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Design and Fabrication of Cell-laden Gelatin Methacrylated Hydrogel Scaffold for Improving Biotransportation

A thesis submitted in fulfilment of the requirements for the degree of

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This thesis is entirely dedicated to them and to you, Grandma.
Abstract

One of the main goals of Tissue Engineering (TE), which has been developed rapidly over the recent years, is to re-create organs or tissues *in vitro or in vivo* with mimicked the anatomy and functions of body systems. Nowadays, replacing damaged tissues or organs has been a main focus in this field for addressing a significant shortage of donor tissues.

Vascularisation plays a crucial role in supplying cells and tissue with essential oxygen and nutrients and removing waste products from the engineered tissue constructs. Any issue in nutrient perfusion and mass transport could significantly restrict construct development to dimensions smaller than clinically useful size, thus limiting the ability for *in vivo* integration.

The main objectives of this study are to develop a novel framework for computational design using topology optimisation and microfabrication of 3D scaffolds using gelatin-based hydrogels (GelMa), allowing artificial vascularisation *in vitro* for testing if the framework is valid through the investigation into cellular viability inside the construct.

In this thesis, computational models were first generated to simulate oxygen transport through solving the diffusion equation. The diffusion models are then used to optimise scaffold topology. By means of microfabrication technologies, hydrogel-based constructs were fabricated to prototype the sophisticated scaffolds.
Cellular viability study was also performed to validate computational simulations and design. The results showed a higher cellular survival rate in optimally patterned constructs than the control.

In summary, the work presented here is not only technically simple and cost-effective, but also establishes an effective approach to the design and fabrication of a vascularised biodegradable and scaffold-free constructs. The proposed methodology will be of considerable implication for engineering bulk tissue constructs which require sufficient ongoing vascularization in the future.
Chapter 1: Introduction

Vascularised scaffolds are currently facing significant challenges in engineering thick tissue due to lack or delayed vascularisation. Despite the employment of new technologies and approaches such as microfabrication, development of new biomaterials embedded with biomolecular prompts, scaffold design and bioprinting that aim to replicate the biological vascularisation remains problematic.

Vascularisation remains as a major technical problem while translating Tissue Engineering (TE) products to clinics at the moment. For the survival of a functional macroscopic tissue constructs with clinically relevant size, a hierarchically arranged network of blood vessels and capillaries must be provided in order to supply consistent metabolic needs of the cells or regions in question.

Having said that, we must consider that the aim of the “pre-vascularized scaffold” is to allow adequate and consistent distribution of oxygen and nutrients to the cells and tissues in the scaffold so that they can undergo remodelling, where the cells would proliferate, host cells would invade the material, the scaffold will be degrade and the host vasculature will mature to integrate (or remodel) the provided vasculature.

The vascularisation issues mainly concern the successful mass transfer of nutrients/oxygen and wastes within the whole construct, which cannot normally occur by diffusion alone. It has been shown that there is significant difficulty of diffusing over a critical distance of 100–200 µm between cells and capillary in order to allow sufficient nutrient and waste exchange.
Moreover, the integration and proliferation \textit{in vivo} of the scaffold within the host tissue, with a proper hierarchically arranged, uniformly distributed pattern of vessels able to provide sufficient amount of blood, still remain problematical in order to achieve desirable chemical and physical modules.

The deficiency in such modules results in an incorrect spatio-temporal gradient of nutrients, thus causing apoptosis and reducing efficiency and proliferation at the core of the construct. Various approaches ranging from peptide/growth factor-tagged substrates, self-assembled endothelial-based cellular bodies, hyper-porous scaffolds, and micro-molding technologies to perfusion bioreactors have been proposed to address this issue, especially in Bone Tissue Engineering (BTE). The main aim was to attain osteointegration of the graft with the hosting tissue, by providing a fully-networked and well-defined vasculature which could help avoid necrosis and lack of integration with the surrounding environment. In spite of advances made with these methodologies, emulation of fully developed and functional vascular systems is still elusive in TE. It has been shown that the diffusion of oxygen and nutrients and waste exchange is restricted to a few micrometers between vessels and cells [1-2]. Proper biotransportation in regenerative medicine and tissue engineering is a major challenge, and various techniques have been attempted in this area, e.g. scaffolding with gradients and vascularised networks at different levels etc.

In recent years, the focus of BTE has been shifted, from the research concentrated on the development of biomaterials for replacing bone, to the design of a structure that includes signaling, porosity and mechanical properties in order to achieve both functionality and arrangement using photolithography techniques, molding, foaming and so on [51,74,78,83].
In addition, advanced materials have been developed, with a switch from polymers to more specialised materials such as elastomers (for biocompatibility and high degradation rates) polyanhydrides and hydrogels (for their capability to be cell-embedded, and high viability over longer time periods) [51,76]. Moreover, many studies have used cells encapsulated in hydrogels combined with growth factors (VEGF) and/or antigens (CD-34) to enhance the vascularisation and the deposition of tissue formation as well as bone mineralization [107]. Despite these advances, due to their high cost, low availabilities and disintegration, hydrogels suffer from some limitations and there is still progress to be made in this field, including its ability in nutrient delivery and waste removal [13,39-41].

As aforementioned, one of the critical issues has been biotransportation inside the hydrogel scaffold. An optimum scaffold should be able to be vascularised and encapsulated with cells. It should be able to deliver biological signals within the whole construct in a hierarchical manner. Biologically, proper gradients inside the construct are desirable to obtain the temporal-spatial transportation of mass essential to cell viability and tissue survival.

Furthermore, combining perfusable vessels into a rigid, hemocompatible hydrogel construct allowing the support of blood pressure and loads is still a desired goal and represents one of the major future directions. Notwithstanding their exciting promise, bulk hydrogels are currently far from these goals due to the reasons mentioned above.

This study aims to design an optimal 3D microstructure through mathematical and theoretical approaches first, in which the biotransportation is considered a critical design criterion for design optimisation of hydrogel scaffold architecture. The optimised scaffold is
fabricated next using soft lithography approach. The optimisation of the parameters during the fabrication of the scaffold and the results of cellular activities are then provided from the dedicated \textit{in vitro} study. Finally, some conclusion is drawn from this new framework of design optimisation, fabrication and cellular study. It is expected that this study provides a new framework for engineering thick tissue where biotransport is essential to the success.
Chapter 2: Literature review

2.1 Blood Vessels: anatomy and physiology

The human body and circulation rely on a highly sophisticated network of blood vessels estimated to be around 100,000 km in length in total [3]. Physiologically, oxygenated blood exits from the aorta into the systemic circulation, while deoxygenated blood is carried to the lungs from the pulmonary artery, allowing the exchange of CO₂ and oxygen.

Blood vessels are generally divided into three categories: arteries, capillaries and veins or venules. Blood vessel walls have a layered organisation [3,4]: a multilayered concentric structure where the interaction between the layers is essential to support circulation function. As shown in Figure 1, the layers are described in detail as follows:

1. **Tunica intima**: coated with epithelial cells to form the squamous endothelium, in direct contact with blood flow;

2. **Tunica media**: coated with circularly arranged smooth muscle cells (MSCs) and elastin, which gives elasticity to the construct, increasing the compliance of the tubule and allowing vasodilatation and vasoconstriction;

3. **Tunica adventitia**: made up of connective tissue and collagen fibres, preventing the collapse of the vessel and playing a role in the mechanical reinforcement.

However, each of these mentioned layers has a different predominance in the various vessels, depending on their function, as shown in Table 1. For example, arteries, which have
to transport blood at high pressures from the heart to the distal areas of the body, need more elasticity and compliance, which result in a thicker tunica media [4]. In contrast, capillaries have only the endothelium bed.

![Diagram of blood vessels](image)

**Figure 1** Concentrical structure of the blood vessels; artery (a), vein (b) and capillary (c). The tubules have the same layered and concentric structure, alternated with elastin (elastic tissue) which give elasticity and increase the total compliance [3].

**Table 1** Blood vessels and their structure

<table>
<thead>
<tr>
<th>Type of Vessel</th>
<th>Tunica Intima</th>
<th>Tunica Media</th>
<th>Tunica Adventitia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arteries</strong></td>
<td>Smooth lining of endothelium</td>
<td>Thick layer of elastic connective tissue with smooth muscle fibres</td>
<td>Fibrous connective tissue, thinner than the tunica media</td>
</tr>
<tr>
<td><strong>Arterioles</strong></td>
<td>Smooth lining of endothelium</td>
<td>Almost entirely of smooth muscle cells</td>
<td>Present</td>
</tr>
</tbody>
</table>
The variation in blood vessel structure is due to the fact that there is a great discrepancy in blood pressure as the blood is initially driven by the contraction of the heart (systolic). As it moves forward to the branches and capillaries, it loses its energy, allowing the exchange of waste products and oxygen. Insufficient blood circulation and perfusion could result in a reduction of this process, damaging the surrounding host tissues and leading to ischaemia, apoptosis and cell death [5].

In the human body, blood vessels are continuously and cyclically exposed to different forces, from mechanical shear stress, stretch, tension, to the pulsatility of fluid flow in the inner lumen. In particular, blood vessels’ walls are subjected to substantial radial and tangential stresses as the laminar fluid flows, in opposition to its transverse pressure, thereby resulting in an expansion or elongation of the vessel itself cyclically [5].

Cells in the layered duct are permanently exposed to these mechanical and fluid dynamic forces, sensed and intercepted by various receptors located in strategic positions (Figure 2). As a result, a series of signal pathways, including vascular remodeling, takes place in order to adapt the shape and composition of vessels to the changes occurring and to restore the original values of residual pressure and stresses [5]. Cytoskeletal proteins, for example, mediate the transmission of signals between integrins, focal adhesion sites and extracellular matrix (ECM) and stimulate receptors situated on the membrane, causing intricate

<table>
<thead>
<tr>
<th>Capillaries</th>
<th>Composed of endothelium</th>
<th>Not present</th>
<th>Not present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venules</td>
<td>Smooth lining of endothelium</td>
<td>Thin layer</td>
<td>Present</td>
</tr>
<tr>
<td>Veins</td>
<td>Smooth lining of endothelium with valves</td>
<td>Thinner than the arteries; little smooth muscle and elastic fibres</td>
<td>Fibrous connective tissue, thicker than the tunica media</td>
</tr>
</tbody>
</table>
sequences of events [5]. Because of these local biological events, blood vessels are defined as autonomous and self-regulated systems, being able to adapt their features in different conditions.

