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Fabrication advances of microvasculature models on-chip

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Abstract
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Despite the technological advances of the last decades, drug development remains a lengthy and costly process with uncertainties still associated with the poor predictive power of the in vitro and animal models. To address this limitation, microphysiological systems have been introduced in an attempt to increase the biological relevance of in vitro devices. One of the current challenges in MPS is the integration of a vasculature network to sustain 3D cultures to closely mimic human physiology. This thesis proposed a new strategy to recreate a more representative vasculature system directly on-chip. As a first step, the 2-photon polymerization was investigated as a 3D printing technique to recreate structures with cell-relevant feature size and resolution. Subsequently, the 2-photon polymerization 3D printing was combined with micromolding to recreate a multi-hydrogel vasculature network integrated on-chip for cell culture. The synergy of the two methods ensured the generation of a high-fidelity multi-hydrogel scaffold for cell co-culture. To preserve the delicate cell culture while still ensuring the sample manipulation for monitoring and analysis, a customized microphysiological system carrier with an integrated heating and perfusion system was also developed. Finally, the possibility of tuning the properties of the 3D-printed hydrogel by controlling the printing parameters was investigated to guide glioblastoma cells to a vascularized compartment. Overall, the thesis not only demonstrated the fabrication versatility of 2 photon polymerization for a vasculature model directly on-chip but also showed the benefits in integrating microphysiological systems on a carrier.

Keywords: 2-photon polymerization 3D printing, multi-hydrogel structure, vascula-ture model, microphysiological systems, organ-on-chip

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To my family

List of Papers

This thesis is based on the following papers:

I. **Federico Cantoni**, Daniel Maher, Eugenia Bosler, Stefan Kühne, Laurent Barbe, Dirk Oberschmidt, Christophe Marquette, Rafael Taboryski, Maria Tenje, Ada-Ioana Bunea. Round-robin testing of commercial two-photon polymerization 3D printers. *Additive Manufacturing.*

II. **Federico Cantoni**, Laurent Barbe, Hannah Pohlit, and Maria Tenje. A perfusable multi-hydrogel vasculature on-chip engineered by 2-photon 3D printing and scaffold molding to improve microfabrication fidelity in hydrogels. *Submitted Manuscript.*

III. **Federico Cantoni**, Gabriel Werr, Laurent Barbe, Ana Maria Porras, Maria Tenje. (2021). A microfluidic chip carrier including temperature control and perfusion system for long-term cell imaging. *Hardware X*; *Volume 10,2021, e00245,*

IV. **Federico Cantoni**, Laurent Barbe, Ananya Roy, Grzegorz Wicher, Karin Forsberg-Nilsson, Maria Tenje. Microengineering a cell co-culture platform for glioblastoma cell migration and intravasation studies. *Manuscript*

Author's contributions

The author's contributions to the papers included in this thesis are as follows:

I. Most of structure design, planning writing and methodology Performed all the experimental work, optimization and data analysis for one printer.

II. Most of conceiving, methodology, planning, data analysis and writing. Performed all experimental work.

III. Most of conceiving (except the heating system), methodology, data analysis, writing and experimental work. Part of planning.

IV. Most of conceiving, methodology, planning, data analysis and writing. Performed all the experiments.

Other work by the author

Review article:

I. Maria Tenje, **Federico Cantoni**, Ana Maria Porras Hernández, Sean S. Searle, Sofia Johansson, Laurent Barbe, Maria Antfolk, Hannah Pohlit. (2020). A practical guide to microfabrication and patterning of hydrogels for biomimetic cell culture scaffolds. *Organs-on-a-Chip 100003*

Relevant peer-reviewed work presented at local and international scientific meetings (presenting author underlined)

I. MPS (2023-06-30): Multi-hydrogel microvasculature by 2-photon polymerization and scaffold micromolding on-chip for perfusable cell co-culture. Federico Cantoni, Laurent Barbe, Hannah Pohlit, and Maria Tenje. *Poster presentation*

II. SMILs (2023-05-31): Combining 2-photon 3D printing and scaffold micromolding for engineering of a perfusable microvasculature on-chip. Federico Cantoni, Laurent Barbe, Hannah Pohlit, and Maria Tenje. *Oral presentation*

III. MNE (2022-09-19): Comparison of commercial systems for two-photon polymerization direct laser writing. Authors: Federico Cantoni, Laurent Barbe and Maria Tenje, Ada-Iona Bunea. *Poster presentation*

IV. SMILs (2022-06-06) Uppsala. Poster title "2-photon polymerization – benchmarking commercial printers to access the nano scale in 3D printing" Authors: Federico Cantoni, Laurent Barbe and Maria Tenje, Ada-Iona Bunea. Poster presentation

V. EUROOCs (2021-07-02), Uppsala (online due to Coronavirus): Poster title"A vessel with an integrated perfusion and heating systems for long-term imaging of microfluidic chips" Authors: Federico Cantoni, Gabriel Werr, Laurent Barbe and Maria Tenje. Poster presentation

VI. EUROOCs (2020-07-09) Twente (Online due to Coronavirus): Poster title "Hydrogel membrane fabricated by 2 photon polymerization in a microfluidic chip for bio-interface investigations" Authors: Federico Cantoni, Ayan Samanta, Hannah Pohlit, Jöns Hilborn, and Maria Tenje. Poster presentation

VII. EUROOC (2018-05-24, Stuttgart): Poster title "Methacryl modified gelatin hydrogels for co-culture of cells to mimic blood-brain barrier" Authors: Federico Cantoni, Ayan Samanta, Jöns Hilborn, and Maria Tenje. Poster presentation

Contents

Abbreviations

2PA: 2-photon ablation

2PP: 2-photon polymerization

AFM: atomic force microscopy

CAD: computer aided design

CAM: computer aided manufacturing

DLP: digital light processing printers

DMD: digital micro-mirror device

ECM: extra cellular matrix

FDA: Food and Drug Administration

FITC: fluorescein isothiocyanate

FRAP: fluorescence recovery after photobleaching

HLF: human lung fibroblast

HUVEC: human umbilical vein endothelial cells

iPSs: induced pluripotent stem cells

OoC: organ-on-chip

MPS: microphysiological systems

NA: numerical aperture

PDMS: polydimethylsiloxane

SEM: scanning electron microscopy

SLA: stereolithography printing

TEER: transepithelial resistance

Introduction and research aim

Launching a new drug in the market is a costly and lengthy process that involves several stages of development[1,2,3,4], Figure 1. The highest failure of the potential candidates is observed in the transition from animal testing to clinical trials due to toxicity or lack of efficacy[5,6].

The screening of chemical compounds as drugs has been performed on a wide range of animals and organisms varying in size and biological complexity and, consequently, physiological relevance to humans. *In vivo* models including C.elegans[7], Zebra fish[8], mice[9], dogs[10] and primates[11] currently represent "the gold standard" for drug development. In the first decade of the 21st century, around 27 million vertebrate animals were used for biomedical research in the US every year[12]. Despite the invaluable contribution to the understanding biological mechanisms and the development of new treatments, there is a consensus in the scientific community that the poor predictability of *in vivo* testing delays drug development while raising ethical concerns related to animal treatments [13]. Animal models are costly due to the specialized techniques to perform the experiments and present a lengthy development over months and years. Moreover, the physiological complexity of an organism entails the influence of unpredictable variables and an inherent non-homogeneity between samples that might affect experiment outcomes[2]. Finally, the human-animal physiology discrepancy causes animal findings to not be directly applicable to humans[14]. Consequently, the introduction of new strategies to design more predictive models is required to counteract the trend and ensure a better understanding of human physiology and more efficient drug development[15].

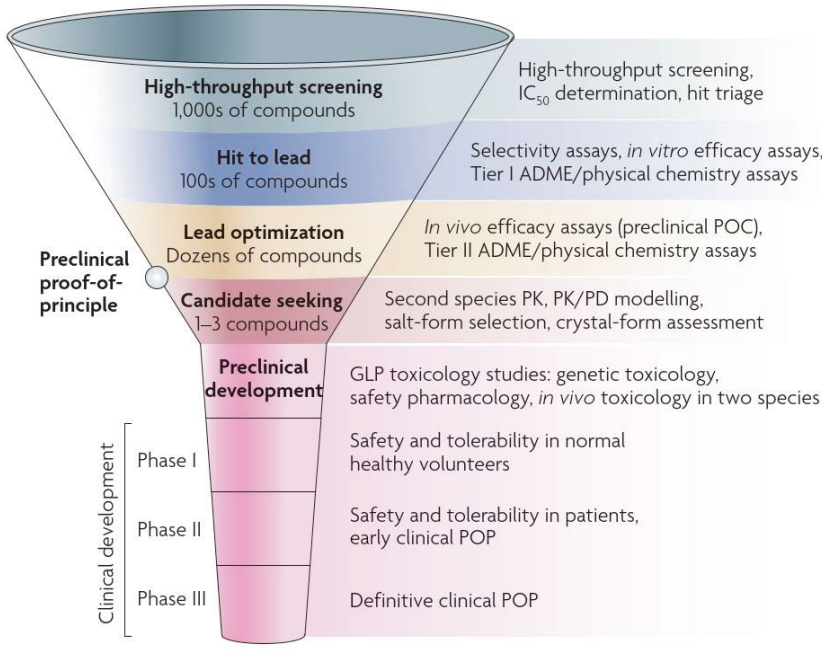

Figure 1: Schematic of the development process for small-molecule drugs. Reproduced from16 with permission from Nature Publishing Group.

As an alternative, *in vitro* models have been conceived to replicate human physiology outside the body. The earliest and still most commonly used *in vitro* substrates are flat plastic-based dishes or flasks with cell culture medium as the counterpart of full blood[17,18]. Higher throughput and standardization were introduced with well plates. The subsequent integration of porous inserts in the well plates provided a two-compartment system divided by a membrane for the first studies of transport across a barrier[19,20] as well as cell migration and invasion[21].

Nowadays, *in vitro* models serve as a pre-screening tool to provide initial indications of the potential efficacy and cytotoxicity of the drug candidate before the *in vivo* testing. Despite the simplicity and reliability, such systems offer poor control over fundamental biological aspects and rely on the 2D cell culture considered far from the human physiology environment[22,23]. Ideally, drug candidates would be narrowed with more predictive models that still preserve a user-friendly interface and cost-effective fabrication.

The recent advances in microfabrication technology and material science have attempted to address such over-simplified models with the introduction

of *in vitro* systems replicating *in vivo* microenvironments more closely to better correlate to human physiology[24,25]. Such *in vitro* models, also known as microphysiological systems (MPS) or Organ-on-chip (OoC), have the potential to recreate in a centimeter footprint device the fundamental components of human physiology to understand the biology of *in vivo* systems and eventually play a role in the investigation of diseases and drug development [26–29]. This technology is currently contributing to a gradual reduction of animal testing reliance with the long-term aim of replacing *in vivo* models.

One of the current biggest challenges that the scientific community is facing is the integration of a functional vasculature network into MPS[30]. The vasculature has an essential role in diseases[31], the delivery of therapeutics[32] and maintaining organ function[33].

In this context, the presented thesis had the aim to develop new strategies to recreate more representative microvasculature *in vitro* models. To achieve this, in **paper I,** the 2-photon polymerization (2PP) 3D printing technique was investigated to generate structures with physiologically relevant feature size and resolution. Then, **in paper II,** the 2-photon polymerization was implemented on a microfluidic platform to develop a new fabrication strategy to create multi-hydrogel vasculature-like structures with the possibility of perfusion. In **paper III,** a carrier to simplify the manipulation of the microfluidic platform was developed to enhance the chip transfer between the incubator and microscope. Finally, in **paper IV**, the influence of the 3D printing parameters over the migration of cells was investigated.

The scope of the PhD thesis is summarized in the following points:

- Investigate 2PP for cell-scale fabrication on-chip,
- Introduce a new fabrication strategy to create a multi-hydrogel structure replicating a microvasculature system,
- Provide microfluidic platform designs to integrate a hydrogel scaffold on-chip for perfusion, screening of hydrogels and printing parameters,
- Improve the printing fidelity with gelatin-based ink for 2-photon applications,
- Develop carriers to simplify the handling of microfluidic platforms,
- Investigate how to tune the hydrogel properties with 2PP 3D printing to control cell invasion.

A summary of the above-mentioned papers can be found in Chapter 5.

Chapter 1: Microphysiological systems

This chapter introduces the vasculature network function, structure and fundamental role in the evolution of *in vitro* models to obtain biologically relevant systems. In addition, the fabrication technologies and the challenges in the MPS are presented in terms of design, perfusion system, handling and transition from 2D to 3D culture.

Towards more relevant *in vitro* models

The selection of chemical compounds as new drugs in the pharmaceutical industry starts with the *in vitro* models pre-screening to investigate the cytotoxicity and efficacy of the candidates[5]. Conventional *in vitro* models rely on testing the potential treatments with cell cultures on flat and rigid substrates. Such 2D-based platforms have the advantages of being highly standardized, compatible with automated labs for high throughput and budget-friendly with no need for highly specialized personnel, i.e., Petri dishes or well plates[34]. These platforms provide a first prediction of the chemical compound-cell interaction to identify the promising drug candidates before animal testing.

Animal models offer a higher biological relevance to investigate more in detail the drug candidate efficacy on a complex biological system closer to the human body.

Although such protocol is currently the golden standard in drug development, it is nowadays widely recognized that 2D in-vitro systems are too simplistic models that fail in recapitulating the key aspects of the *in vivo* environment[14,35]. Consequently, chemical compounds might be wrongly discarded or accepted for the next step, removing possible candidates or generating a waste of resources, respectively.

On the other hand, *in vivo* testing is recognized to not only provide a poor correlation with human physiology but also requires expensive facilities to maintain the animals[4]. These limitations not only have economic consequences but also affect the health of the patients by delaying the development of new treatments.

As a solution to the poor predictability of the current technologies, MPS were introduced with the ambition to provide *in vitro* models recreating the key functions of human physiology[15]. To achieve this, all the MPS share four

fundamental properties: i) A cell arrangement in 3D replicating the native tissue. ii) A multicellular system to recreate the tissue complexity. iii) The integration of biologically relevant biomechanical forces to stimulate tissue development[36]. iiii) The presence of a vasculature-network to sustain the cell culture. The synergy of these characteristics aims to bridge the gap between the traditional 2D *in vitro* cell culture and the complexity of organisms to accelerate drug development and drastically reduce animal testing[37,38].

