Universidade de Aveiro Departamento de Química
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RAQUEL MARIA DA COSTA GONÇALVES

One-step all-aqueous fabrication of tubular pre-endothelized structures

Fabricação em um único passo de estruturas tubulares pré-endotelizadas num ambiente aquouso

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Mariana Braga de Oliveira, Investigadora Júnior na Universidade de Aveiro, e do Professor Doutor João Filipe Colardelle da Luz Mano, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Vasos sanguíneos, Engenharia de Tecidos, sistemas aquosos bifásicos, complexação interfacial, encapsulamento celular, citocompatibilidade, células endoteliais.

resumo

Os vasos sanguíneos são um dos constituintes mais importantes do corpo humano. São responsáveis por manter a função e sobrevivência dos tecidos, fornecendo oxigénio e nutrientes, bem como por fornecer moléculas essenciais e sinalização bioquímica durante os processos de desenvolvimento e regeneração dos tecidos que dependem da formação de novas estruturas vasculares.

A capacidade de desenvolver estruturas ocas e tubulares *in vitro* que visam apoiar a função celular e recriar arquiteturas biológicas de tecidos nativos, como vasos sanguíneos, têm potencial de promover avanços científicos e tecnológicos nas áreas de engenharia de tecidos e medicina regenerativa. Os métodos clássicos para fabricar estruturas tubulares são frequentemente dependentes de pré- e pós-processamento ou de técnicas complexas que por vezes não são facilmente implementáveis, muitas vezes incompatíveis com arquiteturas de forma livre. A criação de materiais tubulares em forma de fibra através de métodos que permitem a sua fabricação direta e a sua deposição em formas e direções versáteis de uma maneira espacialmente controlada e tamanho controlado, pode ser a chave para superar algumas dessas limitações.

Os sistemas bifásicos aquosos (ATPS), que se comportam como emulsões totalmente aquosas, começaram a ser explorados recentemente no campo biomedicina. Esses são usados principalmente como modelos para a geração de biomateriais sofisticados. A complexação interfacial de polieletrólitos de carga oposta tem sido explorada como uma estratégia valiosa para a produção de materiais usando o modelo ATPS. A maioria dos estudos na literatura tem se concentrado na fabricação de materiais de formato esférico para o encapsulamento de cargas bioativas e delicadas. No entanto, a produção de materiais fibrosos com estrutura tubular por esta estratégia tem sido pouco explorada, e sua capacidade de permitir o encapsulamento celular, viabilidade e cultura a longo prazo ainda não foi reportada.

Neste projeto, propomos uma estratégia rápida para fabricar materiais em forma de fibra oca num ambiente totalmente aquoso estabilizado por uma membrana interfacial resultante da complexação de dois polieletrólitos de origem natural e de carga oposta. Estruturas simples ou ramificadas capazes de suportar a perfusão de liquidos foram produzidas, na qual as suas caracteristicas tubulares poderam ser confirmadas por microscopia eletrónica de varrimento. A estabilidade do biomaterial mostrou-se dependente da concentração dos polieletrólitos e do tempo de complexação, bem como do pH do sistema. Além disso, as propriedades mecânicas e comportamento de *swelling* puderam ser ajustadas pelo tempo de complexação, e o seu tamanho foi definido compreendendo diâmetros que variam de escalas milimétricas a micrométricas. O encapsulamento de células-tronco humanas derivadas do tecido adiposo (hASCs) demonstrou a capacidade de suportar a viabilidade e adesão celular até 7 dias, em sistemas contendo sequências adesivas. Fibras heterotipicas contendo hASCs em co-cultura com células endoteliais da veia umbilical humana (HUVECs) contribuiram para a sobrevivência das células endoteliais por pelo menos 14 dias, confirmado por imunocitoquímica. Este trabalho pode representar avanços relevantes na fabricação fácil e em apenas um passo de biomateriais com a capacidade mimetizar tecidos tubulares nativos com relevância biológica.

keywords

Blood vessels, Tissue Engineering, aqueous two-phase systems, interfacial complexation, cell encapsulation, cytocompatibility, endothelial cells.

abstract

Blood vessels are one of the most important constituents of the human body. They are responsible for maintaining tissue function and survival by providing oxygen and nutrients, as well as to provide essential molecules and biochemical signaling during tissue development and regeneration, which depend on the formation of new vascular structures.

The ability to develop *in vitro* hollow and tubular structures capable of supporting cell functionality and mimicking biological architectures of native tissues such as blood vessels have the potential to foster scientific and technological advances in the fields of tissue engineering and regenerative medicine. Classical methods to fabricate self-sustained tubular structures are normally dependent on pre- and post-processing steps, or complex and non-straightforward techniques, often incompatible with the fabrication of free-form architectures. The generation of tubular fiber-shaped materials through methods that allow their direct fabrication and their deposition in versatile shapes and directions in a spatial- and size-controlled manner may be key to overcome some of those limitations.

Aqueous two-phase systems (ATPS), which behave as fully aqueous emulsions, have started to be recently explored in the biomedical field. Those are mostly used as templates for the generation of sophisticated biomaterials. Interfacial complexation of oppositely charged polyelectrolytes has been exploited as a valuable strategy for the production of materials using template ATPS. Most studies in the literature have focused at the fabrication of spherical-shaped materials for the encapsulation of bioactive and delicate cargos. However, the production of fiber materials with a tubular structure by this strategy has been poorly explored, and its ability to allow cell encapsulation, viability and long-term culture has not been yet reported.

In this project, we purpose a rapid strategy to fabricate hollow fiber-shaped materials in a full aqueous environment stabilized by an interfacial membrane resultant from the complexation of two naturally-derived oppositely charged polyelectrolytes. Simple straight or branched structures amenable to be perfused with liquids could be produced, and tubular features of the structures could be confirmed by scanning electron microscopy. The stability of the fabricated material was dependent on polyelectrolytes' concentration, complexation time, and on the system's pH. In addition, the mechanical properties and swelling behavior of the fibers could be tuned by complexation time, and their diameter could be tailored from millimeters to the micrometer scale. Encapsulation of human stem cells derived from adipose tissue (hASCs) demonstrated the ability of the system to withstand cell viability and adhesion up to 7 days, in systems containing cell adhesion sequences. Heterotypic fibers containing hASCs in co-culture with human umbilical vein endothelial cells (HUVECs) enabled endothelial cell survival for at least 14 days, which was confirmed by immunocytochemistry. This work may represent relevant advances on the easy and one-step fabrication of biomaterial-based structures with the ability to resemble native tubular tissues with biological relevance.

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List of Abbreviations

α-SMA – alpha smooth muscle actin

ATPS – aqueous two-phase system

AV – arteriovenous

CAM – chorioallantoic membrane

CDV – cardiovascular disease

EC – endothelial cell

ECM – extracellular matrix

EMP – erythron-myeloid progenitor

EPC – endothelial progenitor cell

EPL – ε-poly-$_{L}$-lysine

ePTFE – extended polytetrafluoroethylene

FGF – fibroblast growth factor

FRESH - freeform reversible embedding of suspended hydrogels

GAG – glycosaminoglycan

GelMA – methacrylated gelatin

hASC – human adipose derived stem cell

hDF – human dermal fibroblasts

HGF – hepatocyte growth factor

hiPSC – human induced pluripotent stem cell

HUVEC – human umbilical vein endothelial cell

LCST – lower critical solution temperature

MSC – mesenchymal stem cell

OEC – outgrowth endothelial cell

PBS – phosphate buffered saline

PCL – polycaprolactone

PDGF-BB – platelet-derived growth factor-BB

PDMS – polydimethylsiloxane

PE – polyelectrolyte

PEG – poly(ethylene glycol)

PEGDA – poly(ethylene glycol) diacrylate

PET – poly(ethylene terephthalate)

PGA – polyglycolic acid

PLA – polylactic acid

PLGA – poly(lactic-co-glycolic acid)

RGD – arginine-glycine-aspartate

SEM – scanning electron microscopy

SMC – smooth muscle cell

TEBV – tissue-engineered blood vessel

TGF-*β* – transforming growth factor β

UV – ultraviolet

VEGF – vascular endothelial growth factor

vWF – von Willebrand factor

2D – two-dimensional

3D – three-dimensional

Chapter I
Introduction

I.1. Background

Tissue Engineering is an interdisciplinary field that integrates biology, material sciences and engineering areas, with the purpose of developing clinically relevant tissue constructs *in vitro* to repair, maintain or improve *in vivo* tissue functions.[1] To create functional tissue analogues, tissue engineering strategies use relevant cells, biomaterials, biochemical/biophysical signals and combinations thereof.[1] Its combination with regenerative medicine applications has enabled the development of advanced strategies to promote tissue regeneration; however, by better mimicking the *in vivo* microenvironment, the number of applications have increased in this field towards the creation of human tissue and disease models for the *in vitro* study of molecular and cellular processes, understanding of underlying mechanisms of certain diseases, and discovery of new drugs/therapeutics.[2]