![Figure 2 Schematic of a typical capillary bed](image)

While most definitions of TE cover a broad range of applications, the term in practice is closely associated with applications that aim to repair or replace portions of or whole tissues (i.e., bone, cartilage, blood vessels, bladder, skin, muscle, etc.). Often, the tissues involved require certain mechanical and structural properties for proper functioning.

Developing the proper methods that allow for engineering tubular tissues with specific three-dimensional (3D) micro-structural arrangements while retaining particular types of cells at specific locations, represents an essential prerequisite for the realisation of structural-tissue mimicry in re-engineering the human body, which has thus far remained elusive nevertheless.
2.2 Mechanical forces and wall pressure

Pressure, wall tension and tensile stress play a fundamental role in the mechanical behavior of vessels and their remodeling, which are related to each other by Laplace’s law [5]:

\[ T = P \cdot r \]  

(1)

where \( T \) is the wall tension, \( P \) the blood pressure (perpendicular to \( T \)) and \( r \) the radius of the vessel.

On the other hand, walls are subjected to circumferential tension, \( C \), due to the shape and elastin present on the duct, given by [5]:

\[ C = \frac{P \cdot r}{t} \]

(2)

where \( t \) is the thickness of the wall [5].

Figure 3 Sequence of vascular response to transmural pressure and shear stress [5].
Shear stress, $\tau$, defined as the frictional force generated by blood flow [5,6] that influences endothelial cells (ECs) in shape, is generally a tangential force exerted by the fluid to the inner walls of the vessel, and is calculated as follows:

$$\tau = \frac{4 \cdot \mu \cdot Q}{\pi \cdot r^3}$$

(3)

where $Q$ is the flow and $\mu$ is the viscosity.

From the above equation it is clear that as the vessel increases in dimension, the shear stress greatly decreases, in converse to the wall tension, $T$. To sum up, two main forces act mechanically in the vessel: the tangential force is directly proportional to the radius of the vessel, and the longitudinal one, aligned with the interface between the blood flow and inner endothelium layer [5].

In 1980, Bomberger et al [6] studied the relationship between the composition of the vessel wall and the arterial pressure. Their results showed that an increase in circumferential stress (and therefore blood pressure) caused a proportional increase in the thickness of the walls due to both Smooth Muscle Cells (MSCs) hypertrophy and an increase in collagen, fibrous protein and elastin in the structure [6,7]. This effect was widely studied over the past years, especially for an understanding of diseases such as hypertension [8]. In this regard, Levy and colleagues used rat models in 1988 to explore the association between vascular structure and hypertension [8]. They discovered that elevated blood pressure in arteries far from the
basic values (100–140 mmHg in systolic regime and 60–90 mmHg in diastolic) induces the release of matrix proteins and collagen, resulting in thickening the layer which, at length, might lead to a collapse and slackened walls (aneurysm) [8].

In summary, natural blood vessels play a significant role in growing and maintaining biological and physiological systems. Significant variations of the basal parameters from normal physiological values can cause a substantial arrangement and modification of the natural vascular structure, leading to health complications, including aneurysm, atherosclerosis and atherogenesis [9].

2.3 Vasculogenesis and angiogenesis

The human body is regulated by the circulatory system, which maintains all the biological parameters included in homeostasis under controlled values: the main role of blood and its circulation is to deliver nutrients and oxygen throughout the body, and remove the metabolic waste products. Moreover, the delivery of biological signals through different tissues and organs (i.e. via hormones or growth factors) is also a key function to physiology [11,13].

In the body, vascularisation is driven by three main processes: vasculogenesis, angiogenesis and arteriogenesis [8-12]. Vasculogenesis is the de-novo formation of blood vessels from the differentiation of progenitor cells (APCs and EPCs) into endothelial cells which proliferate to create a primitive network; while APCs are recruited during embryonic and fetal growth,
EPCs are recruited in post-natal vasculogenesis including wound healing, infarct, ischaemia, atherosclerosis and tumor growth [7,8].

Angiogenesis is the sequential remodeling of this initial vascular pattern, into a more complex one activated by angiogenic growth factors, which exploits the sprouting or intussusception processes. This process can be characterised by matrix remodeling in order to allow the proliferation and elongation of endothelial cells into the gaps created from the degradation of the surrounding matrix. Arteriogenesis is, then, the progression of the network into bigger and enlarged vessels [17-21,23].

Afterwards, this cascade of events is demarcated by two phases, namely stabilisation and maturation, which are strictly linked to each other [10,11]. First, a dilatation of the diameter of the vessel induces a degradation of the basement membrane, allowing cells to migrate under the control of growth factors such as VEGF receptor 2 (VEGFR2) and VEGF A. Second, vessels are stabilised by ceasing proliferation of endothelial cells (ECs) due to pericytes and MSCs and then maturate the vascular network, avoiding its regression and disorganization [10,11].

For instance, intussusception is the phenomenon of the development and expansion from the vascular network through transcapillary pillars, which are able to create connections and link opposite capillary walls [10-12]. The formation of these “bridges” is then followed by a reorganisation of the endothelium by myofibroblasts and pericytes in order to achieve the vascular remodeling. Note that the intussusception is a rapid process but with low proliferation compared to the sprouting process [12].
In TE, the measurement of functional parameters such as oxygen release and consumption or vascular permeability in the construct is an imperative feature to control biotransport and mass transfer in the whole scaffold. Insufficient or higher values of oxygenation in localised areas alone instead of the entire one, will certainly unbalance functionalisation, resulting in apoptosis or hyperoxemia in the region.

2.4 Hydrogels

TE and its branches have been always aimed at developing new classes of materials and structures from research. One class of biomaterials which have been well known and widely used in the fields is hydrogels, or hydrophilic polymers: they are able to switch from the viscous-liquid phase to the gel phase by crosslinking of their chains through ionic, hydrophobic, hydrogen or van der Waals forces, as shown in Figure 4 [9, 10, 13].

Rather promising results of hydrogels have been obtained recently, which clearly demonstrated a wide use of hydrogels in medical device, such as drug delivery and regenerative medicine. One of the most important characteristics is that hydrogels can absorb around 90% of water in the network, closely mimicking the in vivo conditions [9, 11].
Figure 4 Main forces acting in the transition between the liquid-gel phases in hydrogel crosslinking [26].

The versatility of hydrogels is inherently their ability to control the dimensions and shapes of the final structure by using molds before the crosslinking. Importantly, variation of the degree of methacrylation is also tunable during the chemical synthesis, which makes such materials an attractive solution in TE [24-26].

Note that the term “biocompatibility” refers to the ability of a material to be tolerated by the surrounding living environment, without generating fibrous inflammatory response that typically occurs when extraneous artificial materials are inserted into the body. In addition to biocompatibility, researchers have also aimed to achieve desired mechanical properties, degradability and cell-adhesion, which have been recognized the fundamental requirements for tissue formation in vitro and in vivo.
The embedding vascularised wefts within the whole construct is currently a field of research with great interest in TE. It is expected to provide the proper delivery of nutrients and oxygen as well as exchange of waste products. In fact, the viability of cells is strictly related to the presence of interconnected vascular networks which, incorporated in the hydrogel structure, might offer higher mass transport and facilitate cell proliferation.

2.4.1 Natural hydrogels

Natural, synthetic hydrogels and a blend of the two (“hybrid” hydrogels) have been widely used in TE and regenerative medicine as the main matrix to encapsulate cells. The use of such hybrid materials in further studies of scaffolds for tissue replacements have been developed in many different studies [27,28,29].

Collagen is a main element of various tissues, such as skin, bone, cartilage, tendon and ligament [29], which has been widely used as scaffold for regenerative medicine mainly because it satisfies most of the requirements mentioned above. Uses of normal collagen hydrogels (NCH) are affected by various limitations such as poor mechanical properties and their extensive contraction; however, crosslinking the amino acid chains using a chemical approach with glutaraldehyde or diphenylphosphoryl azide might increase the mechanical properties of the gel [29-31]. A study by Helary and colleagues showed that concentrated collagen hydrogels (CCH), instead of normal collagen hydrogels (NCH), might be used in order to tackle these issues, which can also increase cell proliferation, colonisation and neovascularisation, thereby improving integration and successful rate of the constructs in vivo [32].
Elastin possesses a unique feature, elasticity, which demonstrates its potential as a biologically active material for engineering elastic hydrogels in tissue engineering. It is usually found in blood vessels, skin, cartilage and lung as it provides high elasticity and resilience to the whole construct. Recent studies developed in TE and regenerative medicine showed that elastin increases its bonding ability if specific receptors such as glycosaminoglycans (GAGs) and elastin binding protein (EBP) are present in the same environment [33, 34]. Moreover, elastin is able to facilitate the attachment and proliferation of endothelial cells (ECs) during the formation of vascularisation in hydrogel networks [34].

Tissue decellularisation is perhaps one of the most affordable technique used today to generate elastin-containing biomaterials. In addition to partial hydrolysis of peptide bonds and biosynthetic synthesis, the most significant advantage of this technique is that the stability and durability of these tissues are preserved after the process. However, chemical or physical treatments of the tissues with enzymes and batch-to-batch variations make the use of elastin in TE constructs rather difficult with the risk of pathogen transfer and inflammations [34].

Fibrin, a non-globular protein involved in the wound healing process, has been used in surgery as an adhesive and sealant because of its ability to start the gelification process through enzymatic polymerisation at room temperature after adding thrombin to the mixture [35, 36]. As a natural component, no rejection via an inflammatory response or toxic reaction is expected, while its degradation rate can be delayed to 2 days by use of the inhibitor aprotonin [37].
Recent studies showed that the presence of specific growth factors in fibrin gels might enhance cell migration and proliferation [38]. Moreover, seeding of such modules with smooth muscle cells (MSCs) or chondrocytes has been tested in order to engineer and stabilise migration processes within fibrin scaffolds [38, 39].

2.4.2 Synthetic hydrogels

To form hydrogel networks with certain elastic properties, synthetic elastomers are an alternative. Such synthetic materials are used mainly for their ability to form proper 3D crosslinked networks, mechanical properties similar to the native soft tissues, biodegradability, high elasticity and flexibility [31]. Examples of synthetic hydrogels include polyurethane (PU), which is widely used for cardiovascular devices, including cardiac valves, grafts for bypass, and PEG-based hydrogels. Such materials have proven to be extremely versatile materials for TE applications.