After the introduction of the concept of animal on-chip by Michael Shulers *et al.* in 2000[39], the MPS technology experienced remarkable growth following the lung-on-chip system developed by Dongeun Huh *et al.* in 2010[40], Figure 2.

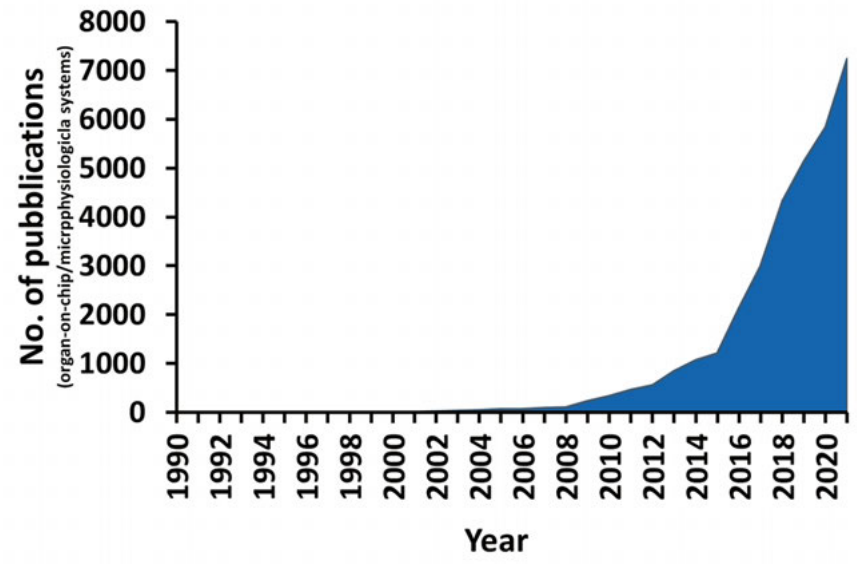

Figure 2: Increase in academic publishing in microphysiological systems (keywords: "Organ-on-a-chip and microphysiological systems"). The amount of publications from the search on Web of Science was plotted from 1990 to 2021.

In the last decade, MPS have been developed for applications in cell biology[41], drug development[42] and personalized medicine[24] by replicating the basic physiology of different tissues including vasculature[43,44], bone[45–47] intestine[48–50], liver[17,51–54], kidney[55–58], lung[59,60,61], heart[62–64], blood vessels[65–67], placenta[68], reproductive system [69,70]and blood-brain barrier[71–74]. The presence of such a heterogeneous pool of systems for different applications highlights the versatility and complexity of MPS technology. However, to sustain the cell culture, all these systems require the implementation of a vasculature-like network that represents one of the current challenges.

Vasculature system

All organ functions are regulated by the cooperation of cells that necessitate nutrients and the exchange of signaling molecules to communicate and interact. In the human body, this transport is mediated by the vasculature system, a hierarchical network of blood vessels to provide each cell with nutrient supply, oxygen, cell-cell signals and waste removal[75]. The vasculature is a fundamental system in the human body with a key role in tissue development and wound repairing[76]. Finally, vasculature dysfunction has also implications in diseases including diabetes[77,43] and tumors[27,78].

Cells are situated within 200 μm from a blood vessel, according to the diffusion limit of the tissue, with a limited number of exceptions, including cartilage[79]. In the demanding task to reach every single cell in the organism, the vasculature forms an intricate network with blood vessel diameter sizes ranging from cm (large vessel) down to a few μm (capillaries). In such a network, capillaries play an essential role as being where the blood-cell exchanges occur[80].

The vascular system is in continuous evolution to adapt to changes induced by tissue development, regeneration or diseases. The generation of new blood vessels is classified into two distinct processes: vasculogenesis and angiogenesis. Vasculogenesis indicates the formation of blood vessels starting from precursor cells, the angioblasts. This vascularization process mainly occurs during the development of the embryo[81]. Instead, angiogenesis consists of new blood vessels sprouting from an existing vessel. Contrary to vasculogenesis, angiogenesis happens during the entire lifespan and it is triggered by the biosignalling of hypoxia and inflammatory states[82].

The fundamental unit block of blood vessels is endothelial cells (ECs) that constitute the inner lining of blood vessels. The EC phenotype is highly heterogenic across the different tissues in the human body[83]. The tissue-specific barrier function of EC is mediated by supporting cells such as pericytes, mesenchymal stem cells, astrocytes, vascular smooth muscle cells and fibroblasts that promote, stabilize and regulate vessel formation and maturation in the different organs[84].

A wide range of microvasculature models has been conceived to replicate blood vessel networks in organs including kidneys[53] and brain[85]. The available strategies are presented more in detail in Chapter 3. In **paper II**, a new cell-friendly fabrication process was presented to create a vasculature model that enabled the co-culture of ECs and fibroblasts as supporting cells.

The ECs cell type plays a key role in determining the relevance of the *in vitro* models. The cell selection depends on the tissue biological mechanism of interest and the availability of the cell type. Consequently, the fit-for-use approach should be chosen to generate the model that satisfies the aims of the investigation.

The variety of cell origins used for vascular biology is classified as cell lines, primary cells and stem cells. The choice of the cell type should be carefully considered, as contributes to the biological answer that the system can provide.

Cell lines are genetically modified cells introduced starting in the 70s after the development of recombinant DNA technology for gene manipulations *in vitro*. Cell lines present a high number of generations before drifting from the original phenotype, a less stringent cell culture protocol and high proliferation when compared to primary cells and ISP cells[36]. These cells also represent a robust model with a well-defined behavior, well-established culture and analysis protocols resulting suitable for reproducible studies[86]. In addition, cell lines from either human or animal sources are readily available since easily accessible from different cell banks.

Despite these advantages cell lines lack the expression of all functions limiting the application for pilot studies or the validation of microfluidic systems. In **paper III**, the mouse brain endothelial cell line (b.End.3)[87] was used to validate a microfluidic chip carrier for continuous transfers between the incubator and the microscope.

Primary cells are collected directly from human tissues. These cells express the full phenotype of human cells providing a relevant model for *in vitro* studies. Human umbilical vein endothelial cells (HUVECs) have been largely used for vasculature *in vitro* models since easily accessible and economical to extract from the umbilical cord that is usually discarded as waste. HUVECs can generate a 3D vascular network *in vitro* and present relevant EC junctional proteins, adhesion molecules and receptors for growth factors [88]. In **paper II**, HUVECs were co-cultured with human lung fibroblasts (HLF) to obtain an engineered 3D microvasculature model on-chip. Fibroblasts were chosen as playing a key role in producing extracellular matrix (ECM) components and growth factors for vessel maturation[84,89]. In **paper IV,** patiend-derived human glioblastoma cells were used to investigate how printing parameters can affect cell invasion in the hydrogel.

Despite the capability to represent the human phenotype, patient-to-patient variability in terms of donor genetics and age can influence the primary cells causing batch variability and poor model reproducibility. In addition, the recovery of some cells is challenging because of the limited availability of tissues. These cells have a limited life span with genetic drift and decay of proliferation over time, limiting the number of passages. A possible solution that might combine the large availability and standardization of cell lines while ensuring the expression of all the primary cell capability are stem cells.

Stem cells represent a recent potential solution to provide a reliable and constant source of cells for biological investigations. Stem cells are classified as either adult or pluripotent stem cells (iPSs). Adult stem cells are directly extracted from the tissue and can differentiate into specialized cell types of the tissue of origin while iPSs are reprogrammed mature adult cells to stem-like

behavior[90]. Stem cells are induced to differentiate in the desired mature cell types by providing specific stimuli[91]. Despite IPSs being a viable remedy to cell sourcing, the current limitation is the lengthy and expensive differentiation process with the additional problem of no standardized procedure for cell differentiation.[36]

MPS-based cell studies

An MPS can be defined as a microfabricated device recreating the physiological cell environment to model the functional units of human tissues. The conceiving and fabrication of any MPS require an interdisciplinary approach in which engineering, biology, chemistry and materials science overlap.
In the following sections, the main considerations when designing and fabricating MPS are discussed.

Microfluidics

Because of the scope of this thesis, more emphasis will be put on the implementation of microfluidics for the generation of MPS. For a more comprehensive overview, the interested reader is referred to the following review [92].

Microfluidics investigates the behavior and manipulation of fluids in devices presenting channel-like geometry with at least one dimension in the microscale range[93]. The device fabrication with features size comparable to the cellular scale enables single-cell resolution monitoring while reducing the amount of expensive reagents and valuable cells.

When moving from macroscale systems, well plates or bioreactors, to microfluidic-based cell studies, MPS, different heat and mass transfer behaviors have to be taken into account[94]. Channels in the micrometre scale experience a laminar flow regime, movement of liquids without turbulences, creating steady streamlines. The orderly nature of the liquid flow ensures accurate control and the possibility to model the fluid flow with a simulation software[95], Figure 3. In **paper II,** the flow inside a microfluidic chip was studied to calculate the shear stress on the cultured endothelial cells.

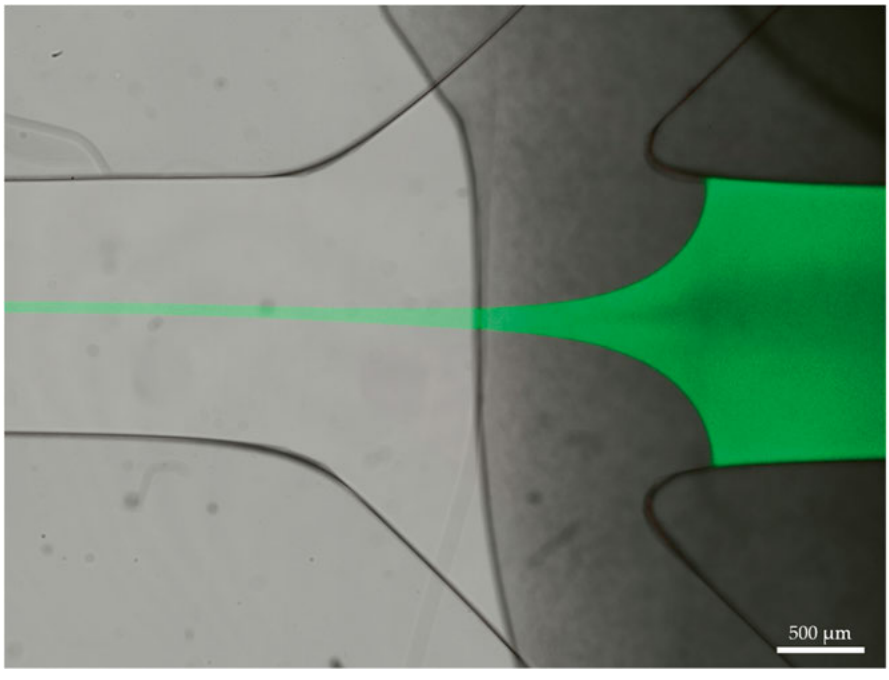

Figure 3:Hydrodynamic flow focusing of a FITC-dextran solution (Green) in a microfluidic device.

The high area-to-volume ratio of the miniaturized channels enables quick heat transfer and uniform temperature across the system shifting the dominant mass transport from convention to diffusion. The synergy of the dominant diffusion mass transport and laminar flow ensures accurate manipulation of molecules to generate controlled gradients of nutrients, growth factors or drugs for cell studies[96]. These aspects combined with optical transparent substrates provide an ideal platform for real-time monitoring with high-resolution imaging of the biological system evolution, **paper II**, **III** and **IV**.

In miniaturized systems, the material surface properties have a higher impact than in macroscale systems. Consequently, the choice of the material as well as the processing technique are fundamental in determining the microfluidic platform properties[97].

Chip fabrication

Different materials and relative processing technologies have been investigated for MPS fabrication. In the platform design process, a trade-off of several aspects not limited to chip design, costs, fabrication time and biocompatibility is required before starting the fabrication of the microfluidic platforms.

As the application determines the technology choice, the strength and limits are presented for different fabrication processes[98].

Silicon and glass

The well-established semiconductor fabrication technology was a natural choice for the first microfluidics device generation[97]. Systems made out of silicon and glass presented mature processing protocols to reach submicron feature size in optical transparent platforms with no absorption of small molecules[99]. The bioinert surface of the silicon-glass-based devices can be improved by surface functionalization to enhance cell adhesion[100]. The technology also benefits from the possibility of integrating sensors on-chip for the continuous monitoring of cell culture[101].

However, such devices present laborious connection techniques and poor gas permeability, limiting the essential exchange of O_2 and CO_2 gases required for cell culture. Moreover, the fabrication process is performed in a cleanroom environment that requires specialized technicians and expensive equipment and reagents entailing high production costs, low throughput and limited use[97].

PDMS and glass

Microfluidics applications experienced a remarkable increase with the introduction of soft lithography in the late 90s[102]. In this strategy, the semi-conductor technology process serves to generate a mold for castable material for replica molding[103]. Replica molding represents a user-friendly and cost-effective technique that do not require cleanroom facilities for faster device production when compared to silicon-glass-based technology. The mold is generated by photolithography with a high-resolution mask to selectively expose a photosensitive polymer layer. In the development process, the exposed area is dissolved (positive photoresist) or maintained (negative photoresists) transferring the mask pattern to the substrate. Finally, a castable polymer is poured on the mold to replicate the pattern[104], Figure 4.