Vascularization is crucial for tissue growth, development and function, since it allows the exchange of oxygen, nutrients, immune cells and essential biomolecules between blood and tissues.[3] As such, one of the main goals of tissue engineering has been to integrate vascular networks within tissue engineered constructs in order to better mimic the biological environment and improve therapeutic efficacy. This focus emerged mainly to bridge the challenging clinical translation of thick and complex tissues such as heart, liver and kidney, due to ischemic complications derived from the lack of vascular supply.[4] Engineered vascular tissues should be transplantable and integrate with the host vasculature or promote the formation of new microvasculature after implantation.[5] To engineer them, it is important to understand the native biology and physiology of blood vessels and tissue microenvironment, in order to rationally choose the adequate combination of cells, biomaterials and biochemical signals. Different cell types are currently being applied in this field, namely endothelial cells, endothelial progenitor cells, smooth muscle cells, pericytes and stem cells.[6] Moreover, in tissues, cells are surrounded by an organized extracellular matrix that provides structural and biochemical support for cell differentiation, proliferation, maintenance and function.[7] In vascular tissue engineering, scaffolding materials (synthetic or natural) are used to provide that support for cells and also biochemical or biophysical cues for vascularization.[8] Fibrin-based materials are widely used in this field due to intrinsic angiogenic properties[9], but the chemical and physical properties of other materials can be modified to support vascularization, by

including, for example, cell adhesive moieties or pro-angiogenic molecules. These molecules are commonly growth factors involved in the angiogenic process, being the vascular endothelial growth factor (VEGF) the most widely used due to its main roles in activating endothelial cell proliferation and migration to initiate the formation of new vessels.[10,11]

Many different approaches have been developed over the years in order to incorporate or promote the formation of vascular structures in bioengineered tissues, based on generation of a pro-angiogenic stimulatory environment, or perfusable tubular channels that recapitulate architectural and physical properties of blood vessels.[4,12] The development of freestanding and self-sustained pre-endothelialized tubular materials has been another studied strategy. Those materials have the ability to be integrated into engineered tissues and promote their vascularization, and at the same time may improve vascular integration with native tissues through the perfusion sources.[13,14] The development of hollow-core fiber-shaped materials (with inherent free-form properties) with ability to be perfused, using a rapid method that allows their direct formation in a single step, may represent a promising strategy to address major constrains of some developed techniques for the generation of freestanding tubular scaffolds mainly comprising the use of templates, multi-step and complex procedures or specialized equipment.[4] Aqueous two-phase systems (ATPSs) have been revealing promising properties for the development of biomaterial structures in an all-aqueous and biocompatible environment.[15] Among the various methods used for biomaterial development, interfacial complexation of oppositely charged polyelectrolytes has been enabling the rapid and one-step generation of versatile structures (mainly spherical-shaped ones such as capsules and particles), with potential application in encapsulation and controlled release of delicate cargos[15,16], and disease modeling[17]. However, the processing of such structures in a fiber form is still poorly explored[18], and the ability of ATPS-based systems to generate cell-laden materials with architectural complexity has never been reported.

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I.2. Strategies for Re-vascularization and Promotion of Angiogenesis in Trauma and Disease

Subchapter I.2.

This subchapter is based on the published Review:
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Strategies for Re-vascularization and Promotion of Angiogenesis in Trauma and Disease

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The maintenance of a healthy vascular system is essential to ensure the proper function of all organs of the human body. While macrovessels have the main role of blood transportation from the heart to all tissues, microvessels, in particular capillaries, are responsible for maintaining tissues' functionality by providing oxygen, nutrients and waste exchanges. Occlusion of blood vessels due to atherosclerotic plaque accumulation remains the leading cause of mortality across the world. Autologous vein and artery grafts bypassing are the current gold standard surgical procedures to substitute primarily obstructed vascular structures. Ischemic scenarios that condition blood supply in downstream tissues may arise from blockage phenomena, as well as from other disease or events leading to trauma. The (i) great demand for new vascular substitutes, arising from both the limited availability of healthy autologous vessels, as well as the shortcomings associated with small-diameter synthetic vascular grafts, and (ii) the challenging induction of the formation of adequate and stable microvasculature are current driving forces for the growing interest in the development of bioinspired strategies to ensure the proper function of vasculature in all its dimensional scales. Here, a critical review of well-established technologies and recent biotechnological advances to substitute or regenerate the vascular system is provided.

Keywords: vascular graft; microvasculature; biomaterials; vasculogenesis; angiogenesis

1. Introduction

Blood vessels are responsible for the delivery of oxygen, nutrients, cells and other molecules to all tissues in the body, supporting its overall function. They comprise a large dimensional range, varying from dozens of millimeters to few micrometers in diameter. Whereas large blood vessels are responsible for blood transport, microvessels are spread in hierarchical ramifications throughout tissues to enable molecular exchanges and waste removal.[1,2] Vascular networks include not only the blood circulatory system but also the lymphatic system, whose main function is to remove interstitial fluid, molecules and immune cells that have diffused from blood to tissue.[3] Vascular diseases are normally associated with the impairment and damage of blood vessels, which may occur at all levels of the hierarchical vascular network.[2] The global incidence of these diseases is very high, especially for cardiovascular diseases (CVD) that remain the leading cause of death worldwide[4], in a tendency that is estimated to continue at least until 2030, according to the World Health Organization.

At the macrovascular level (vessel diameter >1 mm), the mechanisms driving the failure of blood vessels are commonly associated to injury or inflammation, causing the weakening of vascular wall, which can lead to dissections and aneurysm formation, as in the case of syphilitic aortitis. Besides these mechanisms, the most common cause of macrovascular failure is their occlusion caused by plaque accumulation in vascular walls (atherosclerosis), occurring mainly in peripheral and coronary artery diseases.[5] Current clinical therapies are directed either at reducing risk factors related to plaque accumulation (which includes the use of antiplatelet and antihypertensive medications), or culminate in surgical interventions targeted at the restoration of blood flow, mainly comprising balloon angioplasty or bypass revascularization.[6] Catheter-based angioplasty is one of the most commonly performed medical procedures to restore blood flow in narrowed vessels. In this type of surgery, a thin tube is inserted through a blood vessel and, when the tube reaches the narrowed site, a balloon is inflated to push the plaque outward against the vessel wall. A metallic mesh tube - stent - is often needed to support the vessel's wall and keep its lumen open.[7] The use of stents can lead to inflammatory responses provoking in-stent restenosis. This limitation has been addressed with drug-eluting balloons, which provide local delivery of anti-proliferative drugs to treat those lesions in blood vessels' wall.[8] Nevertheless, angioplasty is not effective in many patients, and surgical procedures based

on the insertion of an alternative straight or branched vascular conduit - a graft – to replace the affected section of the vessel are required.

Vascular grafts currently applied in medical procedures may either be (i) made of synthetic/natural origin biomaterials, or (ii) produced from biological sources. The latter include autologous approaches, in which a healthy vessel – typically the internal mammary artery or the saphenous vein[9] - is removed from the patient to be grafted in the injured site; also, macrometer-scale grafts may be extracted from allogeneic or xenogeneic donors, which often requires post-isolation processing to ensure their low immunogenicity.

Complications generated from atherosclerosis and other CVDs, as well as tissue injury caused by trauma, may lead to ischemia in tissues. In this scenario, tissue cells are deprived from appropriate nutrient and oxygen levels for their survival. Consequently, prolonged ischemia or hypoxia may result in tissue dysfunction and necrosis, and ultimately in the need for tissue or organ transplantation. In ischemic diseases including critical limb ischemia (in lower extremities), ischemic heart disease and stroke, surgical bypassing and angioplasty are not always possible, due to the anatomy or the multiple nature of the arterial occlusions, or because the patient is not a surgical candidate due to age or comorbidities.[10] In those cases, the ability to induce the formation of micro-vascular networks in the ischemic tissue through a process named angiogenesis can drive the remodeling and opening of upstream collateral arteries and form a kind of biological by-pass[11], thus restoring blood perfusion and preserving tissue function.[3,7] On the other hand, following tissue injury, the inflammatory and healing responses to restore tissue function are dependent on the restoration of the microvascular system. Current therapies for the treatment of ischemic tissues often rely on the administration of angiogenic growth factors such as the fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF) or hepatocyte growth factor. When these growth factors are locally delivered in the ischemic site, they stimulate the proliferation, differentiation and migration of vascular cells, thus inducing the formation of new capillaries, accompanied by arteriogenesis and consequent perfusion recovery.[7,10] Additionally, other therapeutic angiogenic strategies that offer hope to improve tissue perfusion include the transplantation of stem and progenitor cells derived from bone marrow or peripheral blood, such as endothelial progenitor cells (EPCs) and hematopoietic stem cells.[12] Moreover, angiogenic gene therapies which normally involve the transfer of viral or non-viral vectors containing genes

encoding pro-angiogenic molecules or recombinant proteins has also been a major subject of recent attention.[7,10,13] Angiogenic gene therapies have emerged as a mean to circumvent the short-term effect associated with recombinant protein delivery, by maintaining the expression of the target protein, leading to longer duration of angiogenesis stimulation in the affected tissue.[14] Despite the potential of such therapeutics for the achievement of micro-revascularization, they are facing several challenges which will be discussed in this review.