![Examples of hydrogels](image)

**Figure 5** Examples of hydrogels; normal collagen hydrogels (NCH) showed poor mechanical properties and deformation, being difficult to handle (A), while concentrated collagen hydrogels (CCH) kept their original shape (B). Scale bar= 0.5 cm [32].
Synthetic elastin-based (ELPs) hydrogels have been developed chemically to improve the mechanical and material properties, by modifying characteristics such as chain length and the number of crosslinking sites [49]. Furthermore, the process of obtaining synthetic elastin allows incorporating various peptide molecules into the structure.

Poly(vinyl alcohol) (PVA), a water soluble polymer generally used in glues and paper coatings, was also explored for its biocompatibility as well as tensile strength and flexibility. It is usually produced by alcoholysis, hydrolysis or aminolysis [40], while PVA hydrogels are generally obtained using chemical crosslinking with glutaraldehyde as the chemical agent, exposure to UV-light or freezing/de-freezing (thawing) cycles [41-43]. Despite their advantages, PVA hydrogels suffer from slow biodegradation, making them a candidate for semi-permanent graft or scaffolds, as their degradability may take long-term [44]. Tripeptide growth factors such as glycyl-L-histidyl-L-lysine (GHK) have also been explored in the PVA hydrogels: the results showed successful growth of hepatocyte spheroids in immobilised-peptide hydrogels for 5 days, while no cellular attachment was found in regular PVA hydrogels [45].

2.4.3 Hybrid hydrogels

Significant advances in the field of TE have been achieved by the combination of natural and synthetic hydrogels, because the components of such hybrid materials are somewhat complementary [46, 47]. Specifically, the advantages of natural-based gels can be improved by the features of synthetic gels, such as biocompatibility and absence of inflammatory
response. The main challenge during the synthetisation of such blends is to form a proper hydrogel combination, which must avoid phase separation between the two polymeric networks. Thus, to perform a full integration of different hydrogels, it is essential to use the correct amount of each polymer by balancing their relative concentrations. For example, blending an UV-crosslinked polymer, such as gelatin-methacrylated (GelMA) with a temperature-induced polymer (agarose), relies on an adequate concentration of the polymers' liquid phases before the crosslinking. It is noted that several photocrosslinkable hydrogels containing double C=C bonds with vinyl, acrylic and methacrylic groups can allow co-polymerisation with secondary polymers that also have the modified functional groups [46, 47].

Alginate hydrogels have been recently used in TE combined with different proteins such as collagen and fibronectin to improve cell adhesion, since alginate alone did not show any cell proliferation. In addition, chitosan or PVA added to alginate has been found to enhance its mechanical properties [48].

2.5 Vascularisation of 3D constructs

Various in vivo and in vitro approaches have been developed to vascularise 3D scaffolds, using natural, synthetic or hybrid hydrogels. While these efforts have brought us closer to the achievement of engineering vascularised tissues, a deeper understanding of the relevant methods to manufacture and improve functionally and biologically relevant vascularisation still remains a challenge and needs significant work for realisation.
Over recent years, various approaches have been developed to promote vascularisation in tissue scaffolds, from cell-based techniques, which allow an easy and biologically significant communication between cells, to the recruitment of growth factors of different species via chemical or physical immobilisation, to the fabrication of the constructs via 3D printing or lithography approaches (i.e. soft lithography, photolithography, etc.). This field has seen exciting improvement in the pursuit of artificially engineered vascular tissue [63].

*In vitro* approaches rely on growth and handling of such constructs outside the human body, in either bioreactors or culture dishes. Whereas, *in vivo* approaches are dependent on the ability of the host tissue to form functional and fully perfused vascularisation with the scaffold constructs. *In vivo* study is often preceded by the *in vitro* approach, mainly for ethical reasons and for a proper and fully developed implantable scaffold which might need to be prepared externally beforehand [63].

Thus, on the one hand, cell-based strategies allow mimicking the biological behaviors and physical cues in relation to vascularisation. On the other hand, the use of micro-biofabrication technologies permit control of the spatial organisation of the microchannels, or the reproduction of a complex vascular weave. Likely, a combination of such modules will be required for fabricating fully perfused and biologically controlled scaffolds prior to *in vivo* implantation in clinic [63].
Figure 6 Examples of different vascularisation approaches. (A) Scaffold functionalisation: loading of the scaffold with angiogenic factors, including VEGF, bFGF and PDGF. Inclusion of microchannels within the structure may improve oxygen/nutrient perfusion or cellular alignment. (B) Cell-based techniques: co-cultures may be used to generate capillary-like sprouts when encapsulated in the matrix. Further inclusion of transfected cells to secrete angiogenic factors in the scaffold to induce vessel formation. (C) Bioreactor design. (D) MEMS approach: microfluidic systems seeded with endothelial cells to form vasculature may be used. (E) Modular assembly: cell-embedded hydrogels with a confluent endothelial cell layer may be combined together to form a biological tissue under perfusion. (F) In vivo systems: stacked confluent cellular sheets undergo vascularisation before implantation in vivo. Following arteriovenous (AV), anastomosis are used to vascularise tissues in vivo [63].
2.5.1 *In vitro* approaches

Schematics of the main *in vitro* approaches for vascularisation are shown in Figure 6 [63]. Basically, the TE scaffold can be enriched with pro-angiogenic and vasculogenic growth factors, which include basic fibroblast growth factors (bFGF), platelet-derived growth factors (PDGF) and vascular endothelial growth factors (VEGF), in combination with seeding of ECs or embryonic stem cells into the natural, synthetic or hybrid hydrogel bulk (Figure 6A).

Co-culture of different cell lines with ECs is generally used to develop new vascular branches or sprouts by secretion of angiogenic factors to induce blood vessel formation (Figure 6B). Culture of those constructs in enclosed environments with strictly controlled biological parameters (O\(_2\) and CO\(_2\)), known as “bioreactors”, has also been used to solve mass transport issues in culture; in particular, rotating and perfusion bioreactors have been widely exploited in TE [55].

Black et al. [64] attempted to co-culture keratinocytes, dermal fibroblasts and HUVECs in a collagen-based engineered skin equivalent (ESE). The study showed that co-culture of ECs with pericytes leads to recruitment of ECs to the albuminal layer and formation of the comprehensive capillary structure of the basement membrane, miming the natural structure of blood vessels. From their study, ESE seems to be a candidate for the replacement of skin in injuries and diseases concerning vascularisation [64].

The further studies by Enis and the colleagues showed that culture of HUVECs with an antiapoptotic protein called Bcl-2 in fibronectin-collagen I gels induced differentiation of ECs in artery/venule/capillary-like structures, suggesting an *in vivo* remodeling of the vessels.
The use of such assemblies in ESEs might improve the viability of the graft, thus promoting angiogenesis [55].

Despite these efforts to create capillary-like structures embedded in engineered scaffolds, control of the direct growth of vessels has not yet been demonstrated. In vivo vascularisation for engineering microcirculation can be used to overcome the challenge mentioned above: creating a proper linkage between such in vitro developed networks to the human circulation might be achieved by making use of the body as an incubator attributable to the body’s angiogenic ability [56].

Cellular techniques for fabricating engineered vascularised tissues have been mainly focused on the development of proper network architecture through the secretion of the cells’ own ECM and ongoing remodeling of the environment without external intervention [57].

These approaches might solve some functional and rejection issues, as the seeding of endothelial cells into the scaffold avoids the longer fabrication time need to optimise the usage of specific factors involved in angiogenesis. Issues in the delivery of such molecules as growth factors into the scaffold have been solved by using co-cultures with endothelial cells, due to their ability to produce capillary-like sprouts in the presence of bFGF and VEGF [57,58].
Table 2 Cell-Based Techniques for Vascular Tissue Engineering

<table>
<thead>
<tr>
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<th><strong>Endothelial cell cultures</strong></th>
<th><strong>Endothelial cell co-cultures</strong></th>
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<tbody>
<tr>
<td>Mechanism of vascularisation</td>
<td>Use of endothelial cells to form capillary-like structures</td>
<td>Transfected cells used to modulate angiogenic growth factor delivery</td>
</tr>
<tr>
<td>Tissues and cell types co-cultured</td>
<td>Skin, bone, adipose, and muscle tissue; fibroblasts, neural progenitor cells</td>
<td>Bone, myocardial and dermal tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative measurement of nutrient and oxygen diffusion</td>
<td>Luciferase or other fluorescence-based imaging, fiber optic oxygen sensors</td>
<td>None reported</td>
</tr>
<tr>
<td>Points of functional anastomosis</td>
<td>None, requires ingrowth of host vessels</td>
<td>None, only vascular cell recruitment</td>
</tr>
</tbody>
</table>

Boer and colleagues tested the idea that endothelial cells are able to organise and form a pre-vascular network *in vitro* when co-cultured with hMSCs (osteoprogenitor cells) [69]. Unfortunately, their study did not show any effective perfusion *in vivo* because of the absence of a functional anastomosis in the host vasculature. In addition, the transfection of cells with angiogenic factors has been developed, especially in bone TE. The research on different coatings of scaffolds with hMSCs transfected with or without VEGF plasmid showed that vascularisation, proliferation and osteogenesis were improved in VEGF-transfected and plasmid-coated scaffolds [71].
In contrast to the other approaches, these abovementioned studies have the great potential to demonstrate a controlled release of growth factors over space and time, thereby resulting in a better vascularisation in the whole scaffold.

2.6 3D Engineered Scaffolds for Vascularisation

Tremendous efforts in the field have seen the incorporation of growth factors in the 3D engineered vascularised scaffolds in order to stimulate the creation of capillaries in both synthetic and natural hydrogel constructs. VEGF, basic FGF (bFGF) and PDGF have been widely used to initiate and generate the in vivo vascularisation, despite their inability to guide the proper development of the vascular network directly [57-60].

The scaffold has to be thought of a support to mimic in vitro the in vivo cells’ microenvironment, enhancing their ability of proliferation and differentiation as well as using some molecules and signals to trigger specific cell functions. Common materials used for scaffolding include natural and synthetic polymers [55]. While the former are generally made from extracellular matrix (ECM) components (collagen, hyaluronic acid and fibronectin) which are conducive to cell growth, differentiation and proliferation, they have limitations on degradation rate and mechanical properties. The latter can be synthetised and reproduced readily, controlling ad hoc the main properties mentioned above, though they have shortcomings in the biocompatibility of the degradation products [55].