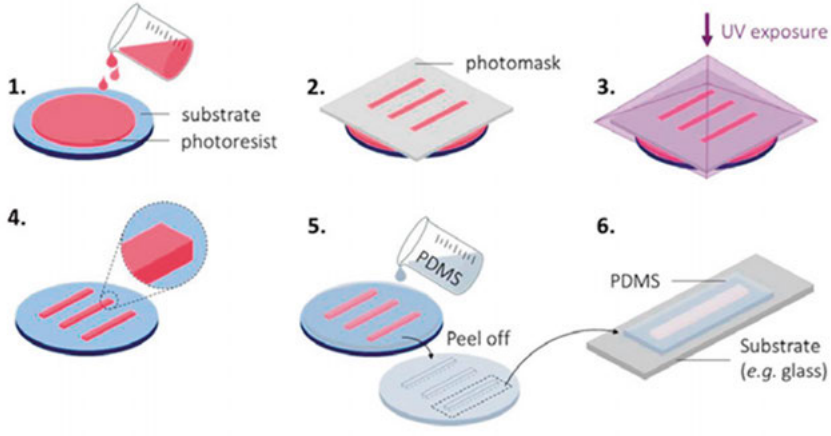

Figure 4: Schematic of soft lithography. The mold fabrication 1-4. The polymer casting and bonding to glass for obtaining a microfluidic chip105

Among the available castable materials, the rubber-like polydimethylsiloxane (PDMS) emerged for the capability to replicate sub-micron features while ensuring an easy and stable bonding to glass surfaces by plasma activation. PDMS also presents other advantages including flexibility, biocompatibility, and optical transparency for high-quality imaging[106,107]. In addition, the rubber-like behaviour allows for active components actuated for instance by air pressure[108]. Lastly, tubing connections for pump systems are easily generated by punching holes with biopsy punchers of different sizes according to the need.

Contrary to glass-based technology, PDMS is gas permeable, enhancing gas exchange between the environment and the internal cell culture. The polymer permeability might cause cell media evaporation with the consequent change in ion concentration and pH shift inside the platform[109]. Such limitation is solved by maintaining the microfluidic platform in a humidified area such as an incubator or top-stage incubator. The PDMS structure porosity also implies the permeability of small hydrophobic molecules limiting applications for drug testing due to the drug uptake of the material or channel cross-contamination [110]. A mitigatory strategy involves coating treatments working as impermeable barriers[111]. However, microfluidics can also benefit from the PDMS sponge-like structure for removing small bubbles from the channel by previously storing the platform under a vacuum[112]. This technique was used in **papers II** and **IV** to remove air bubbles introduced during the ink injection.

Cost-effective strategies for PDMS-glass devices include the fabrication of microfluidic chips by stacking sub-millimetre layers of PDMS and coverslips. The channels and connections in the coverslips and PDMS layer can be

quickly generated by laser and plotter cutting, respectively. Despite the obtained devices displaying poor surface quality and feature sizes and resolutions above hundreds of μm, the cleanroom-free process combined with the possibility to create multilevel platforms in a few hours makes this strategy very attractive for prototyping. In **papers II**, **III** and **IV** this strategy served to realize a microfluidic chip for the culture of b.End.3 cells and HUVECs. The fabrication of microfluidic chips presenting a cover slip for both the bottom and the top resulted essential for the adhesion of 3D printed hydrogel structures to the chip. On the other hand, the use of PDMS prevents the cross-linking of the hydrogel due to the thin layer of high oxygen concentration that is formed close to the material surface[113].

Recent fabrication technologies

MPS are complex systems with several technical and biological challenges. As an alternative to traditional semiconductor technology, new fabrication strategies have been investigated to provide tools with more accessible and versatile materials[114,115]. 3D printing has been explored for both direct microfluidic platform generation and mold fabrication. Microfluidic platforms can be created in a one-step process in a cost-effective approach ideal for rapid prototyping[116]. Alternatively, small complex geometries are directly printed on-chip to add functionalities to the system Figure 5. In **paper I**, the 2-photon polymerization technique was investigated as a possible technology to create shapes and geometries relevant to molds and complex 3D microfluidic devices with submicron resolution[117].

Despite the additive manufacturing potential, the material cytotoxicity, surface quality and optical transparency in the wavelength spectrum of interest are aspects that still need to be addressed.

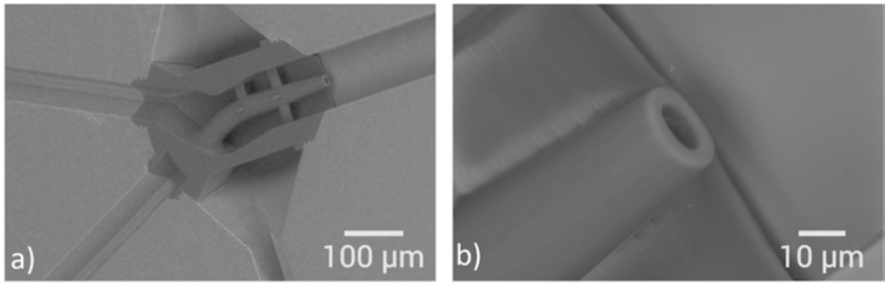

Figure 5: Nozzle for flow-focusing directly printed inside a microfluidic chip a). Details of the 3D printed nozzle are displayed in a). Reproduced from118 with permission from the Royal Society of Chemistry.

Cell culture in miniaturized systems

Besides the choice of the device material and fabrication technology, some other factors must be considered early in the device design process to obtain the desired outcomes when culturing cells. These steps present challenges both in terms of biological and technical aspects[119].

Platform design

Many MPS include channels to access the biological sample for nutrient supply and waste removal. The nutrient supply by flowing liquids exerts mechanical forces, shear stress, on the cells in the culture[120]. Therefore, the channel geometry needs to be designed to create shear stress suitable for the cells. Simulation software are a valuable tool to model the shear stress in case of complex geometries presenting splitting, corners or restrictions, as investigated in **paper II**.

The flow mode and the related cell media refreshment periodicity in the microfluidic chip should be carefully evaluated as this directly influences the removal of waste products and the supply of nutrients. Such an aspect is critical for microfluidic platforms as cell media volume-cell amount ratio is drastically smaller than for the traditional *in vitro* systems[34].

The platform design should not present features that cause air entrapment in the system. Air bubbles change the flow and clog channels blocking the nutrient supply while generating harmful shear stress when moving on the cell culture[121,122]. Similar effects are observed for contaminants such as fibers. Therefore, bubble traps and filters are commonly implemented in the case of perfusion[122]. The bubble trap can be directly integrated into the microfluidic chip, **paper II,** or into the cell media reservoir, **paper III**. In addition, the connection strategy should be leak-free and prevent the introduction of contaminants.

The increasing number of sensors on-chip for continuous monitoring of the cells without the need for a microscope, including TEER electrodes[123], heating systems and temperature detectors[124] impose a further feasibility analysis regarding all the system electrical connections.

For an interested user, the commonly used and commercialized microfluidic platform designs are enlisted in this review[125].

Cell medium

With the understanding of cell-to-cell interaction importance for tissue development and function[126,127,128], MPS have experienced an increasing complexity to accommodate co-culture systems. Each cell type requires a culture medium with specific factors for optimal growth and gene expression. The cell medium is a substitute for blood that maintains the main blood factors relevant for the cells while simplifying the microscopy imaging and preventing clogging[129].

Different approaches can be followed according to the cell type and combination. The cell medium can be a mixture of the cell media in a well-defined ratio that can be screened a priori to determine the optimal conditions. Alternatively, the system is supplied with just one cell medium that satisfies most of the cell needs[130].

In the case of **paper III**, the monoculture of b.End.3 was performed with the supplier-suggested cell media. For **paper II**, a microfluidic platform with cell co-culture was designed. Since the project aimed to study the formation of an endotheliazed layer, only the HUVEC cell medium that presented the optimal growth factor for the endothelium formation was perfused as previously shown by other studies[131].

New challenges in cell media composition are expected as the number of interconnected platforms or more complex biological systems like organoids are growing in applications[132].

Perfusion systems

In the human body, cells often experience a pulsatile or continuous flow pattern, conditions that can be replicated in a microfluidic device but hardly in traditional *in vitro* systems[133]. Especially in the case of ECs, the forces exerted by the liquid flow contribute to tissue development[134,135] . As for all the aspects previously mentioned, the pump system for cell media perfusion depends on several factors, including physiological relevance and the type of study.

The available pumping systems can be divided into two main categories: the passive-driven flow and the active-driven flow[136], Figure 6. The passive-driven flow system usually presents simple designs with no moving mechanical parts relying on basic physical phenomena such as gravity[137] and capillarity[138]. Such system simplicity and reliability led to the integration in different fields, with renowned applications in point-of-care devices for glucose or

more recently Covid-self tests. However, the passive-driven flow technology is not suitable when different flow patterns, recirculation and high levels of automation are required. Consequently, a reader interested in passive-driven flow systems can refer to this review[139].

In **papers III** and **IV,** commercialized pneumatic pumps, specifically a piezoelectric diaphragm in combination with passive check valves, were employed for the cell culture[140]. Such pumps were chosen because combining in a device of a few centimeter footprint several advantages of the more commonly used and cumbersome pump technologies employed for MPS platform perfusion.

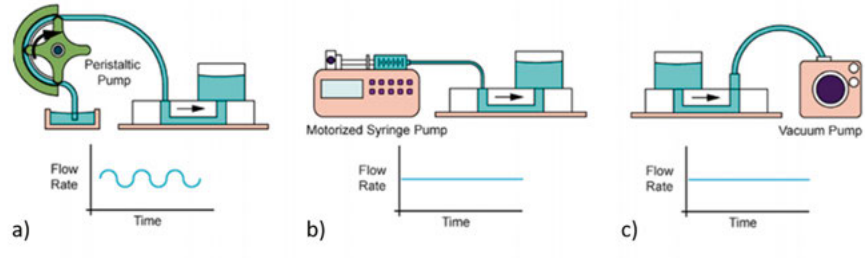

Figure 6: Schematic of a peristaltic (a), syringe (b) and vacuum (c) pump and respective flow pattern. Reprinted with permission from ref.139 Copyright 2017 Wiley.

The pneumatic pumps enable the cell medium recirculation not only by reducing the required volume but also by increasing the concentration of the cell signaling factors over time[141]. The chosen piezo pump presents a pulsatile flow behavior comparable to a high-quality syringe pump. Despite a pulsatile behavior being representative of some physiological studies, for instance, the heart pumping, a fluctuation-free flow protects cells from high shear stresses due to the flow picks of the peristaltic cycle[142].

A pump system should also provide a range of flow rates and modes to accommodate the different needs of tissue maturation. In **paper III,** the piezo pump allowed an initial intermittent flow to enable the cell media exchange in the microfluidic platform while not compromising the formation of the monolayer of a b.Eend.3 layer. After the monolayer formation, a continuous flow was applied to the cells for tissue maturation and tight-junction expression. The piezo pump allowed to cover a wide range of flow rates (50 µl -1000 µl) without the need to change any syringe (syringe pump) or tubing (peristaltic pump).

Integration and portability of microphysiological systems

In the most complex MPS different conditions including pH, temperature, nutrient supply and CO_2 are constantly monitored[143]. All these systems involve

external bulky peripheral components that have a negative impact on cell culture access and microfluidic platform manipulation, Figure 7. Consequently, parallel to the miniaturization of the *in vitro* models there is a need for integrating the peripheral systems into a more miniaturized and user-friendly device[144,145,146,147].

Cell culture is performed in an incubator under controlled conditions for optimal cell culture. To perform manipulations, monitor the cells and perform analytical experiments, the MPS needs to be transferred to different instruments. Small-footprint microscopes, and microscope incubators, can be used directly inside the incubator. However, such microscopes generally lack 3D imaging capability and drastically reduce the available space. In addition, the need for manipulation of the samples puts at risk the other cell cultures causing a repetitive sub-optimal Co_2 and humidity level drop. Alternatively, stage top incubators are implemented directly on the microscope recreating the incubator conditions with controlled Co_2 and O_2. Finally, this solution reduces the access of the microscope, usually a multiuser tool in a lab, until the experiment is complete[124].

As an alternative, in **paper II,** a microphysiological system carrier with an integrated perfusion and heating system was developed for MPS to allow repetitive incubator-microscope transfers. The well plate footprint allowed the system to fit a confocal microscope stage while maintaining the physiological conditions during imaging. In addition, it was observed a drastic reduction of manipulation during the incubator/microscope transfers.

The carried provided direct access to the reservoirs for sample collection or addition of reagents reducing the risk of introducing contaminants or air bubbles in the perfusion system.

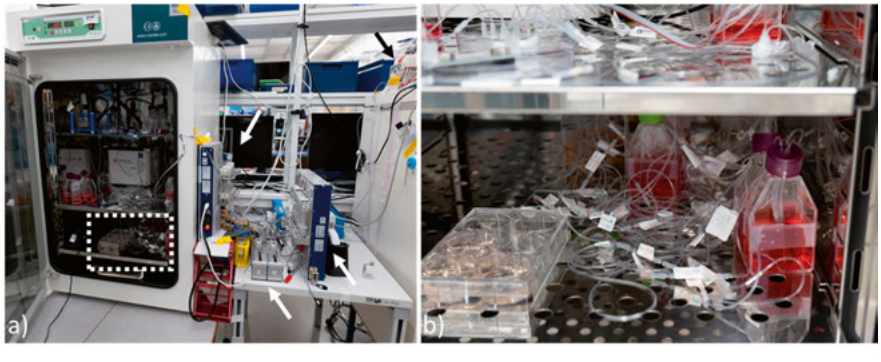

Figure 7: Incubator for cell culture with MPS. a) An open incubator with the connected MPS. The pumping systems are highlighted with arrows. b) Magnification of the tubing and MPS arrangement on the incubator shelves.

Cell culture substrate

Cell behavior is strongly modulated by multiple interactions with the ECM and surrounding cells. As also briefly mentioned in the introduction, 2D cell culture displays different gene expressions and morphology compared to the *in vivo* environment[148]. Consequently, an increasing interest has grown in the development of 3D co-culture models that offer a closer representation of *in vivo* architecture[149]. The ECM is a complex matrix that provides support for cells with a dynamic network of biochemical and biophysical signals to regulate cell behavior including spreading, colonization, differentiation, invasion and proliferation[150,151]. A fundamental step towards the fabrication of systems recapitulating key tissue features is the integration of ECM-like structures inside microfluidic devices. The engineering of matrices resembling the native structure of human physiology is presented in the next chapter while the fabrication on-chip is presented in chapter 3.

Chapter 2: Biomaterials for 3D cell culture

The challenge of integrating vasculature-like networks in MPS raised after the introduction of 3D cell culture systems. This chapter starts with a brief classification of the biomaterial used for the 3D cell culture. Then, more emphasis is put on the hydrogel synthesis, characterization and evaluation that was performed in **paper I**, **II** and **IV**.