Multi-component approaches have been developed as valuable highly controllable multi-parametric systems to treat ischemic tissues. Those often resort to tissue engineering and regenerative medicine approaches, which emerged to regenerate damaged or diseased tissues by using biomaterials, cells and bioactive molecules, in an effort to achieve highly functional tissues after therapy.[15] With few exceptions comprising avascular tissues, engineered constructs need an adequate hierarchically organized vascular networks (comprising arteries, arterioles, capillaries, venules and veins), in order to maintain long-term survival and functionality.[16] The vascularization on *in vitro* tissue constructs can be induced before implantation by (i) the presence of biochemical or biophysical cues, (ii) the incorporation of perfusable channel networks, or (iii) by their incubation in *in vivo* models. Compared to the current therapies that rely on the exogenous administration of free biomolecules or cells, the implantation of (pre-vascularized) tissue constructs offers the possibility to deliver cells and pro-angiogenic factors to the ischemic sites in a spatiotemporal controlled manner.[3]

With the objective to provide a systematic overview of the development of technologies for the treatment of vascular injury, this Review is divided in three main topics. (1) First, the biology and anatomy of blood vessels will be analyzed. Their main components and functions, as well as the processes involved in their formation and maturation, will be addressed. (2) Secondly, different approaches to produce blood vessel substitutes or vascular grafts (macro-vasculature level) are outlined. Those comprise (i) the use of biomaterial-based tubes, and their possible surface treatment and/or endothelization, (ii) scaffold-free approaches that enable the formation of cell-only vessels, and (iii) the formation of tubular fibrotic capsules in living organisms. Examples are provided for each approach considering fabrication methods, as well as the most relevant results considering the mechanical and biological functions of the constructs. (3) Finally, several approaches

to induce the formation of micro-vasculature, either in ischemic tissues or tissue-engineered constructs, are discussed. Those include classical biomaterial-based angiogenic approaches that rely on providing a biomimetic environment through biophysical and biochemical signaling. Alternative scaffold-free approaches relying on the ability of cells to self-aggregate into three-dimensional (3D) microtissues or produce cellular sheets with capillary-like vascular networks before transplantation are also presented. Additionally, the pre-vascularization of tissue constructs through the generation of interconnected perfusable channels in scaffolds that resemble the native microvascular network using specific experimental techniques is also reviewed. Figure 1 summarizes the strategies that will be addressed in the review.

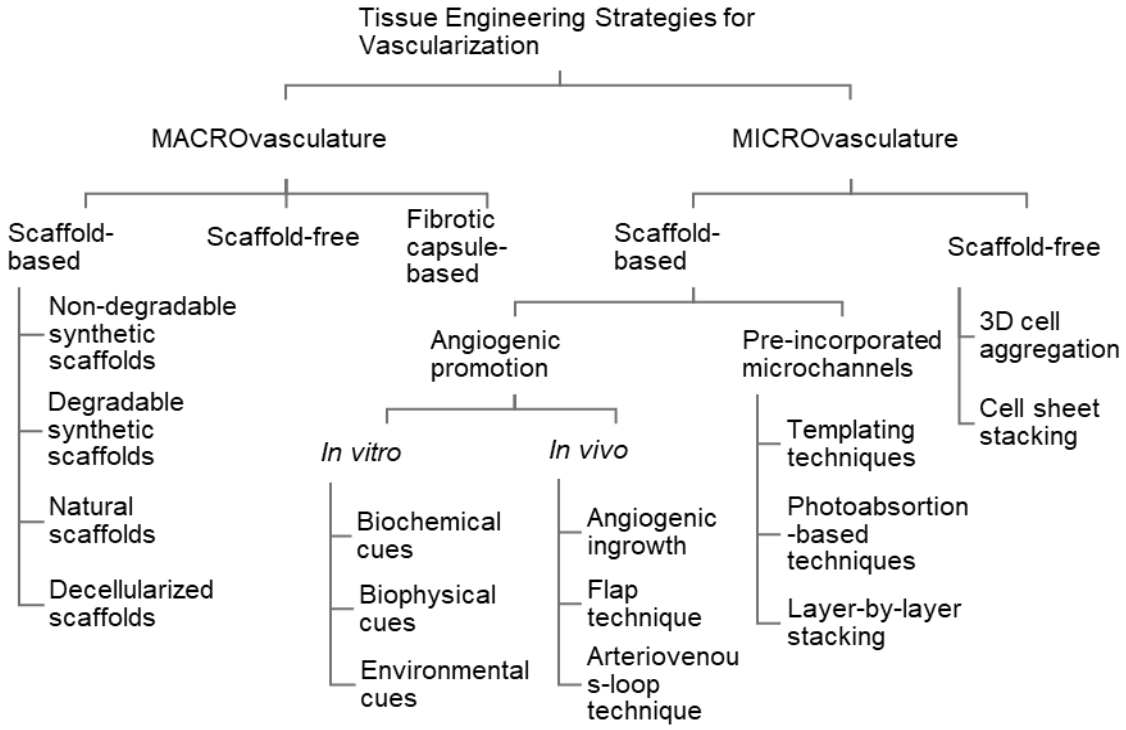

Figure 1. Summary of the various tissue engineering strategies for the generation of macro- and microvasculature.

2. Physiology and anatomy of native vasculature

A fundamental key to develop effective vascular substitutes or neovasculature in the human body may reside in the understanding of its native structure and main functions. The exploitation of these aspects may serve as inspiration to generate more accurate

synthetic functional replicates, thus increasing the probability of success after *in vivo* implantation.

2.1. Structure and function of blood vessels

Although the main function of vasculature is the distribution of blood throughout the body, blood vessels are also responsible for the maintenance of body temperature[17], and can play important roles in organs growth and development[18], as well as in the progression of certain diseases including cancer.[19] Oxygenated blood leaves the heart through the aorta artery (~25 mm of inner diameter) and distributes oxygen, nutrients, hormones, blood cells and fluids to tissues by branching in a hierarchical way into gradually smaller arteries, arterioles (~30 μm of diameter) and ultimately capillaries (5-10 μm of diameter).[20] Due to diffusional limitations of oxygen, effective molecular exchange only occurs within distances of 100-200 μm from blood capillaries.[16] In general, with the exception of the pulmonary circulation, metabolic waste and deoxygenated blood return to heart through the venous system composed by venules and veins.

The structure of arteries' wall varies from species to species, and along the vascular tree.[21] Depending on the composition and thickness of arterial vascular walls, they can be classified as elastic arteries, muscular arteries and arterioles. Larger vessels like elastic and muscular arteries as well as veins have three distinct layers in their walls: tunica intima, media and adventitia. The inner layer facing the vessel lumen - the tunica intima - consists of a single layer of endothelial cells – the endothelium - attached to a thin basement membrane which is primarily composed by collagen type IV, laminins and proteoglycans. Tunica media, in a middle layer, is composed of vascular smooth muscle cells (SMCs) and elastic fibers, while tunica adventitia (outer layer) contain mostly collagen fibers, elastin, nerves, fibroblasts and vasa vasorum which consists in small vessels around the adventitia that supply the vessel wall.[21–23] Additionally, an internal and external acellular layer of elastin, called elastic lamina, may also be present. Smaller arteries such as arterioles normally do not possess some of these layers, and capillaries only have the tunica intima surrounded by pericytes (Figure 2).[3]

The main extracellular matrix (ECM) components of vessel wall secreted by SMCs and fibroblasts, namely collagen and elastin, are responsible for the characteristic mechanical burst strength and viscoelastic behavior of blood vessels.[21] Collagen, that is

present in the form of fibrils, is responsible for the maintenance of structural integrity of the vessel by providing tensile strength, while elastin provides extensibility to withstand the pressure of blood flow, conferring compliance to vessels.[24] Elastic fibers provide the elasticity to blood vessels by stretching in response to blood flow pulses, and then recoiling when the pressure is removed, thereby promoting the transmission of the pulsatile blood wave energy along the vasculature.[9,21,24] Since the structure of blood vessels is sensitive to the magnitude of blood flow and pressure, they can undergo significant deformations in diameter and thickness, because of the generated tensile and frictional stresses in the wall.[25]

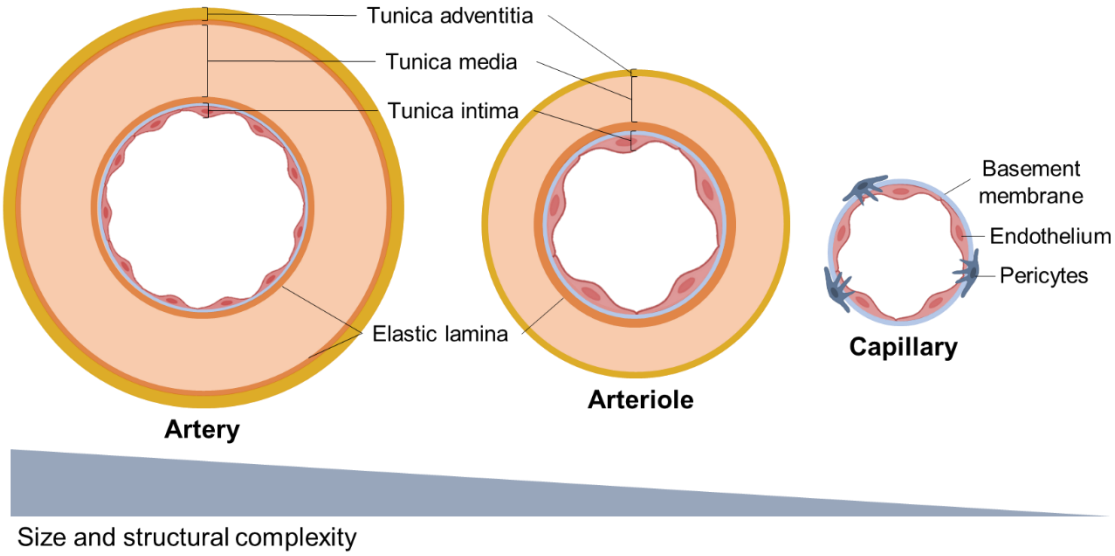

Figure 2. Cross section view of vascular wall structure of blood vessels with different sizes. All vessels have and intimal layer consisting of endothelial cells and basement membrane. Larger vessels still have more two layers containing smooth muscle cells (tunica media) and fibroblasts (tunica adventitia) and extracellular matrix components including collagen and elastin, while capillaries are only surrounded by several pericytes.