Therefore, the need for materials that can mimic biological features both chemically and mechanically is clear to the research community. So far, great attention has been directed to
the use of hydrogels, or other biocompatible materials with natural or synthetic polymer chains being able to contain around 90% water in their network with a high degree of structural and compositional similarity to the biological tissues and ECM [45]. Adhesion, attachment, growth and proliferation of cells within the 3D network have been favoured mostly by the inclusion of RGD peptides in the hydrogel framework.

Tengood et al. [57] showed that bFGF and PDGF combined sequentially with RGD peptides in a cellulose acetate-based scaffold were essential for maturation of vessels. Similarly, Sun and colleagues developed a new hydrogel by combining the polysaccharide dextran with RGD and PEG. The further studies on the mechanical properties of the scaffold revealed a decrease of tissue ingrowth, hydrogel swelling and release of VEGF with a higher crosslinking of dextran [58-60].

Agarose, a polysaccharide polymer extracted from seaweed, has been frequently used in TE. Song and colleagues [61] studied the distribution and viability of cells in such scaffolds by comparing the constructs with different patterns of channels. Their study showed that cell viability decreased by increasing the distance from the channel and, moreover, the dual-channel scaffold provided wider viability outlines.

Furthermore, combination of these materials with new fabrication approaches, such as micropatternning, has also been tested in vitro [62]. An interesting study performed by Raghavan on tubular formation in micropatterned gels showed that these endothelial networks spontaneously assembled after stimulation with VEGF and bFGF for two days, revealing the importance of the initial patterning as a guide for network development [62].
Synthetic polymers have been also used to fabricate 3D vascularised scaffolds, for the ability to tweak and optimise desired material properties, such as degradation rate, porosity and degree of methacrylation. Indeed, synthetic polyesters, such as polylactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone (PCL), are the most commonly used materials [55,63,85].

Porous PLC scaffolds immobilised with heparin and combined with VEGF have been shown to enhance angiogenesis and vasculogenesis in vivo, giving rise to further possibilities for materials to promote vascularisation [63].

Fabrication via Micro Electro-Mechanical Systems (MEMS) is an innovative approach used in many laboratories, in which the constructs are usually made by using molds in silicone or polydimethyl siloxane (PDMS) bonded to glass surfaces by plasma treatment [65,66]. Micropatterning of such modules using hydrogels has been widely explored, offering a designed size and shape with networking and dimensions close to the human vascular systems.

Briefly, the mold in PDMS and the cover glasses are first placed into the plasma cleaner, and a vacuum is applied to evacuate air from the chamber. Then, the plasma is created inside the chamber thanks to the electric coil; a partially ionised gas consisting of ions, molecules and electrons creates reactive surfaces on the glass and device which, when placed together, will form a permanent bond and hydrophilic surfaces that avoid the leakage of liquids. The plasma also sterilises the devices, giving additional advantage to this approach [71,72].
An interesting study from Wenger and colleagues developed a 3D model to investigate angiogenesis during bone formation [66]. In brief, human endothelial cells (hECs), human osteoblasts (hOBs) and human umbilical vein endothelial cells (HUVECs) spheroids were cultured in a collagen-based matrix and their cellular interaction was investigated. The results showed that the distance between hECs and hOBs is pivotal for the modulation of the formation of the capillary-like network in the scaffold. Moreover, HUVECs and hOBs have the tendency to form co-spheroids spatially arranged [66].

Further studies by the same authors Wenger and colleagues developed a 3D co-culture of fibroblasts and ECs in order to improve and enhance angiogenesis, especially in the engineered grafts [67]. Again, the spontaneous phenomenon of the creation of capillary-like structures by ECs in presence of fibroblasts was investigated and a different spatial organisation was noticed. While HUVECs and hOBs were inclined to have a peripheral localisation, ECs were located in the center of the co-spheroids surrounded by fibroblasts [67].

Formation of tubular constructs was also found in co-cultures of endothelial and osteoprogenitor cells in a scaffold for bone TE [68]. In the early work, it was found that pre-vascularisation of such constructs might be a fundamental key to enhancement of the vascularisation in vivo, which showed a higher cellular viability and improved bone formation activities.
Coralline-based substitutes for bone formation and regeneration have also been investigated [68], in which the coral scaffold was coated with control-plasmid DNA (group 1), VEGF-plasmid DNA (group 2), mesenchymal stem cells (BMSC) transfected with control plasmid (group 3) and both BMSC and VEGF plasmid (group 4) in order to consider these different groups in vivo. The effect of angiogenesis and bone formation were investigated by taking X-ray images and comparing the differences in volume in the area chosen. The results demonstrated higher vascularisation and bone regeneration in the presence of VEGF (groups 2 and 4), showing the pivotal role of the growth factors in angiogenesis and healing [68].

Synthetic poly(ethylene glycol) [PEG] has been used intensively by researchers [73,74] to fabricate hydrogel scaffolds and explore the variations of pH levels and mass transport. Cuchiara and colleagues stated that the combination of a microfluidic pattern in the hydrogel with a co-culture that promotes vasculogenesis is essential to trigger mass transport from simple diffusion into convective and extra-vessel diffusion, better miming the biological behaviors [73].

Mooney et al. [75] studied the effects of the combination of both VEGF and PDGF in a degradable PEG-based hydrogel scaffold. Their study showed that release of these two factors alone did not allow a stable and mature formation of vascularisation, while their blend had the opposite outcome.

In addition, Phelps and colleagues [76] combined other molecules such as RGD sequence (L-arginine, glycine, and L-aspartic acid) and VEGF in their Polyethylene (glycol) Diacrylate [PEGDA]-based matrix. Their in vitro and in vivo studies showed that the creation and
spreading of vascularised networks were enhanced by the presence of VEGF and RGD-adhesive molecules, thereby resulting in an enhanced EC proliferation and formation of new blood vessels [76].

Using a blend of such polymers is an alternative approach that can be developed to obtain new materials. For example, Lesman et al. in 2011 [77] developed a fibrin-based PLGA/PLLA scaffold by taking a co- and tri-culture as a platform for improving vascularisation in vitro and in vivo. Their studies showed enhanced mechanical strength compared with the fibrin matrix alone. Moreover, they observed that the variation in the PLLA:PLGA concentrations enhanced vascular maturation and interconnections of vessel-like structures of the vascular networks in the graft both in vitro and in vivo [77].

Despite these advances, there is still a lack of methodological procedure in the design and fabrication of the complex construct architecture for vascular systems. For this reason, new technologies are required to recreate in vitro and in vivo constructs that mimic the desirable vascular network in native tissues.

### 2.7 Microfabrication approaches for 3D vascularised scaffolds

The ability to re-create engineered vascularised networks in 2D and 3D scaffolds depends mainly on the fabrication technologies available to mimic the hierarchical structures of network inside the scaffold. Some fabricating approaches that are commonly used today include soft lithography, direct-ink writing, stereo-lithography, bioprinting and electrostatic
The recently increased ability of scaling a scaffold’s dimensions, with different materials and networks is due mainly to the direct-ink writing technique [52,53,79].

Microengineering approaches based on microfabrication technologies have demonstrated the enormous advantages of being able to control the dimensions and shape of the topological pattern which, when integrated in the microfabricated device, can provide an adequate vascular network for diffusion throughout the scaffold [78]. The importance of microfabrication has been more and more recognised in scaffold tissue engineering.

Soft-lithography is a technique that makes use of (usually) poly-dimethyl-siloxane (PDMS) as a mold, bonded on a patterned photoresist attached to a silicon wafer [78,52-53,114]. After the preparation of the mold, it is immersed in an environment where molecules can attach to its surface. Then, the PDMS stamp is posed on the surface of the new material, which will be patterned as the mold. The term “soft” refers to the use of the elastomer as a mold and it has various advantages, such as low cost of fabrication, high resolution (up to nanometer scale), and without need to use photo-reactive surface, which enables a wide variety of patterns to be made. Despite these features, this approach allows only the use of stamps with regular primitive geometric shapes, such as circular, square and rectangular.

For instance, many 3D structures have been fabricated via photolithography (crosslinking of materials such as hydrogels through a mask to create the desired network), micromolding and 3D printing for realising some predesigned microstructural patterns so that desired mechanical and biological properties can be attained [74].
Stereolithography is considered a rapid prototyping technique that builds 3D structures layer-by-layer. After that, photopolymerisation is carried out in a resin reservoir, after the tracing of the desired pattern from the proper laser. Despite these easy fabrication steps, each layer needs a different mask, leading to higher fabrication timing and costs with this approach [78].

Mapili et al. created the complex biological environment and developed single lines of cells into a multiple ones in a poly(ethylene glycol) dimethacrylate (PEGDMA) scaffold, by using stereolithography to fabricate precise spatial network [78]. They further incorporated the PEG acrylates modified with heparin sulfate and showed that this approach allowed creation of precisely localized molecules within the topological pattern, resulting in a controlled-release of such particles over space and time.

An alternative approach used in TE is direct-ink writing, where a viscoelastic material is dispensed by a nozzle or syringe directly into the wafer or substrate [52]. The automated device has a robotic arm mounted on it, able to move in three dimensions (x-y-z), thus allowing a layer-by-layer deposition of the ink material. The fabrication of the patterned network is performed via deposition of a sacrificial material in the form of a filament. Therriault et al. [53] first demonstrated the fabrication of an interconnected 3D microvascular network using a fugitive ink deposited in a layer-by-layer fashion for a 3D periodic square-spiral design. After the deposition, the whole scaffold was perfused with a resin and cured. After solidification, the matrix was heated at 70 °C in order to liquefy the ink and the vacuum was used from one side of the construct in order to remove the liquid. From their study, the feature sizes showed microchannels ranging from 50–200 µm in dimension.
An interesting work done by Ying and colleagues [79] investigated the effects of the injection molding technique on angiogenesis and thrombosis phenomena. The microchannels created via the lithographic technique in collagen gels were seeded with human umbilical vein endothelial cells (HUVECs) while HUASMCs were encapsulated in the hydrogel’s bulk aiming to understand the relationship between endothelium and perivascular cells. The results showed that the approach used to endothelialise the scaffold is harmonious with the process of the creation of vascularised vessel-like structures and after the cells proliferated up to two weeks, a proper vascular network was created in the hydrogel [79].