Biomaterials

The function of biomaterials is to match the native human ECM to promote tissue formation while minimalizing the immune response. Ideally, the scaffold replicates the physical, chemical and biological characteristics of the native ECM[152,153,154] meeting the following four main requirements: i) Biocompatibility to prevent the immune reaction[155]. ii) The possibility of integrating a vascularized system to sustain tissue formation[156]. iii) The mimicking of mechanical properties and degradation mechanisms of the biological counterpart to promote tissue formation[157,158]. iiii) A cell-friendly fabrication process that preserves cell functions[159,160].

These requirements are common for both tissue engineering and MPS. However, while tissue engineering aims at recreating organs for transplantation[161], MPS focuses on replicating tissue physiology for *in vitro* studies on miniaturized systems[14] . Since tissue engineering is beyond the scope of this thesis, an interested reader can refer to these studies[162,163].

Biomaterials are classified according to the level of interaction established with the cells. Bioinert materials offer an inert substrate with no cytotoxicity to prevent any adverse cell response. This class of materials usually present a high level of processability, i.e. plastics and resins, but lack tissue-specific mechanical properties and chemical stimuli hampering cell colonization and growth[164], Figure 8. In **paper I**, resin materials served to identify the capability of commercially available 2PP-based 3D printers to obtain structures with feature size and resolution at the cellular scale.

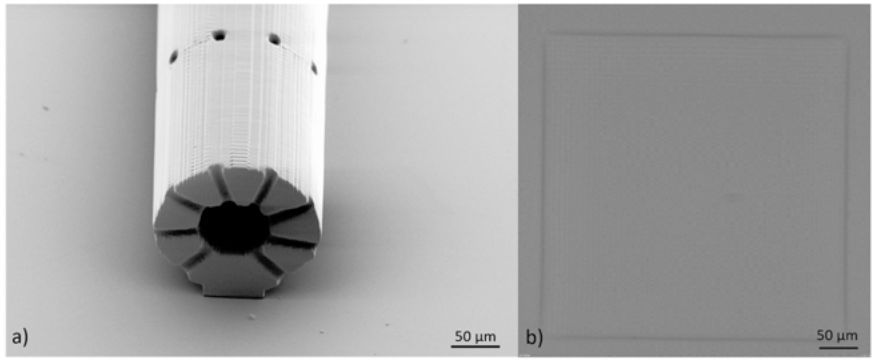

Figure 8: SEM image of a 3D printed channel with a resin (bio-inert material) displaying a vessel-like structure by 2PP a). Confocal image of the top plane of a cube-like structure obtained with a hydrogel b)

Over the past decades, advances in material synthesis resulted in the development of active biomaterials to promote matrix-cell interactions and actively modulate cell function and fate. Such materials not only replicate the mechanical and physical properties of the native ECM but also allow the remodeling of the 3D network over time by cell activity[165,166].

Since human body tissue stiffness varies widely between several orders of magnitude, from sub-kPa in the brain to GPa in the bone, the biomaterial properties have to be specifically tailored for the tissue of interest[167], Figure 9.

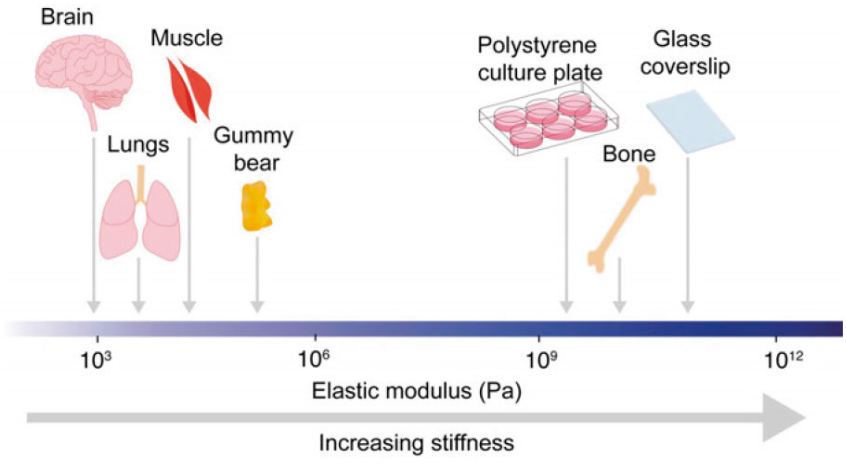

Figure 9: Young´s modulus for different tissues in human body168

Hydrogels

Among the different materials, hydrogels have emerged as one of the most promising alternatives due to their similar mechanical and diffusivity properties to soft human tissues[152,169,170]. The first reported applications of hydrogels in literature date back to the 60s when hydrogels were emphasized as a potential alternative for 3D cell culture[171]. Since then, hydrogels have been employed in fields including soft electronics[172], actuators[173] sensors[174] and bioengineering[175]

Hydrogels consist of a 3D network of hydrophilic polymer chains with water content up to 99%[157]. The small ratio polymer/water weight ratio leads to highly porous networks ensuring solute diffusion and cell-to-cell signaling[176]. The material hydrophilicity and porosity served in a wide range of biomedical applications providing a 3D substrate to understand cell mechanisms otherwise not possible with conventional 2D cell culture substrates[177], Figure 10. According to the polymer precursor source hydrogels are commonly classified as natural or synthetic-based. Each class of material presents advantages and disadvantages that will be discussed more in detail in the coming sections.

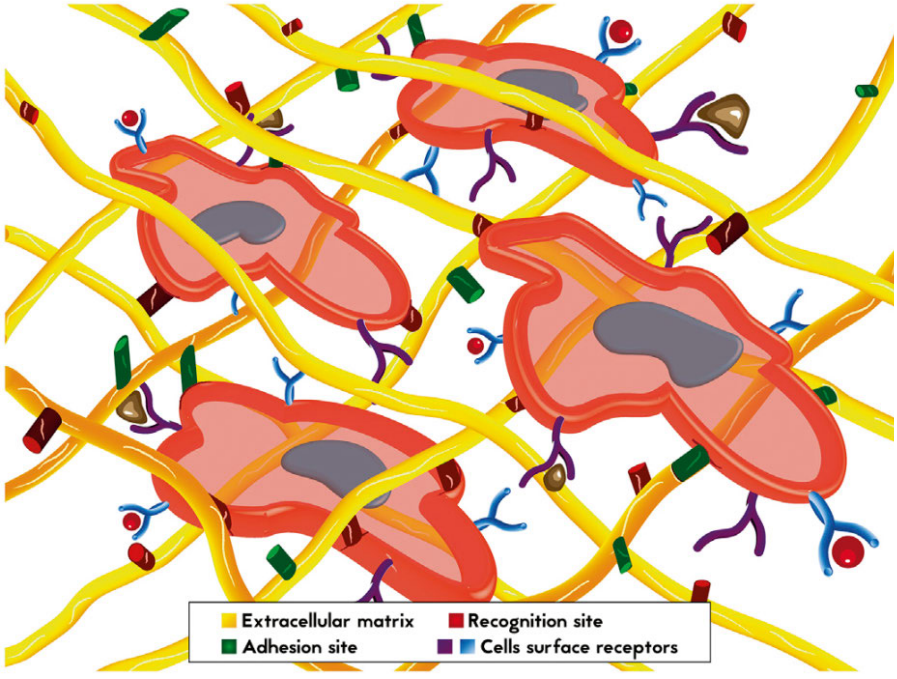

Figure 10: Representation of the cell-matrix interaction in a hydrogel 3D network177.

Natural-based hydrogels

Natural-derived polymers present the benefit of the native ECM components, displaying inherent biodegradable properties and cell-recognition motifs[178]. Collagen[179], fibrin[180], matrigel[181] laminin and hyaluronic acid are naturally found in the human body and have been largely implemented for 3D cell culture. Collagen, for instance, accounts for approximately 30% of human body proteins and represents a suitable scaffold for many different types of cells[182]. The collagen-denatured form, gelatin, is among the most widely used polymers for generating scaffold structures by manufacturing, for more information refer to Chapter 3.

Among the various polymers, fibrin has also been largely exploited in this thesis since suitable for hosting fibroblasts due to interconnected pores and biodegradability as also reported in previous studies[183].

Nevertheless, the origin from a biological source entails limitations in terms of batch-to-batch variability and potential immunogenicity. In addition, the poor mechanical properties[184] and control of the crosslinking kinetics limit the manufacturing process compatibility. In **papers II,** a new strategy to fabricate microvasculature systems in natural hydrogels is proposed and presented more in detail in Chapter 3.

Synthetic-based hydrogels

Contrary to natural-derived hydrogels, synthetic polymers offer higher tunability and processing flexibility[185]. These polymers can be tuned in terms of molecular weight, composition and architecture to match the specific application requirements. The crosslinking process onset can be on-demand and the degradation tuned to match the cell development pace. In addition, the crosslinking density and the associated porosity and mechanical properties of the hydrogel are accurately controlled[153,155,186].

The downside of no intrinsic bioactive properties and lack of degradation sites is compensated by conjugating the backbone polymer with peptides or proteins. Over the last decades, the synthesis and the functionalization of polymers for cell culture have improved, providing a powerful tool for the design of hydrogels with tailored properties. Nevertheless, these hydrogels have not displayed yet the same biocompatibility and biodegradability level as natural hydrogels[187].

Hybrid hydrogels

Hybrid hydrogels combine the biocompatible properties of natural hydrogels with the tunability and process flexibility of synthetic polymers[155].

Natural polymers are composed of saccharides or amino acids building blocks that are available as reaction sites for chemical modifications to create 3D networks. One of the most prominent polymers for hybrid-based hydrogels is gelatin. Gelatin preserves some essential properties of collagen including, high biocompatibility, biodegradation site points, and cell adhesion sites and is recognized as safe by the Food and Drug Administration (FDA)[188]. Gelatin shows reversible temperature-dependent solution-to-gel transition, necessitating the creation of chemical bonds to generate a stable hydrogel at physiological temperature (~37°C)[188]. Crosslinking is induced by polymerization of reactive functional groups that are added onto the gelatin chain backbone[189].

A deep analysis of gelatin modification with functional group and initiator goes beyond the purpose of this study, therefore the interested reader is invited to check this review[190]. In this thesis, a commercialized gelatin-based ink has been widely used for the generation of vasculature-like structures on microfluidic platforms with a 2PP 3D printer, **paper II** and **IV**. A Gelatin-based ink was chosen as displayed good biocompatibility with HUVECs in previous studies[191,192].

Crosslinking strategies

Hydrogel structures are a 3D network of crosslinked polymer chains. The bond between the chains ensures the formation of a 3D matrix that provides a substrate for cell migration, proliferation and growth. The crosslinking strategy is determined by the hydrogel chemistry and should also take into consideration the application. The cross-linking process should be cytocompatible and not create any cytotoxic byproduct[177]. On the other hand, the presence of

encapsulated cells in the precursor solution should not affect the cross-linking process[193]. In **paper II,** the synergy of 2PP 3D printing and micromolding ensured the fabrication of 3D cultures with high cell viability and no cell influence over the 3D printing process.

Physical crosslinking

Physical crosslinking takes place via non-covalent bonds such as ionic/electrostatic interactions and hydrogen bonding. Since the low-energy and the non-covalent nature bonds, the physically crosslinked hydrogel present a lower Young´s modulus than the chemically crosslinked hydrogels[152]. However, the non-covalent nature with low energy bonds displays intrinsic reversible behavior upon environmental condition changes, like temperature, and pH. The advantage of such crosslinking is the absence of chemical modifications of the polymer backbone and the high biocompatibility of the process. For these reasons, physically crosslinked hydrogels are widely used for biomedical applications. Amongst the non-covalent crosslinking strategies, the ionic crosslinking of alginate and the thermal gelation of collagen and gelatin have been extensively implemented in organ-on-chip and tissue engineering[194,195].

Chemical crosslinking

The fabrication of more stable hydrogel 3D matrices is obtained by the covalent bonds between the polymer chains[155]. Several reaction mechanisms have been developed to obtain covalent bonds[196]. Currently, the most widely used strategies can be classified into enzymatic crosslinking, click reactions and radical crosslinking. When compared to physical crosslinking, chemical bonds are generally associated with a higher stability of the polymer matrix and higher mechanical properties. More emphasis will be put on the crosslinking used in the thesis, for an overview of the crosslinking strategies an interested reader can refer to the following review[189]

Enzyme-mediated crosslinking.

Enzymatic catalytic activity is compatible with physiological conditions and offers high substrate-specificity, resulting in an attractive choice to avoid the potential toxicity of chemicals, radicals, or reaction by-products[197].

On the other hand, a small variation in pH and temperature can have a relevant impact on enzyme-mediated crosslinking[198]. Among the different enzymes, Factor XIII, a plasma transglutaminase plays a key role in the generation of the fibrin cloth in fibrin formation. Fibrin has been largely used for cell culture as composed of a network of interconnected pores, that enhances the scaffold colonization while ensuring the diffusion of nutrients and removal of metabolites [199]. In **paper II**, a fibrin solution with encapsulated fibroblasts was molded around a 3D-printed hydrogel structure directly on-chip to create a multi-material cell scaffold.

Light-mediated crosslinking

Upon light absorption, a photoinitiator generates radical species that initiate the polymer crosslinking process. Light-mediated crosslinking is growing in interest thanks to the capability to trigger the reaction on-demand upon light exposure. However, the light dosage not only triggers the reaction but also influences the physical properties of the hydrogel[200]. In **papers II** and **IV,** a light-mediated crosslinking served to create hydrogel structures on-chip. The physical properties of the hydrogel constructs were tuned by screening different energy dosages to control the structure swelling and cell invasion.

Hydrogels swell or shrink after crosslinking according to the polymer nature and the medium osmolarity[201]. The volume change can be tuned by the crosslinking density that has also an impact on the mechanical properties and porosity of the hydrogel[202–204]. The volume change causes a shape deviation from the original design affecting the CAD/CAM mimicry. In high-resolution printing, the change of shape has to be taken into account already during the design of the scaffold[177]. In **papers II** and **IV**, the conceived structure designs mitigated the hydrogel swelling effect on the structure.