2.1.1. Endothelial cell heterogeneity and function

Endothelial cells (ECs) found in the lumen of blood vessels are elongated in the direction of blood flow, representing a large surface area between blood and tissues. These cells are linked to each other with cellular junctions that maintain the integrity of the endothelium and allow intracellular communications, namely tight junctions, adherens junctions and gap junctions. These junctions are important to regulate the endothelial permeability through paracellular-mediated transport between ECs, that highly depends on organ's functions.[26,27] For instance, the blood-brain-barrier has low permeability properties because it is very rich in tight junctions, related to its main role of protecting brain tissue

from dangerous molecules. Moreover, the passage of blood components and circulating cells through vascular wall can also be controlled by transcellular pathways. This passage is mediated by pores in the endothelium named fenestrae, or transport vesicles including vesiculo-vacuolar organelles, caveolae and transendothelial channels formed by vesicle fusion.[26,28]

In a structural perspective, the endothelium can be distinguished as continuous, fenestrated and discontinuous or sinusoidal. A continuous layer of ECs with an intact basement membrane is frequently found in skin, lung, heart and brain, where small molecules (< 3 nm) can easily cross between ECs and larger ones only pass via transendothelial channels or vesicle transport.[26] On the other hand, capillaries from gastrointestinal mucosa, for example, have a fenestrated endothelium, which is characterized by the presence of pores with approximately 70 nm of diameter, allowing increased transport of molecules. Finally, a discontinuous endothelium with larger fenestrations (100-200 nm of diameter) and poorly formed basement membrane, can be found in liver.[26] In fact, several liver pathologies including cirrhosis and hepatic fibrosis, are associated with defenestration of the sinusoidal endothelium.[29]

Besides conferring selective permeability to several blood components, the endothelium plays important roles in vascular homeostasis, angiogenic processes, inflammation, and in the trafficking of leukocytes from blood to sites of injury or infection.[27] In order to maintain blood homeostasis and blood fluidity, ECs express a large variety of anticoagulant (e.g. thrombomodulin and nitric oxide (NO)) and procoagulant (e.g. von Willebrand factor (vWF)) molecules. Blood coagulation (thrombosis) results from the activation of platelets and their aggregation by receptor binding to collagen, and activation of the protease thrombin that converts fibrinogen to fibrin, which is a constituent of blood clots' fibrous matrix.[9,26,30] In normal conditions, an healthy endothelium prevents thrombosis by the secretion of anticoagulant, antiplatelet and fibrinolytic molecules. During injury and inflammation, ECs become activated and express adhesive receptors on the surface, which promotes the adhesion of platelets and immune cells.[31] Moreover, ECs contribute to the regulation of vascular tone by balancing the expression of vasoconstrictor (e.g. endothelin) and vasodilator (e.g. NO) molecules in response to environmental stimuli such as blood flow rate.[26,27] In particular, an increase in flow rate causes dilation of vessels through the release of relaxing molecules such as NO.[32]

2.1.2. Vascular mural cells

Vascular development, integrity, function and remodeling are highly dependent on interactions between ECs and mural cells, namely, vascular SMCs present in large and medium caliber vessels and pericytes in microvessels (i.e. capillaries, postcapillary venules, and terminal arterioles). SMCs express a broad variety of different phenotypes depending on multiple environmental cues, ranging from a proliferative phenotype during vascular development to a mature phenotype found in adult blood vessels. Proliferative SMCs contribute to the synthesis of collagen, elastin, proteoglycans and other ECM proteins, while fully differentiated SMCs are characterized by low proliferation and synthesis rates, and a contractile and vascular regulatory phenotype.[33] Pericytes, on their turn, have essentially regulatory functions, communicating with ECs through paracrine and juxtacrine signaling during stabilization of new microvessels.[34] These elongated cells, often associated to any microvascular periendothelial mesenchymal cell, can directly contact with the endothelial layer through multiple finger-like projections that extend through holes in the basement membrane.[34,35] Like vascular SMCs, pericytes can be induced to express multiple phenotypes *in vitro*. Those have been differentiated into different types of mesenchymal cells, including fibroblasts, osteoblasts, chondrocytes, adipocytes and even SMCs, playing different roles depending on the acquired phenotype.[36] However, even though *in vitro* tests have confirmed the potential of SMCs and pericytes to differentiate into multiple different lineages, the demonstration of this endogenous plasticity *in vivo* has remained controversial, as Ergün and Wörsdörfer recently discussed.[37]

Changes in the structure and function of vascular cells or disruption of endothelial-mural cell crosstalk may result in pathological situations. Various factors may be implicated in such conditions, including: (1) endothelial dysfunction that results, for instance, in altered permeability, imbalanced expression of clotting factors or alterations in vascular tone[31,38]; (2) phenotype switching of SMCs and pericytes in response to abnormal environmental signals or (3) defective interactions between ECs and mural cells.[33,36]

2.2. Formation of blood vessels: vasculogenesis and angiogenesis

The formation of vascular networks in organs and tissues proceeds through two processes: vasculogenesis and angiogenesis. Vasculogenesis refers to the *de novo* formation of blood vessels from *in situ* differentiation of progenitors, while angiogenesis is the process by which new blood vessels are formed from pre-existing ones by sprouting or splitting (intussusception).[39] The process of formation of new blood vessels not only occurs during embryonic development but also in adults. However, since in adults mature ECs and SMCs remain in a quiescent state, this phenomenon only occurs upon onset conditions, such as wound healing, tissue regeneration, or tumor growth and metastasis.[26,33,40]

During embryonic development, the process of vascular structures formation is highly dynamic and dependent on bidirectional interactions between the developing tissue and vasculature.[41] Vasculogenesis begins with the differentiation of mesodermal stem cells into hemangioblasts, which aggregate to form blood islands that then give rise to both hematopoietic stem cells and angioblasts (also known as endothelial progenitor cells (EPCs)). The expression of VEGF promotes the differentiation of angioblasts into mature ECs, that migrate within the ECM and form tubular structures, leading to the development of a primitive capillary plexus. Larger vessels are formed from this plexus and, along with angiogenesis, the vascular network is completed.[40] Beyond ECs derived from mesodermal stem cells, a second origin for the EC-line of blood vessels has been discovered by Plein and colleagues.[42] ECs from the yolk sac give rise to precursors of embryonic blood cells named erythron-myeloid progenitors. Those progenitors can revert their phenotype again to ECs and be incorporated in the mesoderm-derived endothelium of existing vessels, especially in the developing liver, lung and brain.[42,43]

Angiogenesis is a multi-step process highly dependent on cell-cell and cell-ECM interactions, mainly mediated by VEGF expression and activation of various signaling pathways. The formation of new blood vessels may result from internal division of existing vessels, a process known as splitting angiogenesis or intussusception, or by external sprouting of ECs from existing vessels after basement membrane degradation. These mechanisms are followed by lumen formation and vessel maturation.[44] During the early stages of sprouting angiogenesis, blood plasma proteins including fibronectin, vitronectin and fibrinogen, along with interstitial collagen, form a provisional ECM where ECs

proliferate and migrate, thereby promoting lumen and vascular tubes formation.[16,44] The final step is the stabilization of blood vessels, which is accomplished by the recruitment of pericytes and other stromal cells, as well as the formation of basement membrane.[44]

Several mechanisms inherent to EC behavior are fundamental for the formation of new blood vessels, including their orientation, proliferation and migration. The migration of ECs during angiogenesis is a highly regulated process that requires the integration of signals capable of activating specific intracellular signaling pathways, which lead to the remodeling of actin filaments from the cell cytoskeleton. Migration of capillary ECs is essentially driven by: (1) growth factors such as VEGF, basic FGF (bFGF) and angiopoietins (chemotaxis), (2) integrin binding to ECM components (haptotaxis) and (3) flow shear stress (mechanotaxis).[40] VEGF is particularly important in the mediation of several EC functions. After its secretion under a pro-angiogenic environment such as hypoxia, this growth factor elicits the activation of signaling cascades, promoting the migration and proliferation of ECs.

The cellular and molecular regulation of angiogenesis is very complex and its detailed treatment transcends the limits and scope of this manuscript. The interested reader is addressed to one of several excellent reviews[45,46], while here we will provide an overview of some selected therapeutically relevant aspects. The orderly growth of new vascular sprouts requires the balanced specification of ECs in two different functional types in response to VEGF gradients: the migrating tip cells and the proliferating stalk cells.[47] This process is finely regulated by Dll4/Notch signaling through a mechanism of lateral inhibition: expression of the Dll4 ligand on the tip activates the Notch1 receptor in the neighboring cells and instructs them to suppress the tip phenotype and become stalk cells instead.[48]

As described above, pericytes are the second key cellular component of micro-vascular networks and they are recruited to nascent endothelial structures by Platelet-Derived Growth Factor-BB (PDGF-BB) secreted by activated endothelium. The association of endothelium with pericytes is the process of vascular maturation, by which new vessels acquire their physiological functions. In fact, pericytes exchange a complex molecular cross-talk with endothelial cells, comprising principally the TGF-β, Angiopoietins and ephrinB2/EphB4 signaling pathways, through which they regulate endothelial processes such as proliferation, survival and permeability, so that new vessels

can stabilize, persist in the absence of continued factor production and avoid leakage of blood components.[34] Notably, VEGF can also negatively regulate pericyte function by inhibiting PDGF-BB phosphorylation through the formation of a nonfunctional VEGF-R2/PDGF-BB complex.[49] Therefore, the relative dosage/expression of VEGF and PDGF-BB needs to be balanced in order to ensure therapeutic growth of physiological and mature vascular networks.[50]