In another study, 3D microchannels were created by using an electrostatic discharge in a solid matrix such as poly (methyl-methacrylate) PMMA and poly(lactic acid) PLA [109]. In brief, an electron beam irradiation at high energy initiated this electrostatic discharge, which, due to its intensity, dissolved and evaporated regions of the matrix (Figure 7). Regardless of the final network fabricated in the structure, this approach may cause some problems in the following cellular studies. For instance, the high intensity electron beam applied will result in a rapid decrease in cellular viability and the resistance to flow in the vascularised pattern will not be optimal [111].

New efforts to engineer vascularised constructs have been made using 3D bioprinting [80-82], which is the automated deposition of biomaterials embedded with cells to create 3D defined patterns, giving rise to a macroscopic engineered tissue construct. Versatile and sophisticated patterns by exploiting CAD modelling can be generated with a wide range of materials [82]. This strategy currently represents one of the most significant developments
in TE, bringing significant advantages over traditional fabrication techniques, such as easy replication, high reproducibility and low manufacturing times.

In one of the earlier studies, Miller et al. [80] developed a carbohydrate glass-based template system, which was later dissolved by using normal culture medium. To avoid osmotic shock to the cells during this dissolution, the glass lattice was coated with poly(d-lactide-co-glycolide) (PDLGA).

To avoid the release of potentially toxic products, Bertassoni et al. [81] harnessed agarose templates to form hollow cylindrical channels in macroscopic constructs. The following removal of the template through manual vacuum was easily performed, thanks to the poor molecular interaction of the agarose gel with the GelMA (gelatin methacrylated) bulk (Figure 8). The results of this work showed that the technique improved mass transport overall in the scaffold, enhancing cell viability and differentiation up to 7 days. Furthermore, Bertassoni et al.’s study demonstrated the fabrication of different geometries via bioprinting and the formation of an inner monolayer of endothelial cells miming the vessel’s structure (Figures 9–10).
Figure 7 Schematic of the omni-directional printing of 3D microvascular networks in the hydrogel matrix: (a) Deposition of the fugitive ink into the matrix; (b) Empty parts of the networks are filled with a fluid; (c) Crosslinking of the matrix via chemicals through photopolymerisation; (d, e) Liquefaction of the fugitive ink, which is then removed by vacuum exposition; (f) Final fluorescent image of the construct where the network is enhanced by a red dye ink (scale bar: 10 mm) [111].
Figure 8 Schematics of the fabrication steps adopted: a) Dispersion of the agarose via the capillary of the bioprinter; b) Immersion of the agarose fibres in a cell-laden gelatin-methacrylated solution; c) Removal of the template from the hydrogel; d) Final structure, fully perfusible [81].

Figure 9 Photographs of the printed templates in GelMA hydrogels. Template before the removal of the agarose (A-C) and perfusion of a pink microbead dye was performed to show the microchannels (A-C-i). Scale bars 3 mm; microchannels are 500 μm in size [81].
Recent work by Wang and colleagues [82] combined an evaluation of vascularisation using CAD modelling fabrication and in vivo implantation.

First, a set of CAD tools was used to determine the best design and mechanical features of the scaffold via simulation. Second, a polymer resin (PPF) was printed according to the design and specifications of the theoretical studies. This modular approach is thought to be ideal for the fabrication of TE scaffolds as it combines both numerical and experimental methods [82].

3D tubular structures created from microengineering techniques have seen the development of scaffolds containing a confluent and adequate endothelium layer in the inner wall of the vascular network [81-83]. In one of these studies, 3D printing was used only partially in order to dispense a blend of HUVECs and gelatin on a collagen layer, which was
later immersed and totally covered by another deposition of type I collagen [82]. Further
dissolution of the primary sacrificial channel was performed by exposing the scaffold at
37 °C, permitting cell attachment to the inner collagen layer of the construct.

To summarise, the challenges to be overcome include:

- to find the techniques for engineering tubular tissues with specific heterotypic
  cellular micro-arrangements to biomimic the human anatomy;
- to have direct heterotypic interaction between interfacing layers in the structure;
- to guarantee proper values of oxygenation thereby avoiding apoptosis or
  hyperoxemia;
- to use a proper hydrogel blend (i.e. natural and synthetic) to maximise the
  advantages of both;
- to find an alternative approach able for designing and recreating the complexity in
  the architecture of the vascular network.

The aim of this thesis was to establish a new framework in this regard and demonstrated its
capacity and features of solving this critical problem.
Chapter 3: Materials and Methods

3.1 Development of the Optimised Pattern

Failure to engineering thick tissue block is usually due to the difficulty of guaranteeing sufficient nutrient supply, waste removal and uniform cell distribution within the matrix [83]. Previous studies aimed to solve these issues by using modeling methods, in which the design of the scaffold was optimised using feedbacks from computational simulation studies [85-87-115]. The computational tools can be used for creating a best possible design allowing desirable transport of nutrients and removal waste inside the scaffold structure, and eventually vascularising the construct. The optimised the network can then be fabricated via direct or indirect 3D printing technologies. Finally the scaffold is tested via cellular studies in vitro and/or in vivo.

While significant efforts have been made to obtain preliminary design prior to fabrication and in vitro tests [115], the prediction of the effectiveness, behavior and final results of a scaffold remains rather challenge. The design is not always consistent with the outcomes in terms of the biological factors or biotransport processes involved, where nutrient gradients within the scaffold and cellular density and viability may not be attained as expected [84-89].

It is noted that maintaining a proper nutrient gradient in the structure through diffusion remains fairly demanding; though the presence of an interconnected channel network to supply the nutrient may be feasible simply because its direct contribution and consequence have not yet been completely clarified and described [89].
The aim of the study is to design and fabricate scaffolds which replicate the vasculature system. The development of a novel approach to fabricate the scaffolds is the key to obtain a proper device with the desired characteristics.

The main hypothesis is that the vasculature tissue is in a steady-state diffusion condition, where a topology optimisation of the vascular architecture has to be provided in order to avoid hypoxia (Figure 11). The optimised design can be assumed as a two-phase problem with void/fluid as the conductive phase and solid as the less-conductive one (Figure 12). Moreover, nutrients and oxygen maintained at fixed concentration can enter only through a point (inlet) and diffuse throughout the domain. The channel phase, presumably filled with biofluid, was given a nominal diffusivity value of 1 unit whereas the hydrogel phase allowed diffusion to occur at a much lower rate (0.00125 unit). The optimal channel configuration was obtained by minimising the total diffusion compliance (resistance) of the scaffold, $J$, subjected to a hydrogel volume constraint, $\Omega_{\text{gel}}$ (4).

![Figure 11](image)

**Figure 11** Boundary and load conditions in design models with uniform oxygen consumption
The complex scaffold-cell-nutrient interaction has necessitated computational simulation, which has been increasingly incorporated into the design and analysis of tissue scaffolds to investigate the viability of structural designs and the effectiveness of different transport systems [102-104].

The purpose of this topology optimisation is to minimise the diffusion compliance, $J$, expressed as:

$$
\text{Minimise: } J = \int_\Omega \frac{\partial u}{\partial x} D \frac{\partial u}{\partial x} d\Omega \\
(4)
$$

$$
\text{Subject to: } \int_\Omega (1 - \rho_{gel}) d\Omega \leq 1 - \Omega_{gel}
$$

where $u$ is the oxygen concentration, $D$ is the diffusivity coefficient, $\Omega$ the design domain, $d\Omega$ is the volume, $\rho_{gel}$ is the local hydrogel volume, and $\Omega_{gel}$ is the desirable hydrogel volume of the entire scaffold (60% in this case).
The sensitivity of each element to the change in density was determined through the sensitivity analysis, using the following function:

\[
\frac{\partial J}{\partial \rho} = \frac{1}{2} \frac{\partial u^T \partial D(\rho) \partial u}{\partial \rho} \frac{\partial u}{\partial x} \frac{\partial D}{\partial \rho} \frac{\partial \rho}{\partial x}
\]  

(5)

where \( \rho \) is the design variable in terms of relative density, which represents the solid volume fraction (i.e. hydrogel in this study) in each element.

The diffusivity coefficient is related to a volume fraction of the fluid-solid phases: assuming that the fluid and solid phases have diffusivity of \( D_v \) and \( D_s \) respectively, the effective local diffusivity \( D \) of the hydrogel (partially-solid) material can be expressed as:

\[
D(\rho) = D_s + (D_v - D_s) \times \rho_v p
\]  

(6)

where \( p \) is the penalisation factor. This power-law relationship between volume fraction and diffusivity has followed the Solid Isotropic Material with Penalisation (SIMP) principle [104,105].

At the equilibrium, the diffusion system follows the Fick’s law,

\[
-D \frac{\partial^2 u}{\partial x^2} = f_b,
\]  

(7)

where \( f_b \) is the oxygen consumption rate and the value is a constant. The oxygen consumption is volumetric and can be appropriately represented by the body force term.

The topology optimisation framework adopted in this study is shown in a flow chart in Fig. 13, where the initial model is related to the primary inputs and parameters required for initialising individual functions and FEA stands for Finite Element Analysis.
In addition, Fig. 14 plots the history of objective convergence in topology optimisation which indicates a typical convergence.
The resultant 2D model (final model) was then exported as the CAD drawings and evaluated for further process in the manufacturing of the hydrogel scaffold. Figure 15 shows the structure of the channel in the scaffold in various scenarios, each with a different diffusivity of the solid phase. It is assumed that all channels are filled with fluid, which has diffusivity coefficient of 1 unit, while the solid part (not shown) is assumed to have a lower diffusivity.

The model in Figure 15-b was chosen as the similarity of its diffusion constant to the models found in the previous studies [111].