Characterization of biomaterials

Both the physical and biochemical properties of the ECM are key factors in regulating cell behaviors[155]. The characterization of such properties requires the use of different techniques. The main challenge of hydrogel structures fabricated by 2PP is the reduced size, generally below 1mm that is not always compatible with traditional characterization techniques. In line with the aim of the thesis, more emphasis will be put on the specific technology suitable to analyze the hydrogels produced by 2PP.

Swelling/shrinking

The chemistry and crosslinking density of the hydrogel polymers have a strong impact on the swelling/shrinking of the hydrogel[177]. Despite hydrogel swelling being exploited for actuation[205] and drug delivery[206], It represents one of the biggest challenges in microfabrication. The swelling/shrinking upon reaching the equilibrium with the medium generates distortions and in extreme cases loss in the structure functionality. However, the alternative of using pure synthetic hydrogels has the downside of limited diffusivity and no cell recognition sites[207].

Hydrogel swelling has been investigated with the weighting technique[208]. However, the reduced size of hydrogels obtained by 2PP makes this technique not a viable solution. As an alternative, confocal microscopy enables the imaging of the sample to compare the swollen hydrogel size with initial design dimensions[209]. In **paper II,** the fluorescent signal coming from the photo-initiator allows the direct imaging of the sample to correlate printing parameters

to the hydrogel swelling. In case of no fluorescent sample, a fluorophore-dextran solution with a suitable molecular weight can be injected to visualize the profile of the obtained hydrogel structure.

Mechanical properties

The ability of hydrogels to replicate ECM not only lies in the matrix bio signaling but also in matching the mechanical properties of the ECM microenvironment[210–212].

The ECM mechanical properties have been demonstrated to considerably contribute to the cell fate influencing tissue formation[213,214,215]. A hydrogel presents both the elastic and viscous behaviors that are found, with different ratios, in several human tissues[167]. The mechanical energy exerted on biological tissue is dissipated in the viscous behavior and stored due to the elastic component[216]. Both components contribute to the hydrogel stiffness that plays an essential role in modulating cell migration [217], differentiation [218], cell morphology [218] or the development of diseases [219]. In the case of vasculature systems, the alteration of the hydrogel stiffness has a direct consequence on the lumen size and distribution of self-assembled blood vessels. Soft hydrogels (5 kPa) were demonstrated to promote the formation of a more dense vessel network when compared to stiff hydrogels (20 kPa)[220].

The measurement of the elastic and viscous components of a hydrogel is traditionally performed by rheology[221]. However, many hydrogels present heterogeneities across the sample that traditional rheology cannot distinguish. In addition, the size of the 2PP samples, up to 1mm diameter, is not suitable for traditional rheology systems. As an alternative investigation method, atomic force microscopy (AFM) has been employed for surface analysis. The mechanical properties of the sample are extrapolated by measuring the deflection of the probe cantilever due to the tip-sample interaction[222]. For hydrogel applications, with stiffness of a few kPa, AFM requires specific probes for working in an aqueous environment to increase the contact area and prevent penetration into the hydrogel[223].

With a similar measuring technique, nano-indentation presents probes with µm spherical tips coupled with an interferometer-based system for cantilever bending detection[224]. This technology has recently emerged as requiring less sample preparation, no alignment and faster positioning and data acquisition when compared to the AFM systems[225]. The lower lateral resolution (1 µm vs. 30 nm) and deeper indentation (up to a few µm vs. a few nm) are still suitable for scaffold investigation for cell culture studies.

To extrapolate the mechanical properties in the bulk of the sample, micro-rheology by optical tweezers has been used. Optical tweezers enable an accurate displacement of encapsulated beads to extrapolate the viscoelastic behavior of the hydrogel[226]. Micro-rheology by optical tweezers is also a non-invasive measurement that served in monitoring the evolution of fibrin mechanical properties during biological processes such as angiogenesis[227].

Scaffold structure

For the microstructure analysis of the scaffold biomaterial, electron microscopy (SEM) is a suitable choice since providing a resolution of the order of 20 nanometers[228]. In **paper I**, an SEM microscope served to investigate the quality of 3D-printed resin-based structures with sub-micron resolution.
Despite the SEM technology is also commonly used for hydrogel imaging, the required freeze-drying step alters the hydrogel structures limiting the technique for comparative studies[229] . As a possible alternative, cryoSEM prevents the removal of the aqueous solution and collapsing of the structure by performing the analysis in a cryo-chamber [230].

However, the technique requires specific equipment and highly trained personnel to perform the investigation with even difficulties in the sample preparation with a size below 1 mm.

For visual analysis of the hydrogel microscopic architecture, confocal microscope reflection mode or 2-photon harmonic generation has served to visualize fibrous structure in protein hydrogels including fibrin[227] and collagen[231]. Nevertheless, light diffraction limits the structure investigation to comparative studies based on signal intensity and macro structural patterns without any determination of the actual nanometer porosity. In **papers II** and **IV**, the hydrogel swelling and microstructure due to the fabrication technique were investigated by confocal microscopy to determine the optimal printing parameters and diffusion patterns**.**

As previously mentioned in the introduction of the chapter, hydrogel porosity is one of the key factors of a scaffold structure since regulating tissue formation and function. The porosity has a direct effect on the diffusivity of molecules affecting the nutrients, waste and biosignals diffusion and residency time[224]. Fluorescence recovery after photobleaching (FRAP) has emerged as a technique for diffusivity studies in hydrogels, as it is non-invasive and relatively quick to perform. The technique measures the recovery of fluorescent intensity of the material bleached area to determine the molecule mobility and eventually calculate the diffusion coefficient[232] and it is compatible with on-chip investigation. In **paper II** and **paper IV**, FRAP served to obtain the diffusion coefficient of the hydrogel samples obtained with different laser doses.

Characterization of cells in 3D culture systems

Notably 3D cell culture systems are complex systems with different factors that play a role[233]. The characterization of such systems might involve the use of different techniques. In this thesis, the hydrogels and microfluidic chips used for cell culture were transparent and, therefore, all the cell evaluation has been performed with optical microscopy.

However, it is worth mentioning that the integration of biosensor on-chip provides a remarkable strategy to monitor not only the cell function but also the microenvironment parameters. An interested reader is referred to the following review[234].

Microscopy

Microscopy uses lenses to magnify and resolve images of specimens to examine the morphology, structure, and behavior of cells that cannot be seen with the naked eye. Among the different technologies, SEM[228], AFM[225] and XRM[235], optical microscopes take advantage of visible light to visualize samples and are currently the most common tool for 3D cell culture investigation in research laboratories.

Fluorescent microscopy and brightfield microscopy are two different types of optical microscopy that are used to observe specimens. While both techniques utilize light to illuminate and magnify a sample, they differ in terms of illumination sources and the images that are produced[236].

In brightfield microscopy, the sample is illuminated with white light that passes through the specimen and into the objective lens. Brightfield microscopy produces images that are a result of different light absorption of the sample structures. Such technology produces low-contrast images with difficulties in distinguishing the components within cells and hydrogel structures. On the other hand, fluorescent microscopy can generate high-contrast images of the specific structure of interest, Figure 11. Fluorescent microscopy relies on the autofluorescence or labelling of cell structures with fluorophores that emit light when excited at specific wavelengths [237].

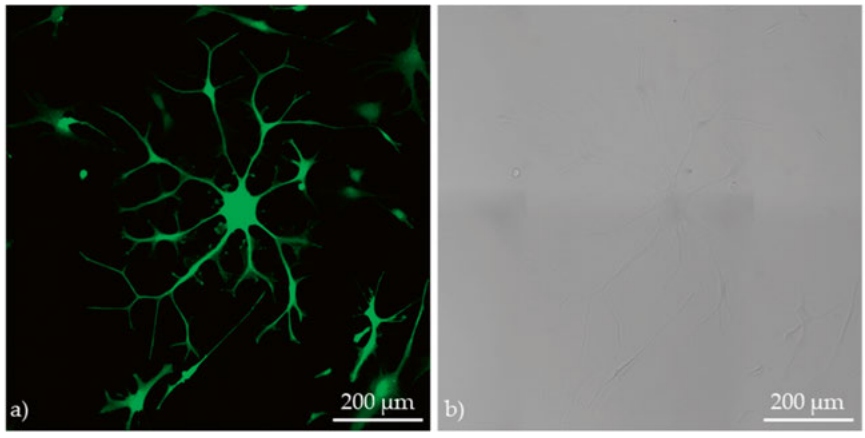

Figure 11: Astrocytes culture after fluorescent labeling a) and in brightfield b)

In this thesis, brightfield microscopy was mainly used for monitoring the general state of the system or identifying areas of interest for further investigation by fluorescent microscopy.

The poor z-axial resolution of a traditional fluorescent microscope is not suitable for 3D system imaging due to the out-of-focus signal. In confocal microscopy, a pinhole allocated between the objective and the detector removes the out-of-focus signal to generate a sharp image of the focal plane. By staking images obtained from different planes, 3D images can be reconstructed to visualize the sample and perform analysis. Confocal imaging was used for the cell analysis of **papers II**, **III** and **IV**.

Live staining

An important aspect of *in vitro* systems is the monitoring and evaluation of the cells as a response to different conditions. When the cells are integrated in 3D cultures the first investigation is the cytotoxicity analysis of the substrate material. Such evaluation includes different assessments including metabolic activity, cell proliferation and cell damage.

The live/dead staining assesses the membrane integrity with the injection of two dyes for labelling the live and dead cells. The live dye is permeable to the membrane and becomes fluorescent upon cell internalization and metabolization. For example, calcein, used in **paper II** and **paper IV**, is converted into a fluorescent molecule by the metabolic activity of the live cell. Meanwhile, the dead dye is instead impermeable to a healthy membrane and can diffuse inside the cell only in a damaged membrane. The cell viability is then determined by calculating the live/death ratio by fluorescent imaging.

An alternative strategy for cytocompatibility evaluation is the monitoring of cell metabolic activity. Such a technique relies on the change of a molecule's spectral properties (emission or absorption) as a consequence of the cell's metabolic activity. The cell viability is then correlated to the different absorbance or fluorescence of the solution. Despite this process being faster due to less sample manipulation it might be affected by the change in cell metabolic activity during the tissue maturation or cell proliferation. Metabolic activity assessment can be challenging in MPS as the reduced cell culture area might provide volumes non-suitable for the absorption/emission analysis. For example, in **paper II** the volume of the endotheliazed channel is less than 10 nl, a volume not suitable for traditional analysis systems.

Cell proliferation and migration can also be investigated by staining the cytoplasm of the cells (Cell tracker) or the nucleus of the cells with live staining.

In **papers II, III** and **IV** live staining of the nucleus served to monitor the proliferation of b.End.3 cells on an MPS carrier, while the cytoplasm staining of U3013 cells ensured the monitoring of the cancer cell invasion and intravasation. Live staining technology has been developed to also stain the cell membrane, actin filaments, tubulins and lysosomes on living samples, Figure 12.

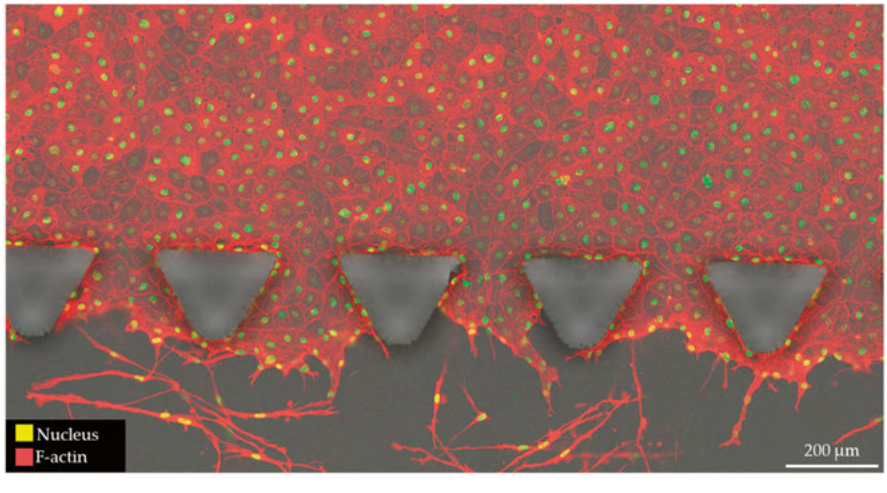

Figure 12: Endothelial cells and fibroblasts stained with live f-actin (red) and live nucleus (yellow).

The live cell staining requires the manipulation of the microfluidic platform for the injection of reagents, washing or collection of the solution sample. This manipulation can have a negative impact especially when frequent monitoring is required. In addition, the staining intensity decreases over time due to the cell´s metabolic activity and cell proliferation. As an alternative, cells can be

genetically modified to generate fluorescent molecules for continuous monitoring without any external reagent. In **papers II** and **IV**, HUVECs expressing GFP were used to monitor the development of a vasculature system inside the microfluidic platform without the need for any further staining.

Immunostaining

Immunofluorescence uses the selectivity of an antibody-binding to label protein correlated to a genotype expression[238]. The antibody functionalization with a fluorophore enables the localization of the protein by fluorescent microscopy. Different components of cells can be specifically labelled providing a map of the cell gene expression by fluorescent microscopy, Figure 13. The fluorescent labelling with antibodies was pioneered by Coons in the 40s[239]. Since the first assays more stable and sensitive fluorophores have been developed to allow multichannel imaging for the simultaneous detection of different proteins. In **paper III**, the tight-junction expression of b.End.3 cells were investigated by labelling the ZO-1 protein participating in the formation of a vasculature lumen for reduced fenestration[240].