An important feature of both VEGF and PDGF-BB function is their interaction with ECM through heparin-binding or matrix-retention domains, which dictates their spatial localization in tissues and regulates the outcome of the angiogenic process. This biological property is critical to generate the concentration gradients that guide ECs and pericytes in vascular morphogenesis to assemble normal vessels, and interfering with VEGF or PDGF-BB ability to bind to ECM causes aberrant vascular formation even at physiological expression levels.[51,52] Angiogenic factor retention in the ECM also has important therapeutic implications. For example, the safety and efficacy of VEGF therapeutic delivery *in vivo* depend strictly on its concentration localized in the microenvironment around each producing cell, and not simply on the total dose delivered.[53] Therefore, delivery methods that lead to heterogeneous factor levels in target tissues with "hotspots" of excessive production (such as gene therapy viral vectors) appear to have a very narrow or absent therapeutic window, whereas a homogenous distribution (such as by factor-decorated fibrin hydrogels) can ensure efficacy at safe doses.[13]

3. Recreating the macrovasculature

Every year, a large amount of small (1-6 mm) and large diameter (> 6 mm) vascular grafts are needed to replace or bypass diseased arteries and veins, generally to relieve lower limb ischemia, or to create a hemodialysis access.[6] Patients who depend on hemodialysis need access vascular grafts to create a connection between an artery and a vein, which can be used to allow blood flow out of the organism through catheters to an external circuit, and return after purification in dialysis machines.[54] As such, researchers have been interested in the creation of novel blood vessel substitutes.

To ensure functional longevity of the grafts and prevent graft failure, those should be non-thrombogenic, biocompatible, resistant to infection, and exhibit adequate compliance and mechanical strength similar to native blood vessels in order to withstand physiological

blood pressures and resist to dilation.[55] In addition, they should be easy-to-handle during surgery, amenable to be sutured and, in the case of degradable grafts, to induce tissue regeneration once implanted.[56] The failure of vascular grafts is often associated with lumen occlusion caused by acute thrombosis or intimal hyperplasia.[55]

3.1. Scaffold-based approaches

With the aim of reproducing the ECM function as a support scaffold for cells, conferring strength, resistance and adequate biochemical signaling, synthetic polymers have been used to produce vascular grafts with adequate mechanical properties to be implanted. In addition, biodegradable polymers capable of being substituted by endogenous cells' ECM, as well as engineered biological materials that mimic more closely the *in vivo* tissue matrix composition, have also been used as scaffolds to support vascular cells.

3.1.1. Non-degradable synthetic scaffolds

Synthetic polymers have been used in clinical applications to replace large diameter vessels since the first attempt by Blakemore and Voorhess in 1951, where textile tubes made from Vinyon "N" were successfully implanted in 10 of 18 patients with atherosclerotic aneurysms.[57] Nowadays, non-degradable synthetic materials made of extended polytetrafluoroethylene (ePTFE) and poly(ethylene terephthalate) (PET or Dacron), are used in clinics as vascular grafts for surgical bypass grafting in areas of high blood flow, when the use of autologous grafts is not indicated.[58] However, the use of purely synthetic vascular conduits is limited by high risk of infection and chronic inflammatory responses, due to incompatibility with the native environment. Moreover, these structures are unable to grow, adapt and remodel when implanted in young patients, leading to graft failure.[58] So far, the use of synthetic vascular grafts has only been satisfactory for the replacement of vessels with diameters higher than 6 mm. The use of smaller diameter synthetic grafts (< 6 mm) is often prone to lumen occlusion due to thrombosis or intimal hyperplasia most caused by compliance mismatch.[56]

A promising way to prevent thrombus formation and intimal hyperplasia is the creation of a continuous endothelial monolayer in the lumen of the vascular graft. The endothelium interface has important roles in the prevention of blood coagulation and acts

as a barrier between blood and intimal tissue due to its characteristic cellular junctions.[55] In 1978, Herring and colleagues created the first endothelized synthetic grafts by seeding venous ECs in 6 mm Dacron grafts, aiming at the replacement of infrarenal aortas of canine models. The grafts exhibited improved thromboresistance and histological resemblance to native endothelium line, when compared to unseeded grafts used as control.[59] A similar approach was reported by Deutsch *et al.* by producing endothelized ePTFE grafts with 70 cm length and 6/7mm inner diameter using autologous ECs. Those constructs were tested in 310 patients who needed infrainguinal bypass surgery and did not have any suitable saphenous vein available.[60] The main purpose of this 15-year clinical trial was to evaluate if *in vitro* endothelization could be provided as a routine service for this specific surgery in a community hospital. Clinical follow-up showed patency rates after 5 years and 10 years, overall, above 60% for both 7 mm and 6 mm grafts. Histologic assessments showed the presence of a confluent layer of ECs in 78% of freshly seeded grafts. However, some grafts have failed primarily because of occlusions that seemed to be related to the presence of inflammatory cells between the ePTFE surface and the endothelium, as well as hyperplastic narrowing.

Difficult cell sourcing, complications on the retention of ECs under physiological pulsatile blood flow, and the poor formation of a confluent endothelial layer remain the major challenges associated with the seeding of ECs on synthetic surfaces.[58] Other strategies have emerged to improve these shortcomings, which include the coating or functionalization of graft surface with (1) adhesive proteins such as fibronectin and collagen, or (2) cell adhesion peptide sequences, such as the arginine-glycine-aspartate (RGD) domain, that have affinity to integrin receptors of the cellular membrane.[58] In addition, since EPCs specifically express CD34 and CD133 antigens on their surface[55,61], coating the surface of synthetic grafts with anti-CD34[62] and anti-CD133[63] antibodies was shown to result in rapid endothelization and reduced thrombogenesis. With the same goal of promoting EC adhesion and proliferation, vascular growth factors have also been immobilized in synthetic surfaces.[64]

Furthermore, coating the luminal surface with heparin or other anticoagulant molecules such as thrombomodulin, P15 and Hirudin proved a promising strategy to reduce the thrombogenicity of synthetic grafts.[1,56] Alternatively, a recently developed method for surface coating that produces slippery and highly repellent surfaces with non-

thrombogenic properties was developed in the search for more biocompatible synthetic vascular grafts. The basis of the method consists of a solid surface infused with a liquid lubricant layer, bioinspired by the *Nepenthes* pitcher plants and their capacity to lock-in a liquid film on the surface of its cupped leaves in order to capture insects by their sliding.[65] The major effects of the presence of the lubricant on the surfaces was the inhibition of non-specific adhesion of cells, biomolecules and microorganisms, as well as the prevention of thrombosis.[66–69] However, due to the repellency properties, the surfaces lack bioactivity and biofunctionality[69], which are critically important in vascular grafts that require targeted EC adhesion to accelerate tissue integration and induce functionality for long-term patency. To overcome this drawback, Badv and colleagues recently developed a method to create stable lubricant-infused ePTFE[67] and PET[68] surfaces by plasma modification, with additional biofunctionality promoted by the antibody anti-CD34. The surfaces underwent oxygen plasma modification to generate functional hydroxyl groups that allowed the coating with fluorinated lubricants, and further enabled antibody functionalization. The presence of the lubricant layer was essential to reduce thrombin generation, blood coagulation and non-specific undesired protein and blood cell adhesion. Moreover, the presence of the anti-CD34 antibody potentiated the anti-thrombogenic properties of the surfaces and allowed specific EC binding from whole blood (Figure 3, A).

3.1.2. Tissue-engineered vessels and grafts

To circumvent the limitations of using exclusively synthetic nondegradable materials, researchers focused their attention on the exploitation of tissue engineering strategies to generate implantable blood vessels for clinical applications. Tissue-engineered blood vessels (TEBVs) or vascular grafts are often produced by combining biodegradable synthetic polymers or natural polymers as scaffolds, vascular cells, and biochemical or biophysical signals.[70]

Traditional techniques to produce TEBVs with tubular shapes include molding, sheet-rolling and lyophilization, electrospinning and 3D printing (Figure 3). The molding strategy involves casting a polymer solution into a mold with an annular structure with pre-defined inner diameter and wall thickness. Other strategies are based on directly forming tubular scaffolds by electrospinning on the surface of a rotating mandrel, or resorting to a computer-aided tubular design by 3D printing.[2,5] Furthermore, the method of rolling

biomaterial sheets around a cylindrical support has also been applied, mostly in crosslinked protein-based scaffolds, in some cases requiring further lyophilization to create a dense and mechanically robust matrix.[5,71]

Many tissue engineered scaffolds have been used to produce biomimetic vascular grafts capable of undergoing remodeling and functional integration with host vessels after implantation. For this purpose, biodegradable synthetic polymers, biomaterials of natural proteins and polysaccharides, or decellularized blood vessels have been considered appealing materials.