**Figure 15** Results of the modelling simulation (a-f), by changing the diffusion coefficient in the hydrogel. Volume fraction (Vf) equal to 60% in all models (a-f); aspect ratio is 1:1 in all models (squared) as is assumed. The diffusivity of the channel is equal to 1 unit, while the diffusivity of the solid part is lower and assumed to be equal to a: 0.00000001, b: 0.00125, c: 0.0025, d:0.005, e:0.01, f:0.025 unit.
3.2 Gelatin-methacrylated hydrogel synthesis

Various studies have demonstrated that GelMA is an inexpensive hydrogel, which is biocompatible and biodegradable over time. In literature, cell-laden Gelatin-methacrylated scaffolds have been fabricated in microfluidic devices to create platforms allowing cells proliferating and migrating when being encapsulated in the gel [90,91]. Importantly, its mechanical properties can be tuned as desired by changing the degree of methacrylation and gel concentration [92]. This nature-based gel is derived from collagen subjected to denaturation and can be obtained from different sources; as shown in Figure 16, when methacrylated groups are added to the amine groups of pure gelatin, the reaction creates a light polymerisable material.

![Figure 16](image)

**Figure 16** Gelatin-methacrylated synthesis. Gelatin macromers containing primary amino groups were reacted with methacrylic anhydride (MA) to add methacrylate pendant groups (A). To create a hydrogel network, the methacrylated gelatin (GelMA) was crosslinked using UV irradiation in the presence of a photoinitiator (B) [92]
It has been demonstrated that cellular encapsulation in this hydrogel network is of excellent biocompatibility, high cellular spreading, elongation, good viability and stability of the structure at 37 °C [92]. Although PEG-based hydrogels are popular in TE for cellular culture studies attributable to their biocompatibility and good mechanical properties, cells cannot adhere and their migration and proliferation can be problematical in the network [92]. For these reasons, GelMA hydrogel was an attractive candidate for our studies and was the unique hydrogel material used in all the experiments [92].

Methacrylated gelatin was synthetised in our laboratory as described in the previous works [85]. Briefly, type A porcine gelatin from skin was dissolved in a 10% (w/v) Dulbecco’s phosphate buffered saline (DPBS; GIBCO) at 60 °C and then stirred until fully dissolved.

Methacrylic anhydride was slowly added in a drop wise to the solution (0.5 mL/min) at 50 °C for 1 h. Addition of warm 5X DPBS at 40 °C was then performed in order to stop the reaction and the solution was finally dialysed against distilled water (DI) using 12–14 kDa molecular weight cut-off dialysis tubing for at least 1 week at 60 °C to remove excess salt and methacrylic acid. After this period, if the appearance of the tubes was cloudy and opaque, further time under the same conditions was allowed until the membranes were found to be clear and colorless.

Lyophilisation of the solution was performed for 3 days to remove all the excess water and to obtain a white and porous foam, which was finally stored at 80°C until further use.
Freeze-dried GelMa macromers were dissolved at 5% (w/v) concentration into sterile DPBS containing 0.5% (w/v) 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959) as a photoinitiator at 80 °C until fully dissolved (Figure 14).

3.3 Polydimethylsiloxane (PDMS) Mold

PDMS molds were prepared following the manufacturer’s instructions. A mixture of silicon elastomer base and curing agent (mass ratio 10:1; Sylgard 184) was dispensed on a Petri dish such that the thickness of the viscous solution was around 1 mm. Following the treatment under vacuum to remove the excess bubbles in the mold, it was cured at 80°C overnight and the material stored at room temperature until further use [72].

3.4 Scaffold Fabrication

In order to implement the optimised topological design, two cover glasses were bonded to a polydimethylsiloxane (PDMS) mold to form a three-layer structure. The mold was previously cut with a “U” shape, and interposed in the glasses paying attention to the right positioning. The plasma treatment with the plasma cleaner was performed following the protocol in Appendix I, as such a method to efficiently bond the glass-PDMS surfaces to each other, avoiding the opening of the device in an aqueous environment (i.e. culture media) and sterilising all the components, as in Figure 17 [80,81].
Laser printing of the model on a chrome and soda lime substrate mask (Bandwidth Foundry International) of 2 cm x 2 cm was performed in order that the smallest feature be 50 µm in size.

For the optimally patterned scaffolds, the chrome/soda lime laser printed mask was then posed on the top of the three-layered device (Figure 17), and 500 µl of 5% (w/v) Gelatin-methacrylated hydrogel was dispensed in the inlet using a disposable syringe.

Following UV light exposure at density of 850 mW/cm² for 20 s allowed the crosslinking of the areas exposed and washed the uncrosslinked areas or other the regions underneath the non-transparent areas of the mask.

The inlet was then perfused with Hank's Balanced Salt Solution (HBSS: Gibco), as shown in the final patterned hydrogel networks in the sample (Figure 17).

The choice of these parameters is due to the fact that the UV light exposure negatively affects the cellular viability in the hydrogel. The higher the timing of exposure, the lower the viability of cells. As the aim was to obtain a fully-crosslinked hydrogel, an intermediate timing value was chosen [93,94].
Figure 17 Device for plasma treatment: plasma cleaner (left) and pump (CC-49 Javac).

Figure 18 Flowchart of fabrication for the implementation of the optimised vascular network in the hydrogel scaffold.

Preliminary experiments showed that, for the sizes of the sample we used, a UV exposure time below 20 s cannot crosslink the hydrogel properly (it was too soft), whereas for
exposure times higher than the value (20 s) showed complete crosslinking for even the smallest areas of the design, attributable due to the effects of light refraction (Figure 18). The control group was fabricated using the same technique and conditions without interposing the patterned mask between the UV source and the cover glasses. The samples of hydrogel block with the same sizes were compared to the optimally patterned ones.

3.5 Cellular studies

Human umbilical artery smooth muscle cells (HUASMC) in M199 (Gibco) supplemented with 10% (v/v) Bovine Calf Serum (BCS; Gibco), 1% (v/v) penicillin-streptomycin and 1% (v/v) L-glutamine were cultured at 37 °C in a humidified and 5% CO₂ incubator. The culture media was changed every 3 days and the cells were passaged once per week. For the experiments, 6 to 7 passage cells were used.

*Figure 19* Comparison between the pattern of the mask and the hydrogel structure; for UV light time exposure higher than 20 s the smallest features were affected from the diffraction of the light which changed the crosslinking of the areas.
The main reason for the choice of this cell line was that smooth muscle cells (SMC) are widely involved in vascularised processes and their migration through the scaffold plays a critical role during the repair of injury sites. This is because when they enhance the release of matrix metalloproteinases (MMPs), cell migration is raised [108].

The previous studies showed that endothelial cells ECs (i.e. HUVECs) spontaneously try to form tubular structures, building intricate capillary-like networks [109]. This phenomenon is also enhanced when peptides are covalently immobilised in the matrix [110]. Encapsulation in the GelMa matrix would have led to a sample with a spontaneous vascularisation, rather than the pattern network as desired.

3.5 DiO-DAPI assay

First, all the flasks of cells were incubated for 1 h with Hank's Balanced Salt Solution (HBSS; Gibco) supplemented with DiO (Molecular Probes) at 1/200 and DAPI at 1/5000 dilution. Following the detachment with trypsin for 5 min at 37 °C, the cells were centrifuged at 1500 rpm and 4 °C for 9 min in order to obtain a pellet.

All the samples (at least 3 per day) were then immersed in the culture media supplemented with DAPI, which was changed every 3 days, in a static condition. In order to quantify cellular viability, we adopted the DiO-DAPI rather than the live/dead assay because it might identify only the cells within reach of diffusion from the channels created. We pre-labelled all the cells with DiO prior to the encapsulation, which allowed us to visualise all cells as fluorescent
green structures. To identify which ones were dead, inclusion of DAPI was then performed in all the steps, thus enabling us to identify dead cells as a green structure with a blue nucleus.

### 3.6 Diffusion

For the modelling, we assumed a constant diffusivity coefficient of \( D = 3.81 \times 10^{-6} \text{ cm}^2/\text{s} \) for the entire scaffold (no evaporation was considered) while for the *in vitro* test the patterned hydrogel scaffold was fabricated as described previously and then perfused with a solution of 0.1% (w/v) fluorescent 20 kDa Fluorescein isothiocyanate–dextran (FITC; Sigma) dissolved in PBS, not necessarily sterile. The fluorescent dye was chosen as an approach to mimic the diffusion of VEGF in the hydrogel matrix [93].

Images were taken with a fluorescent microscope every 3 min after the perfusion, using a magnification of 5X (Figures 23–38).
Chapter 4: Results

4.1 Fabrication of the Optimised Hydrogel Scaffold Samples

Figure 20 shows the realisation of the optimised design of vascular network as a form of the final construct (2 cm x 2 cm), which was perfused with a fluorescent ink. The picture of sample clearly replicated the same network pattern as the fabricated chrome/soda lime mask, demonstrating the effectiveness of the method developed. It must be pointed out that this approach is not only cost-efficient, reproducible and 2D scalable, but also allows fabrication of the final constructs within short time and sizes (around 30 s for a thickness of 1 mm), reducing the likelihood of negative effects on the following cellular studies in terms of viability and proliferation.

Figure 20 Photograph of the Gelatin-methacrylated patterned hydrogel scaffold perfused with a fluorescent ink.
4.2 Diffusion in the Gelatin-methacrylated Hydrogel Matrix

The diffusivity coefficient was calculated using both a modelling simulation in silico and experimental test in vitro. A comparison between these two sets of results was provided in Figure 21.

For the in vitro model it was assumed that as the fluorescent dye diffuses into the hydrogel, the intensity of the image increases. For this reason, the diffusivity was determined by measuring the intensity of the fluorescent in all the images taken using ImageJ. For the in silico model, the diffusivity was calculated using the equation (6).

A 2D diffusion modelling was conducted to simulate the in vitro diffusion of fluorescent dye observed in the physical experiment. The diffusion coefficients of fluorescent dye in water and hydrogel were subsequently determined through the least square technique so that the in silico and in vitro results can match to each other. In the numerical simulation, it was estimated that the dye diffusivity in water was 2.5 times that in the hydrogel; It was assumed that the dye concentration on the left and the right sides of the model was maintained at a constant level. The simulation ran on a finite difference model to simulate a 1.5-hour long diffusion process.

Figure 22 shows a comparison between the simulation in silico and experimental test in vitro, which showed good agreement between the simulated and experimental results for the first 40 minutes of diffusion: the alteration and manipulation of the boundary conditions
(BCs) to achieve computational results consistent with the *in vitro* experiments data over long time was considered as a limitation of computational simulation.

However, the use of the fixed BCs in the simulated model may have overestimated the dye concentration at modelling boundaries. As a result, the dye intensity increased continually and steadily after 40 minutes, which exceeded the dye intensity observed in the experimental study.

