. Bioprinting has been proven to be a useful technique to spatially define the heterotypic interactions between hepatic cells and vascular endothelial cells. This technique was employed to print cell-laden hydrogels within a polycaprolactone (PCL) microfluidic chip, allowing the localized formation of an endothelial barrier on top of the 3D hepatocyte-embedded hydrogel [64]. The composition of the cell-laden bioinks was a mixture of gelatin and collagen type I. The bioprinted liver-on-chip showed high cell viability and increased albumin and urea synthesis, essential functions of the liver, compared to cell culture in static conditions. The same model was updated by including a biliary-like lower channel (Figure 7C) [40]. In this case, liver dECM was used to embed the hepatic cells and support the endothelial barrier on top. In this configuration, liver functionalities such as albumin and urea secretion levels, along with drug metabolism capabilities were further improved. The liver-on-chip model also showed sensitivity to drug toxicity analysis.

Intestinal barrier models. The small intestinal epithelium organizes itself as a 3D compartmentalized barrier, with finger-like structures called villi and tissue invaginations called crypts. Cell differentiation and proliferation occurs along the crypt-villus axis in a dynamic time-

dependant manner. The main functions of the intestinal barrier are to act as a selective absorption site of nutrients and to act as a first defence barrier against pathogens. To properly perform these functions, the multicellularity and 3D architecture of the epithelium are very important parameters, as well as the basement membrane and stromal compartment forming the lamina propria of the tissue [94]. In addition, flow conditions are highly relevant for cell microenvironment and barrier function. For this reason, 3D hydrogels reproducing key structural features of the intestinal epithelium have been adopted to study this barrier *in vitro* [95,96]. For instance, replica molding has been used to produce villi-like microstructures using collagen-I (Figure 7D) [91]. A mechanical stage was then used to expose cells to gravity-driven fluid flow. The combination of flow-induced shear stress and 3D topography enhanced cell polarization and key cellular functions such as metabolic activity and permeability compared to static cell cultures. In another approach, hydrogels have been included in the microfluidic channels mimicking the gut tube to account for the lamina propria compartment with the focus placed on high-throughput testing and easy visualization of the barrier leakiness. A popular approach is surface tension-based patterning of collagen-I, which was loaded and shaped on a central channel, supporting the intestinal epithelial monolayer. This technology has been employed to create enterocyte cell tubules in a multiplexed microfluidic platform for high throughput testing of compounds on barrier integrity using fluorescent dyes (Figure 7E) [38]. The same technology has also been used to model intestinal bowel disease *in vitro* [97]. In there, epithelial cells were exposed to inflammatory cytokines, inducing cell barrier leakiness. It was shown that this inflammatory-induced disruption could be modulated with specific inhibitors, showing its potential to design therapeutic targets. Intestinal organoids represent a very useful modelling tool of the intestinal epithelial barrier. However, their closed lumen and short lifespan restricted their applications and

there has been a continuous effort to find bioengineered solutions to these limitations. In a recent outstanding publication, a perfusable micropatterned hydrogel allowed the formation of a tube-shaped epithelium that maintained homeostasis after long-term culture (> 30 days) [73]. A collagen/Matrigel mixture was polymerized into a microdevice and patterned into a microchannel with crypt-like shapes using laser photoablation. Cells derived from intestinal organoids covered the hydrogel microchannel and self-organized following the crypt-like pattern (Figure 7F). The accessible and perfusable lumen allowed homeostasis with the continuous removal of waste as well as the modelling of a long-term parasite infection. This hydrogel microfluidic chip, that also supported the embedding of stromal cells such as intestinal myofibroblasts and macrophages, has the potential to mimic not only the intestinal barrier but a variety of epithelial barriers.

CONCLUSIONS AND FUTURE PERSPECTIVES

Hydrogel microfluidic platforms have become a rising trend for advanced *in vitro* cell culture models, further increasing the biological relevance in comparison with conventional PDMS-based microfluidic systems. The increasing demand for tissue barrier models that account not only for the cellular barrier but also for the tissue microenvironment, together with the continuous advances on the biomaterial and biofabrication fields, have accelerated the innovation in hydrogel microfluidics. The variety of available biomimetic hydrogels offers a wide choice of functional materials that can both be precisely tuned to display specific properties and support long-term cell culture within the matrix or on their surface. On the other hand, the development of high-resolution microfabrication techniques such as 3D bioprinting has also driven the progress in this field. With these new tools, 3D hydrogel structures and perfusable microchannels

for *in vitro* applications can be fabricated with high precision, combining soft biomaterials, that can faithfully recapitulate cell-ECM interactions, with the spatiotemporal control of fluid flow, which modulates collective cell behaviour. Altogether, hydrogel microfluidics allow to generate *in vitro* models of tissue barriers with multiple cell types and *in vivo* physiological properties such as immunocompetence. These model barriers would provide for improved predictive capabilities of toxicity and efficacy studies as well as for better disease models. Despite this, there are still some challenges that need to be addressed such as the standardization of processes and technologies, the regulatory validation and the low throughput of these platforms.

Looking into the future, we identify an array of opportunities that feed from the continuous advances in the fields of biofabrication, biomaterials and sensing technology. 4D bioprinting arises as the next-generation biofabrication technique, where the concept of time is integrated in the 3D bioprinting. The use of stimuli-responsive hydrogels that can change their properties upon external triggering would mimic the dynamic physical microenvironments that tissues experience *in vivo*. Specific microstructural features of these soft polymers, such as their stiffness and geometry, can be modified in a time-controlled manner upon changes in the environmental parameters. Another important aspect is the quantification of the cell barrier function in real time. For this, new sensing strategies are needed to better understand tissue organisation in physiological and pathological conditions within the hydrogel device. The integration of sensors in hydrogel-based microfluidics entails extra challenges due to the softness of the substrate and the incompatibility with the standard techniques used for the sensor fabrication. Finally, the use of patient-derived cells would facilitate the technology translation into the clinics, providing an effective tool to develop targeted treatments for precise and personalized medicine.

AUTHOR INFORMATION

Corresponding Author

Dr. Elena Martinez

Institute for Bioengineering of Catalonia (IBEC), Department of Electronics and Biomedical Engineering, University of Barcelona (UB), Barcelona, Spain

https://orcid.org/0000-0002-6585-4213

Email: emartinez@ibecbarcelona.eu

Dr. María García-Díaz

Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain

https://orcid.org/0000-0002-4794-5437

Email: mgarcia@ibecbarcelona.eu

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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