Biodegradable synthetic scaffolds. Biodegradable synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL) and copolymers thereof are normally processed into 3D tubular scaffolds to generate TEBVs or vascular grafts. Those degrade over time, enabling the previously seeded cells (or body's own invading cells) to proliferate and produce their own ECM, and thus remodel into constructs with greater similarity to native tissues in terms of cellular and structural compositions [58]. Polyurethanes are other class of particularly relevant polymers used in vascular tissue engineering to produce compliant vascular grafts [55]. Polyurethanes are copolymers comprising three different monomers: a hard domain derived from a diisocyanate that provides strength, a chain extender, and a soft diol domain that provides flexibility. The first generation of polyurethane vascular grafts used polyester and polyether monomers; however, they showed hydrolytic instability and oxidative degradation in clinical trials.[72] Further development led to a new generation of more stable polyurethane-based grafts namely the poly(carbonate-urea)urethane vascular grafts, that exhibit similar compliance to that of human arteries.[58]

The first clinically applied TEBV as a vascular graft was reported by Shin'oka *et al.* in 2001. A vascular graft with 10 mm diameter made of PCL-PLA copolymer reinforced with PGA was seeded with autologous cells from peripheral vein, and later implanted in a child with pulmonary atresia - a congenital heart disease - to reconstruct the pulmonary artery. No studies were performed to confirm if the scaffold was remodeled into a complete biological blood vessel; however, 7 months after implantation there were no signs of graft occlusion or dilation.[73]

Despite their promise as valuable components of tissue engineered vessels, the efficiency of autologous ECs or other human-derived primary vascular cells has been clinically hampered by their limited accessibility, finite proliferation capacity and time consuming culture periods, reducing their immediate availability.[24] To overcome these challenges, recent studies explored the potential of human induced pluripotent stem cells (hiPSC) in generating TEBVs on biodegradable synthetic scaffolds. These reprogrammed cells show properties of self-renewal, high proliferation rate and differentiation into functional SMCs and ECs in large quantities.[24] In a clinical perspective, the use of these cells has been considered as promising because they can be efficiently derived from somatic cells of a patient, and then be differentiated into vascular phenotypes or be directly seeded onto tubular scaffolds, to generate personalized autologous vascular grafts. However the clinical safety of these reprogrammed cells still needs further clarification.[24] Generali *et al.* recently used hiPSCs derived from human peripheral blood mononuclear cells (PBMCs), and promoted their reprogramming into SMCs and ECs. Both cell types were co-cultured on tubular PGA scaffolds coated with poly-4-hydroxybutyrate, used to provide additional strength to the artificial vessel.[74] After a maximum of 9 weeks of culture under static and/or dynamic conditions, histological analysis demonstrated the presence of a thin luminal layer of vWF-positive ECs and an interstitial layer of smooth muscle actin (α-SMA)-positive SMCs, comparable to native vessel wall structure. Moreover, it was observed that, compared to static conditions, dynamic culture enhanced cell infiltration, tissue formation and scaffold degradation (Figure 3, B). Interestingly, this study reported the first use of PBMC-hiPSCs-derived SMCs and ECs on biodegradable scaffolds towards the generation of patient-specific small diameter vascular grafts; however *in vivo* studies are still needed to test the patency and functionality of these grafts.[74]

Despite their biodegradability, polymers applied for the development of artificial vessels often have a synthetic origin, and thus often lack bioactivity and cell binding sites. Functionalization with biological components, including bioactive peptides, cytokines and antibodies, are reported approaches to improve the grafts' integration and remodeling capability in the host vasculature.[55] For example, Zheng *et al.* functionalized the surface of small-diameter (2.2 mm) PCL electrospun tubular scaffolds with the RGD domain, to examine their compatibility in a rabbit carotid arterial implantation model.[75] In comparison with non-modified PCL grafts, RGD-functionalized PCL grafts exhibited reduced platelet

adhesion, improved endothelization and SMCs infiltration *in vivo*. Despite the excellent patency of RGD-modified grafts observed after 4 weeks of implantation, this period was not sufficiently long to evaluate the long-term behavior of the graft *in vivo*, including PCL degradation and vascular remodeling.

Natural scaffolds. Natural proteins such as collagen, elastin and fibrin, have been widely used to produce biomaterial-based scaffolds, normally gels, for vascular tissue engineering applications. Moreover, collagen-elastin blends[76] and in some cases also containing glycosaminoglycans (GAGs)[77], have been also studied as protein scaffolds for engineering blood vessels. In fact, the presence of elastin can alter mechanical and biological properties of collagen scaffolds enhancing their viscoelasticity and cell activity.[76] Additionally, hyaluronan – a GAG present in native tissues including blood vessels – and corresponding oligomers, have been also used and combined with collagen and biodegradable materials to improve biological properties of vascular grafts including hemocompatibility, endothelization and smooth muscle regeneration.[78–80] Other natural polymers that have been used for tissue engineering blood vessels include silk fibroin[81,82], gelatin[83], chitosan[84] and composites[85]. The utilization of natural proteins and polymers may be advantageous compared to synthetic biodegradable polymers. While they also exhibit biodegradability, some of them have bioactive properties due to the intrinsic presence of chemical groups and physical features able to promote cell responses.[70]

Since collagen is the major ECM component of blood vessels, Weinberg and Bell reported in 1986 the first TEBV based on collagen hydrogels as a support biomaterial for cells.[86] In this approach, SMCs and fibroblasts were cultured on collagen matrices, and injected in annular molds to form a tubular structure. A solution containing ECs was injected in the lumen, in order to mimic the native multilayered structure of arteries. Although this model allowed the formation of a functional endothelium with vWF expression and permeability barrier properties, a Dacron mesh was needed to be incorporated to enhance the mechanical properties of the hydrogel lattices.

Efforts to improve mechanical performance and circumvent the incorporation of synthetic supports in hydrogel-based blood vessels have focused in three main aspects: (1) supplementation of the culture medium where SMCs and fibroblasts grow with inducers of ECM deposition, such as ascorbic acid, and stimulation of cells with growth factors and other biomolecules in order to promote gel compaction and remodeling; (2) chemical,

enzymatic or photo-crosslinking of the biomaterials, and (3) using specialized bioreactors that apply mechanical forces including tension, shear stress and cyclic strain, for fiber orientation and SMC alignment in the circumferential direction of the tubular construct.[5,58]

Fibrin gels can be easily formed through the polymerization of fibrinogen in the presence of thrombin. For clinical applications, both fibrinogen and thrombin can be isolated from a patient's own blood plasma and purified, thereby preventing immune rejection on implantation.[5,24] However, it is difficult to control the polymerization rate of fibrin, and this protein is rapidly degraded by enzymatic proteolysis and therefore protease inhibitors, such as aprotinin, are normally needed.[5] Fibrin-based TEBVs have been demonstrated to stimulate the synthesis of collagen and elastin after implantation in an extent compared to native blood vessels.[87,88] Nonetheless, strategies to improve mechanical properties of fibrin gels have been developed, due to the limited stability of fibrin. Tranquillo and co-workers developed a pulsed flow-stretch bioreactor where tubular constructs (2 or 4 mm of inner diameter), made of fibrin gels embedded with human fibroblasts, were exposed to a pressure wave throughout the lumen, resulting in graft distension.[89] The pulsed cyclic flow of the bioreactor allowed burst pressures 73% higher than the ones of grafts exposed to constant flow as well as significantly higher collagen production. After 7 to 9 weeks of culture in the bioreactor, the grafts exhibited burst strength and compliance comparable to the native femoral artery and circumferential alignment of collagen fibers that likely improved the graft's mechanical properties. On the other hand, Aper *et al.* developed a new method to generate highly stable fibrin tubes based on a specialized high-speed rotating mold that promotes the compaction of fibrin.[90] With increasing rotation velocity, fibrin fibrils became thinner, more ramified and densely compacted resulting in an increase in tensile strength as well as burst pressure. The stabilization of the fibrin network was also enhanced through the addition of factor XIII which, when activated by thrombin and calcium, cross-links fibrin fibers. To evaluate the performance and potential remodeling of the grafts *in vivo*, fibrin tubes (inner diameter = 5.6 ± 0.2 mm) seeded with isolated and expanded ECs and SMCs, were implanted in sheep models as carotid artery replacement grafts. The grafts remained patent after 6 months and remodeled, demonstrating ECM protein production and wall structure similar to native vessels (Figure 3, C).

During the last years, 3D bioprinting technology and its more recent innovation - freeform reversible embedding of suspended hydrogels (FRESH) -, emerged as promising manufacturing processes to produce straight or branched natural-based tubular structures with spatiotemporal control.[91,92] For instance, Gao and colleagues engineered a biomimetic vascular tubular structure – "bio-blood-vessel" – using a 3D coaxial printing technique and a hybrid bioink composed by a mixture of decellularized ECM of porcine aortic tissue and alginate. This bioink was encapsulated with EPCs and poly(lactic-*co*-glycolic) acid (PLGA) microspheres loaded with a proangiogenic drug (atorvastatin). After *in vivo* implantation, the constructs significantly improved the treatment of ischemic limbs of mice models.[93] On the other hand, in comparison with classical 3D printing strategies that rely on the printing of a soft material in an additive manufacturing manner, in FRESH, viscous bioinks are printed in a support medium that behaves as a rigid material at low shear stress to maintain the desired tubular structure in a 3D geometry while the nozzle is moving during the printing process.[94] This technology circumvents the lack of structural integrity and eventual structure collapse associated with the traditional printing process, allowing for the fabrication of more complex tubular structures that mimic more effectively native vessel geometry.[92] Combination of layer-by-layer and sacrificial templating technologies has been also used to produce hollow tubular structures. By the alternate deposition of chitosan and alginate on sacrificial tubular templates and subsequent template leaching, multilayered tubes with tunable wall thickness and flexibility were fabricated. When immobilized with fibronectin, these tubes promoted adhesion of ECs in the lumen and SMCs in the outer surface.[95]