*Figure 21* Comparison between the hydrogel scaffold and the simulation: microscope image of the construct at 5X (a) and the simulated sample (b).
**Figure 22** Minimum and average dye intensity over time: a 9 min delay in the simulation was used.

**Figure 23** Fluorescent image of the channel after perfusion taken at t=0 min. Scale bar 50 µm.
**Figure 24** Fluorescent image of the channel after perfusion taken at $t=3$ min. Scale bar 50 µm.

**Figure 25** Fluorescent image of the channel after perfusion taken at $t=6$ min. Scale bar 50 µm.
Figure 26 Fluorescent image of the channel after perfusion taken at t=9 min. Scale bar 50 µm.

Figure 27 Fluorescent image of the channel after perfusion taken at t=12 min. Scale bar 50 µm.
Figure 28 Fluorescent image of the channel after perfusion taken at t=21 min. Scale bar 50 µm.

Figure 29 Fluorescent image of the channel after perfusion taken at t=18 min. Scale bar 50 µm.
**Figure 30** Fluorescent image of the channel after perfusion taken at t=21 min. Scale bar 50 µm.

**Figure 31** Fluorescent image of the channel after perfusion taken at t= 24 min. Scale bar 50 µm.
Figure 32 Fluorescent image of the channel after perfusion taken at t=27 min. Scale bar 50 µm.

Figure 33 Fluorescent image of the channel after perfusion taken at t=30 min. Scale bar 50 µm.
Figure 34 Fluorescent image of the channel after perfusion taken at t=33 min. Scale bar 50 µm.

Figure 35 Fluorescent image of the channel after perfusion taken at t=36 min. Scale bar 50 µm.
Figure 36 Fluorescent image of the channel after perfusion taken at t= 39 min. Scale bar 50 µm.

Figure 37 Fluorescent image of the channel after perfusion taken at t= 42 min. Scale bar 50 µm.
Figure 38 Fluorescent image of the channel after perfusion taken at t= 45 min. Scale bar 50 µm.

4.3 Cellular Viability: DiO-DAPI assay

Cellular viability was evaluated using a DiO-DAPI assay: DiO (Molecular Probes) is carbocyanate lipophilic green fluorescent dye used in TE to label cell lipids and cell membranes [96], while 4',6-diamidino-2-phenylindole (DAPI; Sigma) is a blue fluorescent dye used in microscopy to label mainly the cell nucleus. DiO is mainly used in low concentration as addition to the culture media in order to pre-label cells and monitor their proliferation [96]. DAPI, instead, is usually used in cellular studies to label the nucleus of all the cells (i.e. Actin-DAPI assay, for the quantification of proliferation) but previous studies reported that, if used in low concentration, it can label the dead cells as because the cellular membrane is weak and allows the passage of fluid into the cells, as shown in Figure 39 [96,98].
Preliminary studies with the DiO-DAPI assay showed a viability around 80% at day 1, increasing over time to 97% at day 7 (Figures 40 and 41). Although these results appeared fairly promising, the cellular concentration initially used was too low in this preliminary test (37 x 10^4 cells/mL). As shown in Figure 40, few green and blue spots relative to the viable and dead cells were visualised in the fluorescent images.

**Figure 39** Cell staining methods for viable and dead structures.

**Figure 40** Fluorescent images overlapped to brightfield of the DiO-DAPI assay taken at day 1, 3 and 7. Magnification 5X; scale bar 50 µm.
Since cell density affects cellular interaction, activation and expansion [95], it was decided to repeat the experiment with increased cell density (concentration) in the hydrogel in this study [79,90,98].

**Figure 41** Quantification of the viability (low cell density) at day 1, 3 and 7.

**Figure 42** Fluorescent DiO-DAPI images (patterned) taken at days 1, 3 and 7 showing high cellular viability in the whole construct. Cellular density $5 \times 10^6$ cells/mL. Magnification 10X; scale bar 50 µm.
Figure 43 Fluorescent DiO-DAPI images in the network-free hydrogel block of 2 cm x 2 cm (control) taken at day 1, 3 and 7 showing medium-high cellular viability in the whole construct. Cellular density $5 \times 10^6$ cells/mL. Magnification 10X; scale bar 50 µm.

Figures 42 and 43 provide the results of the DiO-DAPI assay with higher cell concentrations, both for the patterned scaffold and the control. It is clear that besides encouraging results of the assay for the control, the patterned hydrogel scaffold has a greater viability than the control scaffold.

As shown in Figure 44, the quantification of these results showed high viability which increased over time, genuinely as a result of cell proliferation in the hydrogel. As predicted, the viability of the patterned construct was found to be higher than that of the control (non-patterned hydrogel scaffold), demonstrating the diffusive effectiveness of the optimised topological pattern in the hydrogel construct (Figure 44). In other words, the fabricated network of vessels was found actually to improve nutrient transportation within the construct, as suggested by the modelling simulation.
Furthermore, all the steps included in the fabrication (i.e. injection of the hydrogel in the void space, manufacturing timing, etc) of the scaffold did not negatively affect the viability results, an added advantage of the approach developed.

A single factor analysis of variance (ANOVA) was performed to have statistical results of the groups: the statistical significance was not sufficient to validate the hypothesis as a p-value equal to 0.4744 was calculated ($p > 0.05$), but at the same time the F-statistic suggested not to reject the hypothesis as $F < F_{crit}$.

![Figure 44](image)

**Figure 44** Quantification of the construct viability using a DiO-DAPI assay at days 1, 3 and 7.
4.4 Cellular migration

3D cellular migration is of a fundamental role in TE and related studies, being a natural process which occurs biologically in the scaffold. It is a complex phenomenon comprised of cellular adhesion, integration to the substrate and activation of a sequence of signals, which occur mainly when cells are able to move inside the extracellular matrix (ECM) in order to repair wounds or damaged sites, breaking down the proteins that make up the gel via enzymes [106].

On the one hand, the hydrogel scaffold needs to provide a stable substratum and microenvironment where cells can adhere and exchange cues. On the other hand, the scaffold has to supply a certain porosity, providing void spaces for cells to move around [106]. As a matter of fact, biochemical and biophysical factors are present when 3D migration is required. It has been widely shown in literature how difficult it is to replicate this process in pure PEG-based hydrogels [92]. However, when conjugated with growth factors such as bFGF covalently immobilised in the matrix, alignment and migration are enhanced, especially if a gradient in their concentration is present [107]. Nevertheless, extreme migration of smooth muscle cells (SMC) might lead to pathogenic diseases such as hyperplasia, because of the uncontrolled growth and proliferation of SMC within the matrix [108].

In this thesis study, Gelatin-methacrylated (GelMA) hydrogels were chosen for their biocompatibility, degradability and good mechanical properties. As shown in the literature
[90], the benefits of GelMa hydrogels are related to the spontaneous migration in the matrix, as confirmed by the results of the DiO-DAPI assay.

Figure 45 shows a fluorescent image of a channel inside the scaffold at day 7, which is initially void as cells are encapsulated only in the hydrogel matrix. The presence of cells in that region is an indication of the migration in three dimensions. However, it was not possible to measure how far cells migrated as the technique used to label them is not selective for one cell only.

![Fluorescent image of the DiO-DAPI assay at day 7, focused on the channel; the presence of cells in this region confirms the migration in the construct. Magnification 10X. Scale bar 100 µm.](image)

**Figure 45** Fluorescent image of the DiO-DAPI assay at day 7, focused on the channel; the presence of cells in this region confirms the migration in the construct. Magnification 10X. Scale bar 100 µm.
Chapter 5: Discussion

5.1 Artificially Patterned Vascular Network

Despite the great promise of the techniques described before, none of the studies found in literature have achieved a perfect combination of the modular and theoretical approach with experimental counterpart. In particular, the existing research gap in TE concerns the lack of a proper modelling and optimisation techniques relating to the development of a 3D vascularised scaffolds. Although the interesting results from Wang et al. [82], a consistent and standard methodology has not been implemented. Cellular encapsulation or seeding as well as measurement of the viability are missing in their study. Moreover the absence of an optimisation related to the diffusion in the scaffold makes this work incomplete [82].

The goal of this project is to merge diffusion-based topology optimisation, biomaterials synthesis, photolithography technique at microscale and cellular study for developing an approach able to generate vascularised tissues constructs. For the first time it enables the cellular study in a more predictive fashion with mathematical modeling and optimisation, which is expected to form a procedural tool for the future studies in the relevant areas.

While the optimised topological vascular system developed in this study has a non-uniform network, it maximised the diffusion of the domain, allowing more nutrient and oxygen to be transported in the entire scaffold via natural diffusion. In contrast, other studies in literature largely adopted uniform network for vascularisation [80-82], which did not guarantee the
maximization of diffusion in the entire domain, to a certain extent restricting the biotransportation and vascularisation in the constructs.

The relevance of the study is demonstrated by simulating the possible diffusion outcome before the in vitro experiments. This will better guide the fabrication with more closely predictable results obtained from the in vitro tests. From this regard, a novel framework was proposed here by integrating mathematical modelling, computational design optimisation into fabrication and in vitro testing, thereby better tuning and attaining in vitro and in vivo experiments for maximising the outcomes. It should be pointed out that the nature of this project, which integrated diffusion-based topology optimisation with microfabrication of cell-laden hydrogel scaffolds for in vitro studies, might form a more feasible framework for future studies.

Another fundamental requirement in this field is the architectural design of the scaffold for attaining the desired characteristics. In addition to the mechanical properties, biocompatibility and conjugation of specific molecules, the scaffold should also provide a proper environment for the ingrowth of cells, translating to specific pore size and shape as well as their interconnectivity, to allow migration and proliferation of cells within the matrix to promote dynamic vascularisation processes. The results obtained in this study demonstrated such a feature as shown in Chapter 4, where the capacities of nutrient diffusion and cell migration have been validated via the in vitro tests.
5.2 Microfabrication for Hydrogel Tissue Constructs

It is clear that microengineering approaches based on microfabrication techniques are important in scaffold tissue engineering to control the dimensions of the microstructural pattern for providing a guide for cellular growth [62,78]. The method developed in this thesis study enabled us to realise the desired vessel-like topological architecture from the following aspects.