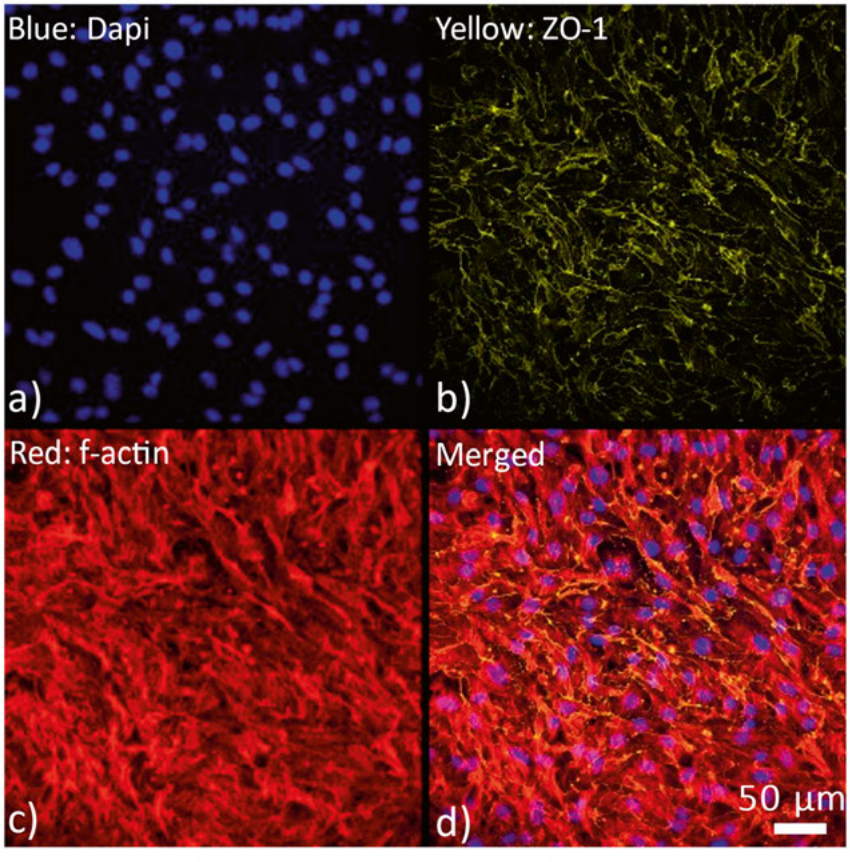

Figure 13: The confocal imaging of b.End.3 cells. Nuclei staining (blue). ZO-1 staining (yellow). F-actin staining (red).

For all the presented staining technologies some considerations have to be taken into account when performed on a 3D cell culture on-chip. Since small changes in osmolarity can have a relevant impact on the hydrogel swelling/shrinking the staining solution has to be carefully chosen. In addition, hydrogel porosity might reduce and even prevent the diffusion of some antibodies. Consequently, the time protocol of incubation and washing has to be optimized accordingly, especially for very thick samples without vascularization. In **papers II** and **IV**, all the hydrogel characterization was done directly on-chip to efficiently perform the washing and drastically reduce the volume of reagents. Finally, the choice of fluorophores needs to consider the possible autofluorescence of the hydrogel and cells.

Chapter 3: Manufacturing on-chip vasculature systems

Despite bioactive polymers can be designed with mechanical and chemical properties close to the ECM, the hydrogel scaffold needs also to replicate the characteristic microstructure of the biological counterpart[233,241]. Particularly, the absence of an internal vasculature quickly induces the formation of a necrotic core as the physiological conditions are maintained by solute diffusion within a distance of only 200 μm[28]. The lack of vascularization is associated with the formation of necrotic areas in thick hydrogels, spheroids and organoids[242]. However, the engineering of functional and healthy tissues needs both a blood vessel network as well as the perfusion of cell medium to ensure the replenishment of fresh media and the removal of metabolites. While the first challenge relies on a technology capable to generate hollow structures with sub-100 μm feature size (**papers I**, **II** and **IV**), the second deals with the soft-hard material interfacing between the hydrogel and a microfluidic platform for the connection of a pumping system (**paper II and III**).

The generation of a functional microvasculature network represents one of the major challenges in MPS. However, as the MPS evolve toward the development of multicellular systems with physiological cell densities, the perfusion of cell media via an integrated vascularized network becomes fundamental. Nowadays, the generation of vasculature systems in hydrogels can be categorized into a nature-driven approach and an engineering-driven approach.

Nature-driven approach

Initially observed in *in vitro* in the 80s, the nature-driven approach relies on the inborn capability of ECs to self-assemble in vascular networks[243]. The generation of new blood vessels is classified into two distinct processes: vasculogenesis and angiogenesis. Vasculogenesis indicates the formation of blood vessels starting from precursor cells, the angioblasts. This vascularization process mainly occurs during the development of the embryo[81]. Instead, angiogenesis consists of new blood vessels sprouting from an existing vessel. Contrary to vasculogenesis, angiogenesis occurs during the entire lifespan and it is triggered by the biosignalling of hypoxia and inflammatory states [82]. Many studies reported the vessel formation using pro-angiogenetic factors including

vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF)[244,245].

A perfusable vasculature occurs by combining ECs with supporting cells like fibroblasts, smooth cells and pericytes that produce fundamental ECM components to stabilize new vessels[131]. The encapsulation of HUVECs with supporting cells in hydrogel including fibrin, matrigel and collagen to obtain microvasculature-like systems has also been explored on microfluidic devices [246,29,247]. This strategy enabled the engineering of micro-vessels with diameters close to the size of native capillaries[248]. However, the poor control over the vasculature formation in terms of vessel density and size combined with the lack of anastomosis sites to provide flow hampers the comparison of different studies[249].

Engineering-driven approach

The engineering of blood vessels inside *in vitro* systems enables the fabrication of well-defined hollow structures in scaffolds when compared to the nature-driven approach. The control over the new vessel geometry ensures the simulation of the fluid flow in the vasculature while providing systems with a consistent and repeatable network suitable for comparable studies[88]. Among the different technologies to fabricate vasculature networks, more emphasis will be put on the strategies that allow direct integration on a microfluidic platform. Such strategies are here grouped into two main categories.

Molding

Simplified vasculature hierarchical structures can be generated by molding the hydrogel precursor solution on a patterned template in a similar fashion presented to obtain the PDMS microfluidic chips[250,251]. Despite the technique being suitable for a large variety of hydrogels and the crosslinking process cell-friendly without any adverse reaction[252], the limitation in the replication resolution and bonding to a substrate hinder the applications. Moreover, the hydrogel mechanical properties cannot be freely tuned as the hydrogel serves for both the cell culture and structural support of the device itself.

Alternatively, a subtractive approach can be implemented to generate hollow structures in the scaffold. Most of the earliest models of engineered vasculature generated simplified versions of artificial vessels with the removal of capillaries, thin filaments or spirals from a 3D hydrogel to form a channel[253,254,255,256]. The capability of recreating hierarchical vascular structure was achieved by dissolving or melting encapsulated sacrificial material in the hydrogel scaffold[257,258]. Such technology is suitable for any hydrogel as long as the sacrificial material does not interfere with the crosslinking process of the hydrogel precursor solution[31] or the cell viability[259].

In a similar subtractive approach, hollow structures can be created inside a hydrogel precursor solution by injecting a low-viscosity fluid in a technique called viscous fingering[260]. Such technique benefits from the Taylor instability when injecting a fluid in a moderate to highly viscosity solution[261]. The fabrication strategy does not require any expensive equipment and can be obtained by simple liquid injection. However, the technique is limited to straight hollow structures and diameter size above 100 μm.

Despite the simplicity of such strategies being attractive, the lack of design freedom and the poor capability to replicate a biologically relevant microvasculature prevent the mimicking of physiological conditions.

3D printing

3D printing, also called additive manufacturing (AM), is a bottom-up technology that fabricates an object layer-by-layer starting from a model generated by computer-aided-design (CAD) software. Such an approach introduces some advantages including, less waste of material, a high degree of design in 3D and on-demand creation of complex structures (fast prototyping). Since the first time 3D printing was introduced in 1984 by Charles Hulls[262], the technology has found its way into both academia and industry. To satisfy the different application needs a wide range of materials and technologies have been developed encompassing a wide range of techniques. The description of all these technologies goes beyond the aim of this study and an interested reader is referred to the following review[263]. 3D printing has been extensively used for biological applications with previous studies in the fabrication of complex structures with controlled porosity[264–266], well-defined mechanical properties[267] and cell migration[265].

Considering the main focus of the thesis, extrusion-based and light-based 3D printing strategies are presented. However, an interested reader in biofabrication is referred to the following reviews[268,269].

Extrusion-based 3D printing

Extrusion-based systems extrude a hydrogel precursor solution ink through a nozzle onto a printing substrate[270]. The technology is currently the most widely spread strategy to fabricate hydrogel constructs due to its simplicity, compatibility with different types of gels and fast printing.

The formation of vascular structures is obtained by multi-material printing using a coaxial needle where the core is a sacrificial material to create a hollow structure. Such structures have an inner diameter above 100 μm and can be connected to needles to be placed inside a microfluidic chip for perfusion[271,272]. The coupling of the printed structures with needles for perfusion requires delicate manipulation of the structure entailing a high risk of the hydrogel construct damages[273]. Moreover, the print is performed on open microfluidic platforms that present concerns in terms of contamination and hydrogel

structure dehydration during manipulation[274]. These aspects combined with the difficulty in recreating branching systems with a diameter below 100 μm have limited the technology use for microvasculature fabrication[275].

Light-based printing

In light-based 3D printing techniques, the precursor solution is crosslinked upon light exposure. The crosslinking reaction on-demand allows the fabrication of intricate geometries providing customization and flexibility[276]. Over the last decades, the advancement of both polymer and photoinitiator chemistry provided a wide range of bioinert and bioactive inks with a discrete versatility for the 2D and 3D cell culture[277].

Single photon 3D stereolithography (SLA) relies on laser scanning to fabricate 3D structures by the selective crosslinking of the liquid photosensitive precursor solution by UV light exposure[177]. The technique achieved biologically relevant resolution down to 5 μm feature size in the XY plane but with a small throughput. The integration of micro-mirror arrays (DMD) enabled to shift from a point-by-point scanning to a single plane projection with a considerable reduction of printing time[278]. Despite the technology benefitted from the increase in printing speed the poor resolution (around 100 μm) along the Z axis still prevents the use for microvasculature model fabrication[279,280].

Recently, the printing rate has drastically increased with the introduction of volumetric 3D printing[281]. In this technique, the ink is simultaneously exposed to different beams from multiple angles. The superposition of the optical fields creates volumes with energy above the crosslinking threshold with the consequent formation of the object[282]. Besides the higher throughput, volumetric printing reduces the structure anisotropy associated with layer-by-layer fabrication and removes the need for supports in overhanging structures[283].

Despite the technology representing a valid bioprinting strategy[284,285], the poor axial resolution is not suitable for creating the smallest features of tissue architecture and reduces the compatibility for on-chip fabrication.

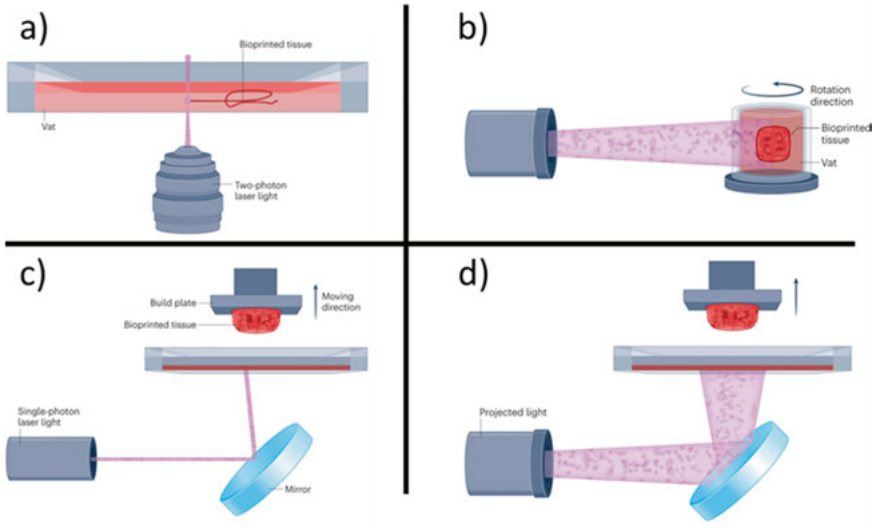

Figure 14: a) 3D printed object obtained by 2PP laser rastering b) 3D printed object by volumetric part generation with simultaneous irradiation from multiple angles c) 3D printed object generated by single photon laser rastering d) 3D printed object obtained by subsequent exposure to single photon light patterns. Reproduced from286 with permission from Nature Publishing Group.

If 3D printing opened to the fabrication of free-form objects and rapid prototyping, 2PP 3D printing has extended its ability to generate arbitrary designs with unmatched submicron feature size and resolution[287]. Despite being a young technology, the first system was commercialized in 2007, the 2PP technology has captured the interest of both academia and industry bringing advances in the fields including, microoptics[288] microfluidics[289] and bioengineering[265,290–293]. In contrast to conventional sub-micron fabrication techniques, 2PP introduces the third dimension while simplifying the process steps by circumventing the need for a clean room facility[294], Figure 14.

Theorized in the 30s by Göppert Mayer for being experimentally validated in the 60s by Kaiser the design freedom of 2PP relies on the crosslinking confinement into the laser voxel due to nonlinear absorption of photons[295]. Consequently, free-hanging structures and complex geometries are created by scanning the laser voxel into the resin without the need for material supports or photoabsorbers[276], Figure 15.