Decellularized scaffolds. In order to produce tubular vascular grafts with reduced immunogenicity, biological scaffolds produced by the decellularization of native blood vessels have been suggested. Decellularization consists on the removal of cells from tissues, resorting to combinations of detergents, protease inhibitors and enzymes.[1] This procedure has already been performed with native human vessels such as umbilical and femoral arteries, in animal blood vessels including porcine and bovine carotid arteries, or in TEBVs.[1,58] Moreover, alternative tissues with native tubular shapes have also been explored as biomaterials to construct large and small diameter vascular grafts since the end of the 1980's. Those include the acellular submucosa layer of porcine small intestine[96–98], which is primarily composed of collagen type 1, and the decellularized ureters of pigs.[99]

One advantage of decellularized arteries is that the native architecture of the vessel ECM is mostly preserved, thus allowing the production of grafts with mechanical properties similar to native vessels.[2] However, the major advantage of this process is the removal of cellular antigens present in vascular allografts, xenografts and TEBVs after the decellularization processes, leading to the prevention of immunologic responses that may drive graft rejection.[1] The CryoLife company has developed a decellularization method (SynerGraft technology), with the aim of reducing the immunogenicity and antigenicity of cadaveric vessels. This technology has already been employed in human allografts which were assessed clinically as hemodialysis access grafts in patients with inadequate autologous vessels, showing reduced antibody production after implantation.[100]

In addition to native vessels, tissue-engineered grafts produced *in vitro* have also been decellularized. For instance, engineered fibrin-based arteriovenous grafts encapsulated with human dermal fibroblasts, were decellularized and tested as hemodialysis access in baboon models. Some of the grafts rapidly occluded due to early thrombotic events but, excluding those, a patency rate of 60% after 6 months was identified, with no signs of immunological responses.[101] Niklason and colleagues performed a clinical trial using human decellularized tissue-engineered grafts, with 6 mm in diameter, as hemodialysis access in 60 patients with end-stage renal disease. Those grafts were made by seeding SMCs derived from deceased organ and tissue donors in a PGA scaffold, followed by a period of maturation of 8 weeks in bioreactors with pulsatile cycles. The developed vessels were then decellularized and implanted in patients. Grafts showed mechanical robustness, with burst strength similar to the one of human vessels. After implantation, those structures were repopulated by host cells, suggesting their integration with the host vasculature and remodeling.[102]

The idea of using animal-origin blood vessels, namely the ones retrieved from bovine and porcine sources, became appealing due to their wide availability compared to human tissues. Moreover, their mechanical properties were shown to be similar to the ones of human vessels.[2,58] However, the use of animal tissues in humans is highly prone to severe immune responses and graft rejection, which encouraged the employment of adequate decellularization methods to reduce these responses. However, the complete removal of immunogenic foreign biomolecules in animal tissues by decellularization techniques remains ineffective.[2] A suggested solution is the treatment of tissues with glutaraldehyde

because, by crosslinking tissue proteins through aldehydes reaction with side-chain groups of proteins, antigens may be masked for antibody recognition, thus reducing immunogenicity. However, beyond the possible problematic cytotoxicity of glutaraldehyde, when xenografts receive this treatment, intimal calcification can be enhanced *in vivo* due to aldehyde bonding to calcium.[103] Recently, alternatives started to emerge in the genetic engineering field with the aim of enhancing the survival rate of implanted xenografts, by genetically modifying the genes responsible for the human immunological responses to the animal-derived tissues. In the last decade, the CRISPR-Cas9 technology has started to spark more interest in the scientific community as a gene editing tool. As such, this system has been explored to create genetically modified animals with improved compatibility with humans, by inactivation or mutation of genes that encode immunogenic molecules such as the porcine procoagulant vWF, or the galactose-alpha-1,3-galactose glycoprotein that is present in all porcine cells, but not in humans.[104]

Efforts to create TEBVs with mimetic features of native blood vessels have fostered the development of devices with improved function and performance after grafting. Several clinical applications have demonstrated the potential of such TEBVs as vascular grafts for bypass surgeries or hemodialysis access. However, the development of these structures is not yet free of challenges. Due to low toxicity, reduced immunogenicity, and biodegradability, scaffolds prepared from some natural materials seem to offer a higher potential for the long-term formation of vessel-like tissues than their synthetic counterparts. However, most TEBVs prepared from natural materials have been associated to a set of common limitations, including poor mechanical performance and lack of stability. A range of engineered bioreactors have been developed seeking the promotion of proper TEBVs remodeling, as well as their strengthening and stabilization in adequate maturation periods. This last feature is crucial for functional longevity of the graft, preventing dilation and aneurysm formation, and consequent graft failure. Excluding decellularized blood vessels that preserve ECM components, the effective deposition of elastin in TEBVs remains challenging, with a small number of studies demonstrating its effective production by cells. In native vessels, elastin is thought to act as a provider of vascular compliance, and its absence in TEBVs may lead to excessively elastic vessels with poor performance.

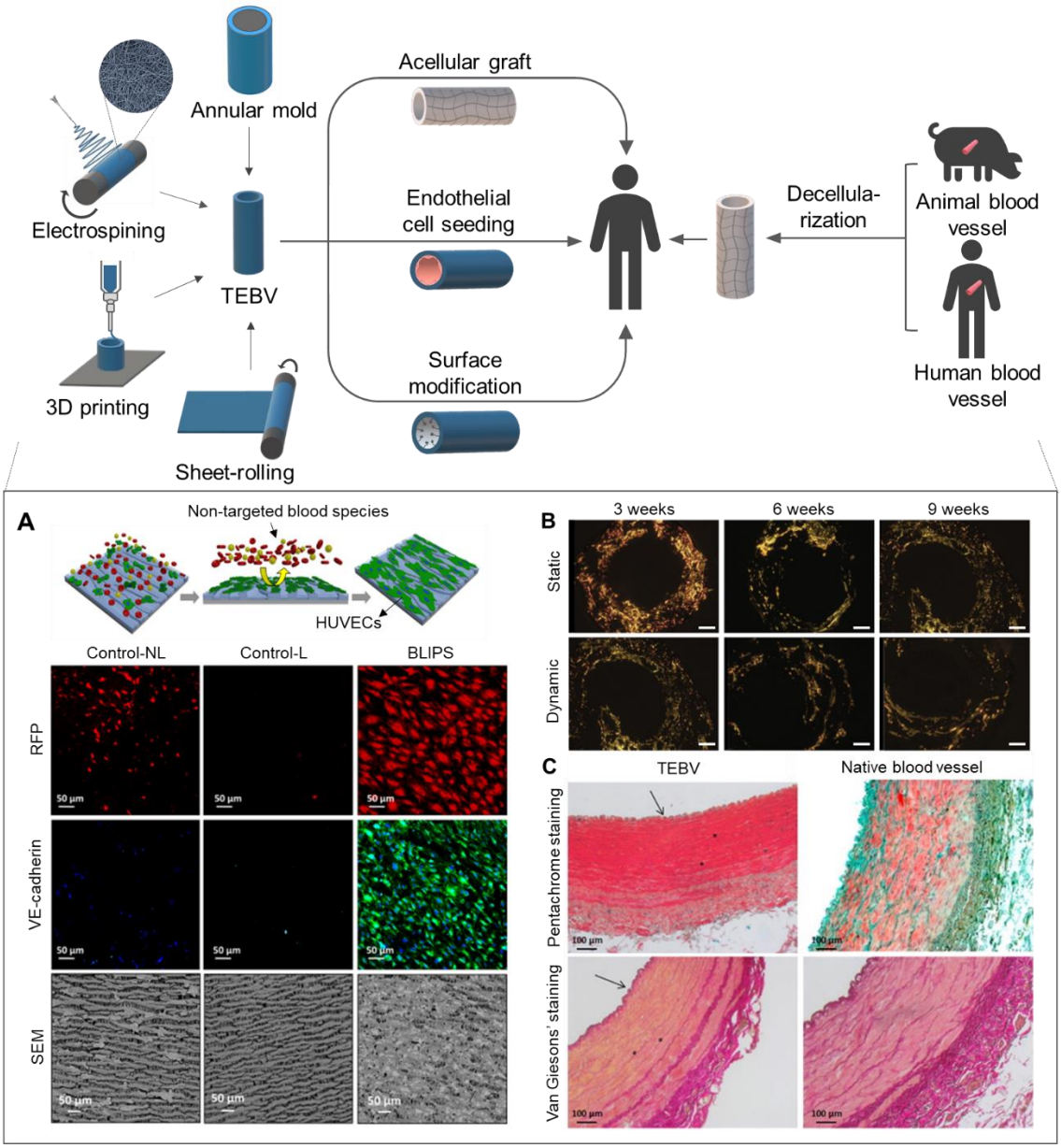

Figure 3. Scaffold-based approaches to produce biomaterial tubular structures (tissue-engineered blood vessel (TEBV)) by molding, electrospining, 3D printing and sheet-rolling techniques using synthetic or natural biodegradable polymers (with ou without encapsulated cells). Those TEBVs may be then decellularized, seeded with endothelial cells or surface modified before implantation. Moreover, native blood vessels derived from human or animal sources can be decellularized and used as vascular grafts. (A) Lubricant-infused ePTFE vascular graft functionalized with anti-CD34 antibody capture endothelial cells from whole blood. Fluorescence microcopy and SEM images showed that lubricant infused surfaces functionalized with anti-CD34 (BLIPS) were capable of capturing and adhere significantly more red fluorescent protein (RFP)-expressing HUVECs after 4 days of incubation with whole blood, in comparison with solely ePTFE grafts without modifications (control-NL) and grafts lubricated but without antibody functionalization (control-L). The positive immunostaining for VE-cadherin (green) confirmed the HUVECs phenotype of adherent cells, and provided evidence of a confluence monolayer with cellular junctions.[67] (B) Polarization microscopy of the PGA/P4HB tubular scaffold over 9 weeks, under static and dynamic conditions, showed that dynamic culture favored scaffold degradation more extensively allowing

tissue remodeling. Scale bars: 500 μm.[74] (C) Remodeling of fibrin-based TEBVs explanted after 6 months as carotid artery replacement in a sheep model. Histological analysis showed high structural similarity of TEBVs to native carotid artery, containing collagen deposition (pentachrome staining: yellow, van Giesons' staining: red), a confluent cell line representing the endothelium (arrows), and a thick layer of SMC (pentachrome staining: red, van Giesons' staining: yellow).[90]

3.2. Scaffold-free (or self-assembly) approach

The ability of cells to produce and assemble their own ECM and form cell-sheets *in vitro* has been exploited to generate biomaterial-free blood vessel mimetics. Those mono- or multilayers of cells are detached from the cell culture substrate, wrapped around a mandrel that serves as support to allow the construction of tubular structures, and then cultured for graft maturation (Figure 4).