First, no heating of the hydrogel matrix at high temperature [53] or electrical discharge [111] are required to realise the final topological hydrogel pattern fabricated through crosslinking under UV exposure. Instead, a quick washing with culture media has been found effective. As a result, no added stress on cells is involved in the fabrication of construct; and for this reason, the viability is not affected negatively as shown in the results.

Second, there is no automatised device (such as bioprinter) required and thus there is no associated issues with determination of the optimised parameters for scaffold/construct printing [74, 80-82]. Therefore, shorter fabrication timing is required (we measured in 20 s of UV exposure for the crosslinking and 5 s for the washing).

Third, the proposed technique is simple, scalable in 2D and flexible. The scalability is due to the fact that with the same approach it is possible to fabricate scaffold in different dimensions for applications as skin tissue constructs (2D). However, 3D applications can be exploited only with 3D printing or layer by layer crosslinking. While the flexibility is due to
the fact that different UV light sensible hydrogels (i.e. PEG, PEGDA) could be used with the same approach [73-75].

Fourth, a significant advantage of the proposed microfabrication approach is its effectiveness and low cost. Each sample needs 500 µL of Gelatin Methacrylated (GelMA) solution, which is polymerised under UV-light exposure for realising the topological pattern in the hydrogel constructs and no sacrificial mold is required [81].

Finally, the study demonstrated how this proposed diffusion based topology optimisation and microfabrication procedure improved upon the limitation of biotransportation inside the construct and sacrificially molded micro-channels without the use of complex machinery. It has been also shown that the control of the replica of the patterned vascular channel allowed implementing the optimal design with considerable advantages in the fabrication of the microchannels within the construct.

5.3 Cellular Studies

The thesis proposed a novel procedure ranging from design optimisation, microfabrication to cellular studies aiming to tackle existing problem of vascularisation in engineering bulk tissues. Different from the previous studies, an optimised artificially-patterned vascular network is provided here for promoting cell viability, differentiation and proliferation so that more sustainable tissue constructs can be generated for in vivo implantation.
The *in vitro* cellular testing using an alternative assay (DiO-DAPI) validated the hypothesis and expectations of the topological optimisation and microfabrication of the desired vascular network. The results showed a more proper diffusion of nutrients and O₂ in the entire scaffold in the optimised hydrogel (group A); while a lower diffusion was present in the control (group B). In other words, the topology optimisation for maximising diffusion of the structure does allow better delivery of nutrients to the central region of the constructs.

Imaging of the samples with Human Umbilical Artery Smooth Muscle Cells (HUASMC) embedded in the GelMA hydrogel matrix confirmed the improved outcome of cellular activities, where the cellular viability was higher in group A over days 1, 3 and 7. From this study, it thus seems that the both uniform distribution of cells in hydrogel matrix and topology optimisation of the vascular network are the key to assess the biotransport of nutrients in a thick scaffold. Also, the results of the diffusion in modeling simulation *in silico* and experimental test *in vitro* have been found agreed with each other (Figure 20).

The cellular study suggested that the topology optimisation of vascular network did promote the biotransportation for enhancing cellular viability and proliferation inside the tissue construct. The fabrication approach established not only enabled us to build micro-patterned vascular network in the hydrogel matrix, but also does not affect cellular survival, proliferation and migration capabilities (Figure 41). It is also demonstrated that the fabricated microvascular network endows cell-laden tissue engineering constructs with improved mass transport and oxygen uptake, thereby resulting in a higher viability and metabolic function.
5.4 Limitations

Although our data provides some encouraging findings by combining modeling simulations, topology optimisation, microfabrication and cellular study for TE, different procedures might be attempted in order to have a more complete view and better understanding of how the vascular network can best placed for enhancing the cellular behavior, organization and orientation.

One of the main limitation of this study is the lack of a comparison of the diffusion results and cellular viability (using the same assay and conditions) between different geometries with the same volume/void fraction and cell types. Although control group enabled us to show how the optimised network improved results, how other network (e.g. uniformly regular channels) could be performed and compared quantitively, thereby further confirming the effectiveness and significance of optimisation and microfabrication.

Another limitation is relative to the fabrication of the mask, which was written with a tolerance of ± 250 nm; since the mask was laser printed and etched with a chrome enchant, the principle of “underetching” may be present in the final mask. Furthermore, the soda lime substrate mask used stops the transmission of the light below 360 nm; this is an added limitation to the study as no materials photocrosslinkable with wavelength below this value can be used.

Moreover, since we used photolithography, the technique developed need to be suitable to materials that are photosensitive and is only restricted to the fabrication on planar surfaces.
Note that soft-lithography however is able to solve this issue as it makes use of physical contact on a patterned stamp [114].

Finally, the approach does not guarantee the feasibility of fabrication for the layer-by-layer structure of the blood vessels (Figure 1). In other words, really 3D vascular network should be studied in the future. The lack of the control over the positioning of the different cell types does not allow the creation of the concentric anatomy typical of vascularisation.
Chapter 6: Conclusions and Future Directions

6.1 Conclusions

Motivated by the lack of a consistent design and fabrication of reproducing a vascular system that allows sufficient nutrient supply for cells to remain functional, this study was carried out to determine if the integration of topology optimisation with microfabrication is effective for generating the proper vascular network in building tissue constructs. The results associated with the developed approach for re-creating the same conditions defined in the modelling simulation (i.e. with one inlet to allow the diffusion of the nutrients in the entire construct domain) were not statistical significant after the ANOVA test (p-value > 0.05) and for this reason did not validate the hypothesis related to the diffusion of nutrients in the hydrogel matrix. But at the same time the F-test suggested us not to reject the hypothesis (F < Fcrit) and very similar outcomes have been achieved in the in silico and in vitro experiments.

We also conclude that a proper topological optimisation of the network prior to the fabrication is essential to ensure a full diffusion of a certain construct domain for the survival of the cells encapsulated in the matrix. A lower and partial diffusion of nutrients and O₂ might result in cellular hypoxia and death. An effective assay for the investigation into the cellular viability was also performed by using DiO-DAPI instead of the live/dead assay. Through comparing with the control group, it was found that the optimised vascular network performed better results in terms of cell viability.
Furthermore, cellular migration of HUASMC in the matrix has been showed as spontaneous phenomena naturally occurring in GelMA hydrogels [90]. The cell migration has been observed in the optimised network of hydrogel tissue constructs.

In summary, the work presented here is not only technically simple and cost-effective, but also establishes an effective approach to the optimal design and fabrication of a vascularised biodegradable and scaffold-free constructs.

6.2 Recommendation of Future Research

The future studies may investigate not only the attachment, proliferation and differentiation of the endothelial cells (ECs), but also their degree of alignment and elongation inside the artificial vascular network. ECs are supposed to be directed along the axis (e.g. longitudinal direction) of the network, as occurs physiologically in formation of natural blood vessels. A periodic perfusion of the void part of the scaffold (patterned network) with HUVECs could be performed in order to exploit the self-assembly of capillary-like constructs [81-83]. As such, a scaffold with an endothelial layer embedded in a smooth muscle cells (SMC) hydrogel matrix as mimicking the natural structure of vessels might be fabricated. In other words, a similar approach to the one used by Bischel and the colleagues could enhance tubular formation of blood vessels [112].

Optimisation of the abovementioned perfusion conditions can be performed in order to find the best possible rate (mL/min) as well as the inclusion of mechanical stimulation in the experiments. It should also consider exploring the differences between a static and a
dynamic fluid flow (e.g. on-off cyclic tension tests in bioreactor) for vascularization, thereby assessing if or not an artificial vascular network remains a better performance in such conditions.

As abovementioned, the formation of new blood vessels is a spatio-temporal cascade of events. Specific materials associated with molecules activated at precise timing and distance are fundamental for achieving biological modulation of the process. An alternative approach could be to develop a new gradient-based hydrogel, able to control the release of such substances in localised areas and sequential time through a cascade of events.

The use of the same approach to fabricating controlled release scaffolds characterised by a time-dependent release of a drug (i.e. growth factor) could be explored by manufacturing an optimised topological vascular network of hydrogel with different concentrations of the gel, different degradation rates and functionally-graded properties. It would be interesting to study the sequential release of growth factors from different areas of the vascularised scaffold, which may have a synergistic effect on enhancing nutrient transportation.

The 'sequence' issue as well as the exact order of release could be explored by further optimising the vascular pattern and varying the porosity of the gel. For example, if we suppose three different growth factors (A, B and C) where the exact sequence of release from the matrix is expected to be A-B-C, it may be achieved by encapsulating A in the external layer, B in the middle and C in the internal layer with the optimised patterned network. To avoid the simultaneous degradation (and thus release) of growth factors, the
porosity and vascular network in the gel may be varied, as the higher the porosity and surface areas are, the faster the degradation rate is.

The focus of future research can also be the development of new matrix materials with desirable mechanical properties closer to the biological counterpart. Future efforts could increasingly concentrate on the development of tissue engineered products accomplished to safety standards, as well as new sources of cells and tissues, quality control, and ethical and clinical evaluation. To better address the biotransport issue so that the cell and tissue can survive and function properly, there is a need to integrate all different techniques available, including mathematical modelling, design optimisation, 3D printing and cellular studies, for creating a best possible vascular network inside tissue constructs for desired in vivo applications.
Appendix I

Plasma Treatment Protocol

1. Turn on the power switch;
2. Close the chamber door;
3. Turn on the pump switch;
4. Close the valve such that the arrow rotates of 90°, in order to create vacuum inside the chamber;
5. Wait for 6 min;
6. Turn up the “RF” level to “HI”;
7. Plasma forms inside the chamber (pink color);
8. Turn the valve such that the arrow rotates of another 90°
9. Wait for 2 min
10. Turn off the “RF” level
11. Turn off the pump switch
12. Turn off the Power switch
Appendix II

Cell Counting and Merging pictures

1 After opening Fiji-64 software, open the image to modify
2 Subtract the background going to the tool bar, then “Process”; change pixels to get better results and to avoid a blurry background
3 Adjusting the brightness/contrast, then “Apply”
4 Save image as “modified”
5 Adjusting threshold as all the cells are red
6 Process binary watershed
7 Analyze particles from the tool bar “Analyze”
8 Summarise the results, choosing “35-infinity, outlines”
9 Display results, summarise include holes, record starts

To merge Images:

1 Open green modified image and change it to an RGB type color image
2 Open red modified image and change it to an RGB type color image
3 Process: image calculator
4 Save as merged Image
References


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