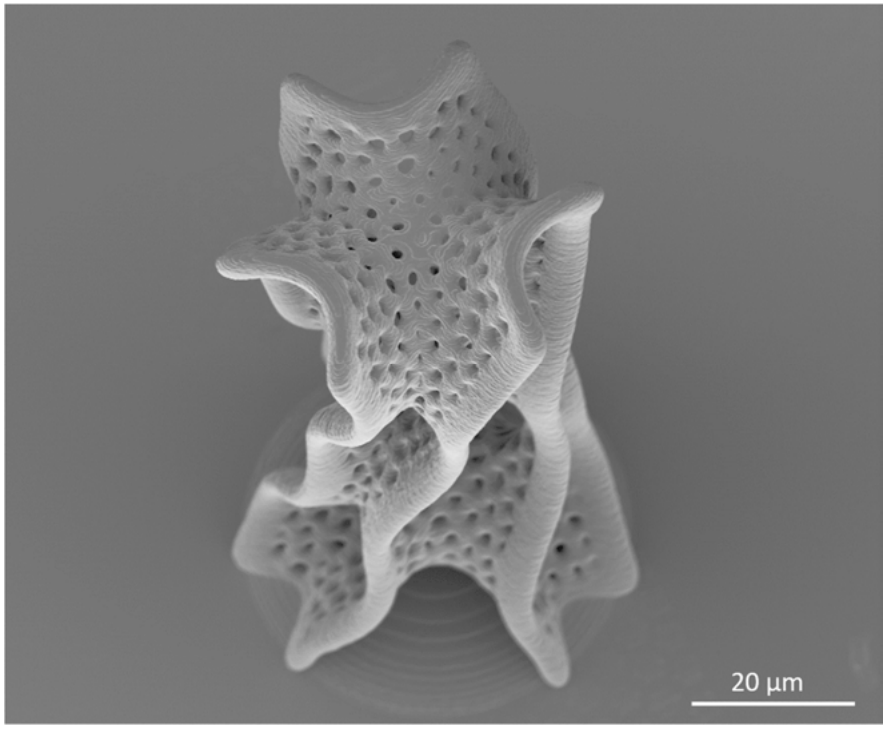

Figure 15: A Voronoi structure obtained by 2PP printing.

The voxel displays an ellipsoidal shape with a size that strongly depends on different factors including the ink, laser wavelength, objective numerical aperture, energy dose refractive index matching and printing mode[296,297]. In **papers I** and **IV**, the influence of these parameters were investigated to compare 3 commercially available printers and the physical properties of a hydrogel, respectively.

The polymerization mechanism and rate are strongly influenced by the photo-initiator[298,299]. The common photoinitiators used in single photon cross-linking require high energy doses due to the poor 2–photon absorbance resulting in not being suitable for cell encapsulation, Figure 16.

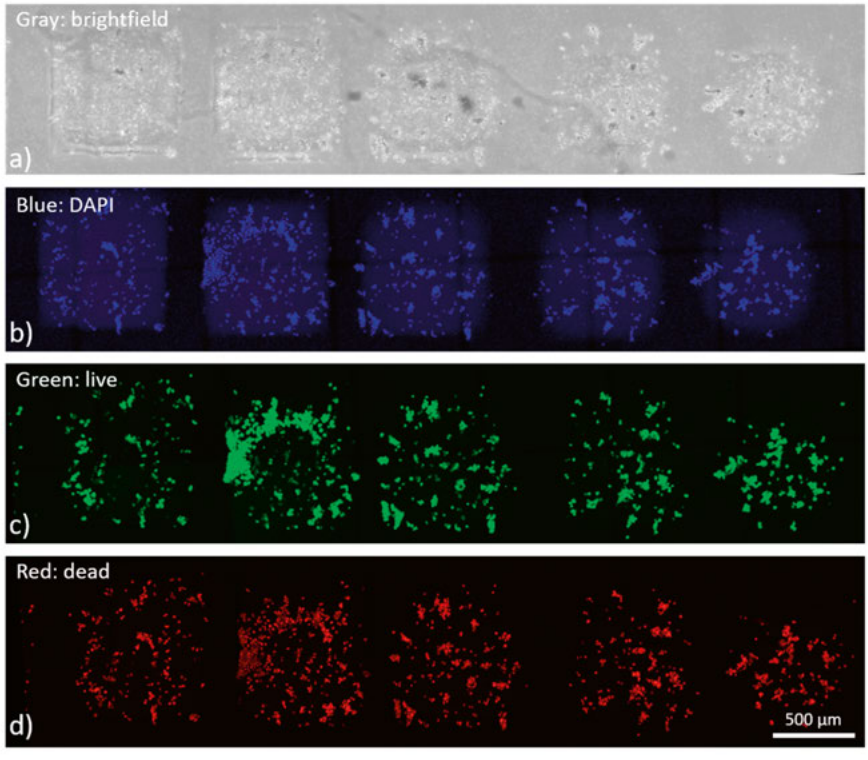

Figure 16: Confocal imaging of b.End.3 cells encapsulated in a GelMA hydrogel obtained by 2PP. Brightfield (grey). DAPI (blue). Live staining (green). dead staining (red).

One of the biggest challenges in the implementation of light-activated hydrogel precursor solutions is the development of efficient, cell-friendly and water-soluble photo-initiators for biological applications. However, the unprecedented opportunity to closely replicate biological tissues with native feature size resolution[300] to model human physiology has stimulated the development of new biocompatible polymers and photoinitiators[301,302,303].

Complex construct architectures with well-defined mechanical properties and porous architectures have been fabricated to investigate cell-matrix interactions in 3D[304–306]. In **paper IV**, The invasion of glioblastoma cells in a hydrogel membrane was investigated by screening different 3D printing parameters. The submicron resolution has also drawn interest for the fabrication of microvasculature-like networks[307] with the possibility of direct integration on-chip reducing any extra manipulation of the delicate hydrogel constructs[293,207].

The 2PP high-printing fidelity with submicron accuracy has the downside of a lengthy fabrication process. Over the years, several methods have been developed to accelerate printing speed. The technology has been developing

more efficient photoinitiators, more powerful lasers and investigated the use of multiple laser voci obtained by diffractive optical elements or micromirror arrays (DMD)[308].

To overcome the lengthy and potential toxicity of the 2PP process, multiphoton absorption (2PA) is used to ablate the material and create small cavities in an already-formed hydrogel[309,310]. The strategy provides resolution down to 1 μm in collagen while confining the process toxicity to the cells in the ablated volume[66]. In a similar process, the fabrication speed is improved by inducing photo-cavitation to the detriment of the process resolution[311]. The printing resolution of both 2PA and 2-photon cavitation enabled the engineering of vasculature-like networks that can be populated with ECs[312,66].

However, the presence of encapsulated cells in the hydrogel causes scattering effects limiting the achievable resolution[313]. In addition, the limited capability to position cells in the scaffold limits the creation of multicellular systems[25].

In **paper II**, we addressed these limitations proposing a new strategy to tackle the engineering of multicellular systems in multi-hydrogel scaffolds with integrated vasculature networks. The proposed approach combined 2PP and micromolding to create high fidelity structure while ensuring high cell viability. The capability to replicate a microvasculature model with the flexibility to freely choose the cell concentration in the hydrogel offers new possibilities for systems recreating physiological conditions.

The presented technologies still involve tradeoffs in terms of scalability, resolution, print time, cell-process compatibility and materials. However, the recently experienced advancements in the field of 3D printing and the potential to be integrated and combined with other technologies, as shown in this thesis, pave the way to an exciting journey towards the creation of more relevant *in vitro* models in the future.

Chapter 4: Conclusions and Outlook

In this PhD thesis, a new fabrication approach to advance the fabrication and handling of microvasculature models was investigated. After exploring the capability of 2PP printers in **paper I,** the possibility of combining 2PP with micromolding to obtain a multi-hydrogel vasculature network directly on-chip was demonstrated in **paper II**. The usability of the MPS for analysis and handling was enhanced with the fabrication of a carrier with integrated perfusion and heating systems, **paper III.** Finally, the versatility of 2PP to tune hydrogel properties was demonstrated in **paper IV.**

Currently, the process of developing new drugs relies on the pre-screening of drugs on 2D cell cultures, followed by animal testing, and eventually trials on humans. This process is lengthy and often does not produce the intended outcomes due to the limitations of 2D cell cultures and the poor correlation between animal and human physiology.

MPS have the potential to address these issues by recreating key aspects of human physiology and offer a more relevant *in vitro* model in the interest of the patient´s health, **paper II** and **IV**. The implementation of MPS has also the promise of reducing the use of animals for research purposes aligning with the "3Rs principles" for the replacement, reduction, and refinement of *in vivo* testing.

In the last 2 decades, MPS has already achieved remarkable milestones. Despite the first MPS being introduced in 2010, already by 2016 the technology was declared by the World Economic Forum as one of the top ten emerging technologies. A few years later, in 2021, a commercialized microphysiological device (Emulate) was demonstrated to be more reliable than animal testing. Finally, in 2022, the FDA declared the "modernization act 2.0," opening to alternatives to animal testing for drug approval. Considering the significant impact of the regulations surrounding animal testing, the potential implications of MPS for drug development are significant. The growth of interest in MPS technology is also highlighted by the recent upsurge of startups as well as institutes and programs focused on the bench-to-bedside translation of MPS by some of the biggest players in the pharmaceutical industry.

Although many opportunities lie ahead, MPS as an emerging technology also face challenges. One of the current technical hurdles is the integration of the microvasculature network inside the scaffold for the cell culture. The mi-

crovasculature plays a key role in mediating the interaction between the different cells of the human body and represents one of the accesses for therapeutic delivery. However, the following challenges were identified: i) A suitable technology for microvasculature fabrication. ii) A hydrogel capable to provide mechanical stability while ensuring a suitable ECM for different cell types. iii) Scaffold integration on-chip. iiii) Microfluidic chip handling.

In **paper I,** commercially available 3D printers based on 2PP technology were benchmarked to investigate the capability to create objects with feature size and resolution in the cellular scale. Then, in **paper II**, a new strategy to fabricate a multi-material hydrogel scaffold with integrated channel network was developed on-chip. The technique enabled high-resolution printing with a resolution down to 10 µm while not affecting cell viability. In addition, the fabrication directly on-chip ensured the perfusion of the system to sustain the cell coculture. The proposed technique is also suitable for multiple tubular structures found in the human body including nephrons in the liver and the lymphatic system. Different organs, present different biological mechanisms and cells that a scaffold fabrication technique should adapt to. The versatility of tuning the properties of the obtained 3D printed structures was investigated in **paper IV** by tuning the printing parameters to investigate the invasion of Glioblastoma tumor cells inside a hydrogel membrane. The integration of the membrane on a microfluidic chip allowed the monitoring of the HUVECs and Glioblastoma interactions during cell intravasation.

Further developments are required to ensure high throughput, reproducibility, and compatibility with industrial manufacturing. This entails not only the MPS optimization and standardization to better compare studies across laboratories but also the miniaturization of all the peripheral control systems to perform the experiments. In **paper III**, a miniaturized piezo-pump was directly integrated into a well plate footprint carrier to obtain a more user-friendly setup for running MPS.

Noteworthy, MPS offer a more ethical and efficient alternative to animal testing. However, the technology still heavily relies on animals and human material. As representative examples, fetal bovine serum (FBS), commonly added to cell culture media, is obtained from bovine fetuses. Matrigel, a widely used hydrogel for 3D cell culture, is generated by inducing a tumor in mice. This reliance poses both supply and ethical problems even if less severe than those associated with animal testing. Alternative solutions have been developed to further decrease the *in vitro* testing dependence on animal-sourced material. Nevertheless, the mid and long-term goal of MPS is not only to gradually substitute animal testing but also to provide relevant data for in-silicio models, which can ultimately contribute to eliminating the use of animals and human material in drug development. Another aspect, often associated with new technology is to not create false expectations with catchy but potentially misleading terms. In this thesis, the term microphysiological system was pre-

ferred to organ-on-chip because considered more representative of the performed studies and less probable to create confusion with organoids and tissue engineering.

In this PhD thesis, 2PP was identified as a suitable technology to recreate channel structures replicating a vasculature-like network in a hydrogel scaffold on-chip. 2PP resulted in a technology not only able to achieve submicron feature size and resolution but also to selectively tune the properties of the scaffold to tune the material-cell interaction. In parallel to this work, the benefit of developing carriers and platforms with integrated miniaturized peripheral systems to sustain the cell culture was explored.

As displayed in this work, the potential of the emerging MPS technology is encouraging and the current technological progress is addressing the challenges to capture the complexity of human biology for future *in vitro* models.

Chapter 5: Summary of the included papers

Summary of paper I

The versatility of 3D printing has revolutionized the fabrication of components in different fields. However, only the recent introduction of 2PP technology has opened to the creation of structures displaying arbitrary geometries with submicron resolution and feature size. The growing interest in high-printing fidelity for microfabrication has accelerated the development of faster systems and inks with application-specific functionalities. Considering the consequent upsurge of commercially available systems, the presented study investigated the strengths and weaknesses of 3 3D printing systems (NanoOne$_{250}$, PPGT+ and FemtoLAB).

Accounting for the wide range of potential applications, 6 representative structures were designed with features replicating recurring geometries of different fields to assess the capabilities of the printers. The sample geometry was observed to have an influence over the printing fidelity of the systems for feature sizes below 1 µm. In contrast, feature sizes above 1 µm were printed with high fidelity aside from the sample geometry. The printing angle was also found to play a key role in determining the printing fidelity. Tilted planes presented lower printing performance due to the voxel shape. Finally, a higher NA aperture objective ensured faster printing to the detriment of minimal feature size and resolution.

All the systems successfully printed free-hanging structures demonstrating the capability to print suspended structures without the need for any support. The versatility to fabricate multi-material constructs with different NA objectives demonstrated the possibility of printing features on preexisting patterns with µm resolution alignment. The FemtoLAB printer allowed the fabrication of large objects without the need of stitching lines while the NanoOne$_{250}$ displayed the fastest prints without compromising the printing quality.

The study demonstrates the strengths and limitations of the analyzed systems while indicating the potential future applications of 2PP-based 3D printing.

Summary of paper II

One of the current challenges in microphysiological systems is the incorporation of a vasculature network to sustain 3D cultures and enhance the physiological relevance in an *in vitro* environment.

In this study, we propose a new fabrication strategy to combine the high-printing fidelity of 2PP with the high cytocompatibility of hydrogel molding to create perfusable vasculature-like structures. The 2-step process consists of the 3D printing of a channel network with a gelatin-based ink before the injection of a second hydrogel to surround the structure, in this case, a lung fibroblast-laden fibrin hydrogel. HUVECs cells were injected inside the printed structure after the fibrin crosslinking. The multi-hydrogel structure was directly printed inside a microfluidic chip to ensure a stable interface for the pumping system connection. The channel geometry with thin walls reduced the hydrogel swelling influence over the printing fidelity while enabling the coupling of the 3D printed structure with a natural-based hydrogel, fibrin and collagen. The strategy served to print perfusable channels with an inner diameter comparable to capillaries (10μm). The diffusivity of different molecular weights 4, 40, 70, 250 and 500 demonstrated a diffusion cutoff for molecular weights equal to 500kDa. An analysis of the microstructure of the hydrogel constructs revealed a crosslinking density along the radius of the printing fibers associated with the energy gradient of the voxel.

No relevant differences in cell growth and proliferation were observed on the HUVECs cultured on the 2PP hydrogel and glass surface coated with the adhesion-promoting solution provided by the cell supplier.

Finally, a 10-days co-culture of HUVECs and human lung fibroblasts were maintained in straight and branched channels to demonstrate the strategy capability to sustain cell culture.

The proposed fabrication strategy successfully combined 2PP and hydrogel micromolding to create a multi-hydrogel perfusable hollow scaffold directly integrated on-chip for cell co-culture.

Summary of paper III

Despite the increasing use of microphysiological systems for biomedical applications, the development of affordable and user-friendly devices for the handling and transferring of microfluidic chips is still lacking.

In this paper, a customized microfluidic carrier with an integrated perfusion and heating system was developed to simplify cell perfusion and monitoring inside a microfluidic chip. The carrier simplified the fluidic platform manipulation minimizing the cell culture disturbance in terms of temperature and flow fluctuations during the system transfer from the incubator to the microscope for cell imaging. The integration of a pump system ensured different perfusion patterns (intermittent and continuous) with tunable flow rates ranging from 50 µm/min up to several ml/min of recirculating cell media. The carrier design with a well-plate footprint fits standard microscopy stages ensuring easy installation for continuous cell monitoring on the microscope stage while preserving optimal temperature due to an integrated heating system. The possibility to perform staining directly on-chip allowed cell monitoring (b.End.3) over 10 days of culture. The formation of a tight monolayer covering the entire microfluidic channel was visualized with the immunofluorescence staining of ZO-1 proteins, nucleus and f-actin staining.

Overall the paper has shown the benefits of interfacing microphysiological systems on carriers to ensure a more consistent cell culture and continuous monitoring.

Summary of paper IV

The invasion and intravasation of tumor cells are key aspects for understanding the spreading of tumors. However, the investigation of the tumor-vascular interactions remains a challenge.

In this paper, a microphysiological system was developed to investigate the interaction between endothelial cells (HUVEC) and glioblastoma tumor cells (U3013). The system consists of a hydrogel membrane separating two microfluidic chip channels. The membrane design reduced the structure deformation due to the hydrogel swelling providing a valid substrate for the seeding of HUVECs and U3013 cells. The tumor cell invasiveness through the membrane was investigated by tuning the 3D printing parameters. The cell behavior was associated with the physical properties of the hydrogel that displayed higher diffusivity and swelling as the 3D printing energy dose decreased. During the energy screening, an influence of the structure architecture was observed on the cell invasion pattern. Finally, the MPS successfully enabled to observe the tumor cell invasion toward the side of the endothelial cells and the monitoring of the HUVEC-U3013 interaction during the tumor cell intravasation.

In line with paper II, this study demonstrated the versatility of 2PP to create arbitrary structures with tunable properties. The integration of the hydrogel membrane in a microphysiological system enabled the monitoring of both tumor-scaffold and tumor-vascular interface interactions.

Popular Science Summary

The large majority of people have experienced, to a certain extent, a therapeutic treatment in their lives, underlying the large impact of drug development on modern society. Drug discovery is a complex process, consisting of several steps, to select chemical compounds suitable to treat diseases. After identifying a potential chemical compound, a long journey starts to determine the efficacy and safety of the candidate drug. Each development phase is heavily regulated to ensure the patient´s safety and be aligned with ethical aspects. The drug development process starts with a prescreening of the drug candidates on cells cultured on flat plastic surfaces (*in vitro* models) to quickly identify the efficacy and toxicity of the chemical compounds. For the successful candidates, the next step consists of testing on organisms, such as nematodes and fish as well as small and medium-sized animals, like dogs and monkeys. Ideally, the closer the drug gets to the human trials the higher the model's resemblance to human biology to predict the drug efficacy and toxicity. Finally, the compound is tested directly on human volunteers in clinical trials to test the efficacy and toxicity before commercialization. On average, for each commercialized drug, the development process requires 10 years and investments of around 1 billion dollars. The biggest barrier for the drug candidates is commonly observed in the transition from animal models to human trials with a failure rate above 90%. One of the main reasons for the high failure rate is the poor resemblance of the used models with human biology. The traditional *in vitro* models are too simple to capture the key fundamentals of biological mechanisms while animal biology is not always representative of human physiology. In addition, the complexity of the organisms hampers the isolation of the specific disease mechanisms of interest, limiting the extrapolation of the drug effects. These limits not only affect the health of the patients by delaying the development of new treatments but also pose ethical concerns about the sacrifice of animals.

The recent advances in microtechnology and material science have paved the way for the development of devices capable of recreating the key aspects of human tissue architecture and function. These devices, microphysiological systems (MPS), are an “upgrade” of the traditional *in vitro* platforms. The long-term aim of MPS is to closely replicate the human body environment and accelerate the drug development process while reducing, potentially even removing, animal testing. The development of such systems encompasses a

large variety of scientific fields, including engineering, material science, chemistry and biology. Up to now, different MPS have been developed to study tissues such as the heart, kidney, liver, brain, fat, muscles and bones. All these systems share a similar design concept based on a network of channels and chambers with sizes comparable to human hair. The reduced volume of these compartments enables the creation of small devices with the size of a coin where fluid can be precisely and accurately controlled to recreate the conditions for the proliferation of cells. In these systems, cells are cultured in a sponge-like structure, hydrogels, that mimics the environment surrounding the cells in the human body, the extracellular matrix (ECM). Cells can colonize the hydrogel and assembly to generate tissues and organs. However, human tissues are maintained functional by a complex network of blood vessels not only capable of providing each cell of our body with the required nutrients and waste removal but are also critical in cell-to-cell interaction, disease development and drug delivery. Despite the hydrogel properties can be tuned to accommodate different cell needs, one of the current biggest challenges is to recreate the microvasculature network inside hydrogel-cell systems.

In this thesis, a 3D printing technology based on 2-photon polymerization was investigated to recreate the geometry and function of the microvasculature. As a first step, the printing quality of commercially available 2-photon polymerization 3D printers was assessed by printing structures with dimensions comparable to the cell scale, **paper I.** After identifying the capability of the technology, the 3D printing technique was combined with hydrogel molding to create microvasculature-like structures on a microphysiological system. The presented strategy created a more cell-friendly process and drastically reduced the printing time. In addition, high printing fidelity was achieved with a resolution down to 10 µm (size comparable to a capillary and equal to around 1/10 of the human hair). The fabrication directly on the microphysiological system enabled to perfuse nutrients in the vasculature-like structure and sustained a cell co-culture of HUVECs and fibroblasts for up to 10 days, **paper II**. In this way, we defined a strategy to create vasculature-models directly on-chip. Parallel to this study, a platform with integrated heating and perfusion systems was developed to simplify reagent injection and sample collection in a microphysiological system. The proposed platform allowed multiple transfers from the incubator to the microscope for periodic imaging during 10 days of culture of mouse brain endothelial cells, **paper III**. Finally, the effect of the 2PP printing parameters on cell migration in a hydrogel membrane was investigated. This work enabled to guide the tumour cells towards an endothelial layer obtaining a model to study the glioblastoma invasion, **paper IV**.

In this PhD study, the versatility of the 2-photon polymerization technique in tuning hydrogel properties and in the fabrication of hollow structures in hydrogel scaffolds to recreate vasculature-like systems is shown. In parallel, the possibility of simplifying the microphysiological system handling was shown by developing a carrier with an integrated perfusion and heating system.

Svensk sammanfattning

De flesta av oss har någon gång genomgått en terapeutisk behandling, vilket visar på den stora roll läkemedel och läkemedelsutveckling spelar för människor och samhälle. Läkemedelsutveckling är en komplex process bestående av flera steg, med målet att hitta kemiska föreningar som är lämpliga för att behandla sjukdomar. Efter att ha identifierat en potentiell kemisk förening börjar en lång resa för att fastställa effektiviteten och säkerheten av det potentiella läkemedlet. Varje utvecklingsfas är hårt reglerad för att säkerställa patienternas säkerhet och uppfylla etiska krav. Utvecklingsprocessen startar med en förscreening av läkemedelskandidaterna på celler odlade på plana plastytor (in vitro-modeller) för att snabbt identifiera de kemiska föreningarnas effektivitet och toxicitet. För de framgångsrika kandidaterna är nästa steg att testas på organismer, som nematoder och fiskar samt små och medelstora djur, som hundar och apor. Ju närmare läkemedlet kommer de mänskliga försöken, desto större likhet är det mellan modellen och den mänskliga biologin. Slutligen testas föreningen direkt på frivilliga människor i kliniska prövningar. Varje kommersialiserat läkemedel har genomgått en utvecklingsprocess på i genomsnitt 10 år och investeringar på cirka 1 miljard dollar. Den största barriären för läkemedelskandidaterna upptäcks vanligtvis vid övergången från djurmodeller till försök på människor, där över 90 % av läkemedlen inte har önskad effekt. En av huvudorsakerna till att så många läkemedel inte fungerar på människor är att de tidigare modellerna inte varit tillräckligt lika den mänskliga biologin. De traditionella in vitro-modellerna är för enkla för att fånga viktiga grunder för biologiska mekanismer medan djurbiologi inte alltid är representativ för människans fysiologi. Dessutom hindrar organismernas komplexitet isoleringen av specifika sjukdomsmekanismer, vilket begränsar förståelsen av läkemedlets effekter. Att så många läkemedelskandidater inte klarar det slutgiltiga testet gör att utvecklingen av nya behandlingar fördröjs, vilket påverkar de många patienter som blir utan läkemedel, och är också etiskt problematiskt då djurförsöken varit till ingen nytta.

De senaste framstegen inom mikroteknologi och materialvetenskap har banat väg för utvecklingen av modeller som kan återskapa nyckelaspekterna av den mänskliga vävnadens uppbyggnad och funktion. Dessa modeller, mikrofysiologiska system (MPS, från engelskans microphysiological systems), kan ses som en uppgradering av de traditionella in vitro-plattformarna. Det långsiktiga

syftet med MPS är att göra en så naturtrogen modell som möjligt av miljön i den mänskliga kroppen, och därmed påskynda processen för läkemedelsutveckling samtidigt som man minskar, eventuellt till och med helt kan ta bort, djurförsök. Utvecklingen av sådana system omfattar flera olika vetenskapliga ämnesområden områden, så som ingenjörsvetenskap, materialvetenskap, kemi och biologi. Hittills har olika MPS utvecklats för att studera vävnader som hjärta, njure, lever, hjärna, fett, muskler och skelett. Alla dessa system delar en liknande struktur baserat på ett nätverk av kärl och hålrum med ungefär samma storlek som människohår. Den begränsade storleken av dessa kärl och hålrum gör det möjligt att skapa små MPS-enheter, så kallade chip, med storleken av ett mynt där vätska kan kontrolleras med noggrannhet och precision för att ge de rätta förutsättningarna för celler att föröka och sprida sig. I dessa system odlas celler i en svampliknande struktur, hydrogel, som efterliknar miljön som omger cellerna i människokroppen, den extracellulära matrixen. Celler kan kolonisera hydrogelen och växa samman för att skapa vävnader och organ. Mänskliga vävnader hålls funktionella av ett komplext nätverk av blodkärl som inte bara kan förse varje cell i vår kropp med de nödvändiga näringsämnena och avlägsna avfall utan är också avgörande för interaktion mellan celler, sjukdomsutveckling och läkemedelsleverans. Trots att hydrogelens egenskaper kan ställas in för att tillgodose olika cellbehov, är en av de nuvarande största utmaningarna att återskapa detta blodkärlsnätverk inuti MPS-chippet.

I denna avhandling undersöktes möjligheten att återskapa strukturen och funktionen hos blodkärl med hjälp av 3D-skrivare baserade på 2-fotonpolymerisation (2PP). 2PP är en typ av laserteknik med extra hög precision. Som ett första steg utvärderades utskriftskvalitén hos kommersiellt tillgängliga 3D-skrivare med 2PP genom att skriva ut strukturer med dimensioner jämförbara med cellskalan, artikel I. Efter att ha identifierat förmågan att skriva ut strukturer i liten skala testades 3D-utskrifts metoden för en mer praktisk applicering. En blodkärls-liknande hydrogel-struktur skrevs ut med 3D-skrivare och täcktes med ett andra lager av hydrogel för att skapa en stabil struktur på mer cellvänligt sätt. Denna två-stegsprocess möjliggjorde hög utskriftskvalité, med en upplösning ner till 10 μm (storlek jämförbar med en kapillär och lika med 1/10 av ett människohårstrå), då hydrogelen lätt sväller och hålet i blodkärlet blir för litet, dessutom minskades utskriftstiden drastiskt av den tunnare strukturen. Tillverkningen direkt på MPS-chippet gjorde det även möjligt att tillsätta näringsämnen i den kärlliknande strukturen, och en samkultur av HUVEC-celler och fibroblaster kunde växa i chippet i upp till 10 dagar, artikel II. Parallellt med denna studie utvecklades en plattform med integrerat system för värme och tillsättning av vätska, för att förenkla tillsättningen av näring och färgreagens samt provtagning i ett MPS-chip. Denna plattform gjorde det möjligt att ta proverna från inkubatorn till mikroskopet för att kunna doku-

mentera cellernas tillväxt under en 10 dagars period, artikel III. Slutligen undersöktes förmågan hos tumörceller att röra sig in i blodkärlsstrukturen, genom att göra vissa delar av strukturen svagare, artikel IV. Detta är första steget i att designa ett MPS-chip för att studera invasion av cancerceller och testa behandlingar.

Den här doktorandstudien visar på mångsidigheten hos 2PP-tekniken för att anpassa de fysiska egenskaperna hos hydrogelen och att skriva ut strukturer i hydrogel men hög precision. Parallellt visades möjligheten att förenkla MPS-hanteringen genom att utveckla en bärare med integrerat system för värme och tillsättning av vätska vilket gör det lättare att dokumentera cellerna i chippet.

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Federico

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