L'Heureux *et al.* pioneered this strategy by reporting the first completely biological TEBV in 1998.[105] In this seminal study, a tubular structure with 4 mm diameter was built using exclusively cultured human cells. SMCs and fibroblasts were cultured in elevated conditions of ascorbic acid for a 30-day period to enhance the secretion of ECM by cells. The sheets were wrapped around an inert cylindrical support, presenting, in the end, three different layers: an acellular inner membrane produced by dehydration of a fibroblast sheet, a middle layer made of SMCs sheet, and an "adventitia" layer of fibroblasts sheet. The acellular internal membrane was used to act as a barrier to prevent intimal hyperplasia by the migration of SMCs into the lumen.[106] After 8 weeks of maturation in a bioreactor, the tubular support was removed, and ECs were seeded inside the structure forming the intimal layer. This model exhibited structural similarity to native tissue presenting various ECM proteins and a functional endothelium. In addition, it exhibited adequate mechanical properties, with burst strengths similar to the ones of human vessels, as well as blood compatibility and an easy surgical handling and suturing.

With the purpose of translating this sheet-based technology to the clinic, Cytograft Tissue Engineering, Inc. (Novato, California, USA) was created, in 2000.[106] To enable this translation, the initial L'Heureux's technique was altered with the removal of the medial layer of SMCs to simplify the process, and with the use of human-derived fibroblasts. Considering these modifications, in 2006, the Lifeline grafts were established and validated in preclinical models, and further tested as autologous hemodialysis access grafts in patients with end-stage renal disease.[106] In 2014, Wystrychowski *et al.* reported the first clinical application of a devitalized allogeneic Lifeline graft as arteriovenous shunts for

hemodialysis access, using fibroblasts derived from two donors with end-stage renal disease. The grafts showed no sign of aneurism for up to 11 months; however, stenoses at the anastomotic sites were observed as well as thrombosis-related failure.[107]

Based on the sheet-based technique, Jung *et al.* produced blood vessels with a smaller diameter using human mesenchymal stem cells (MSCs) and EPCs.[108] MSCs were used for their capability to differentiate into different vascular phenotypes, including a contractile SMC-like phenotype when co-cultured with ECs and under exposure to flow. Aligned MSC cell sheets were prepared on a nanopatterned polydimethylsiloxane (PDMS) mold to mimic the organization of SMCs in native blood vessels, and four of those sheets were wrapped around a glass mandrel to form a tubular structure (inner diameter = 1 mm) (Figure 4, A). After 2 weeks of culture in a rotating wall bioreactor, the mandrel was removed, and EPCs derived from umbilical cord were seeded in the lumen, using a perfusion bioreactor. Under increasing flow rate, the TEBV dilated and increased levels of NO were observed, which suggested that the endothelium was functional. The functionality of the endothelium was also confirmed by the adhesion of monocytes onto the ECs, which were activated by the presence of the pro-inflammatory cytokine TNF-α (Figure 4, A).

Cell sheet rolling seems to be a promising technique to generate commercially available scaffold-free vascular grafts with a fully biological nature. One may deduce that, due to the biomimetic vascular architecture of these grafts, the remodeling process after grafting may be favored. However, long maturation periods and difficult cell sheet manipulation still need optimization for these structures to be used as readily available autologous vascular grafts.

3.3. Fibrotic capsule-based vessels

Previously described approaches normally utilize *in vitro* bioreactors with perfusion systems designed to mimic the pressure and shear stress caused by native blood flow in the lumen of blood vessels, in order to examine the vascular conduits' performance and remodeling under these conditions, or to promote *in vitro* cellular maturation.[109] The use of the *in vivo* environment as a bioreactor has been explored as an alternative approach to produce autologous vascular substitutes within the recipient's own body (*in situ*). This strategy, also known as "the *in vivo* bioreactor approach", relies on the mechanism of

foreign body response to an implanted biomaterial, which is divided in several steps: first, protein adsorption occurs seconds after implantation and then a provisional matrix, composed by fibronectin and other bioactive components, deposits around the material. Afterwards, acute inflammation followed by chronic inflammation occurs. In a third stage, the foreign material is isolated in a fibrous capsule that contains proteins, cells (mainly myofibroblasts) and matrix deposited by cells (mainly collagen).[110] In this context, the *in vivo* formation of vascular grafts is based on the concept that: after the implantation of a foreign cylindrical device, the host's organism will respond allowing the growth of a tissue capsule around it, which can be further used as a vascular graft (Figure 4). Fibrotic capsules developed in the context of an immunologic response primordially have a protective function. As such, its composition will naturally be different from the one of native vessels. To attain the various requirements necessary to ensure long-term functionality of these capsules as vascular grafts, the control over the graft remodeling by cells may be the key to achieve structures resembling native blood vessels' features.[110]

The application of vascular grafts generated *in vivo* was pioneered by Sparks in the late 1960's. A prosthetic annular tube, composed by an inner steel mandrel and an outer Dacron tube, was implanted in the rib cage or subcutaneously on the chest wall of preclinical models in order to produce an autologous vascular graft.[111] This technique was also applied in patients in need for vascular replacement, where 5 to 12 weeks after implantation on the rib cage, a reinforced autogenous vascular conduit was removed and used to bypass the obstructed arteries.[111] Other clinical experiments were performed using a silicone mandrel method[112]; however, his technique was not widely adopted due to late graft failure associated to thrombotic and stenotic occlusion, and formation of aneurysms due to poor mechanical properties.[110]

Campbell and colleagues utilized the peritoneal cavity of rats and rabbits (1999)[113], and dogs (2004)[114], as an *in situ* bioreactor for the creation of tubular grafts. Their studies reported that the implantation of foreign materials in this cavity triggered host responses resulting in the formation of a fibrous capsule composed by layers of myofibroblasts and an outer layer of mesothelium which have some functional similarities with the endothelium.[58] When a silastic (inert silicone elastomer) tubing was placed in the peritoneal cavity of the recipient rat and rabbit models, two weeks after implantation, a tubular tissue capsule was formed and used as an arterial interposition graft after the

removing of the silastic tubing. After 4 months, the grafts showed an overall patency of 67% in rats and 70% in rabbits, and graft remodeling was confirmed by the presence of structures that resemble elastic lamina and intense levels of smooth muscle myosin heavy chain, suggesting a phenotypic switching of myofibroblasts to SMC-like cells; however, only 50% of the developed capsules were suitable to be subsequently used as vascular grafts.[113]

Implanted materials used to trigger a foreign body response may be tailored so the developed immunologic response leads to a tissue capsule (later used as a vascular graft) with adequate biological and physical properties.[110] Most of the early studies utilizing living hosts as bioreactors for the generation of engineered vascular grafts were based on the use of silicone. However, recently, polymeric rod models made of the poly(ethylene oxide terephthalate)–poly(butylene terephthalate) (PEOT/PBT) copolymer have been widely used due to their tunable mechanical and physical properties. For example, Geelhoed *et al.* implanted subcutaneously PEOT/PBT (55/45) rods in the neck of goats, resulting in the formation of a tissue capsule around the implanted material, after 1 month (Figure 4, B).[115] The formed tissue capsules (diameter = 6.8 mm) were grafted on the same goats, during 1 and 2 months, as arteriovenous conduits. Prior to grafting, tissue capsules exhibit mechanical strength to withstand physiological pressures and a dynamic compliance inferior of that of native artery which was explained by the low expression of elastin. Two months after grafting, histological assays revealed the presence of SMC-like cells, elastin, a well-defined endothelial monolayer as well as a functional contractile phenotype, suggesting tissue capsule remodeling into a vascular phenotype. In addition, the non-thrombogenic properties of the endothelium were confirmed by using a specially designed *ex vivo* perfusion system exposed to whole blood flow from the goats. Nonetheless, intimal hyperplasia associated with stenosis was detectable near the venous anastomosis and venous outflow tract.

Overall, the *in situ* development of TEBVs would be advantageous as the tissue capsule formed around the implanted device is completely autologous, and produced vascular grafts are non-toxic and cause no immune responses. However, beyond the long periods of incubation needed to generate the tissue capsule, the high invasiveness of this method remains the major drawback for it application, since at least three surgeries are needed: one to implant the foreign material, another to harvest the newly formed tissue

capsule, and finally the grafting on the defected vessels. Also importantly, the response of the organism to an implanted device may be different depending on the gender, age and the presence of pathologies.[110] Therefore, the clinical translation of this method may be limited by the biological variability of the tissue capsules between patients, leading to poor reproducibility.

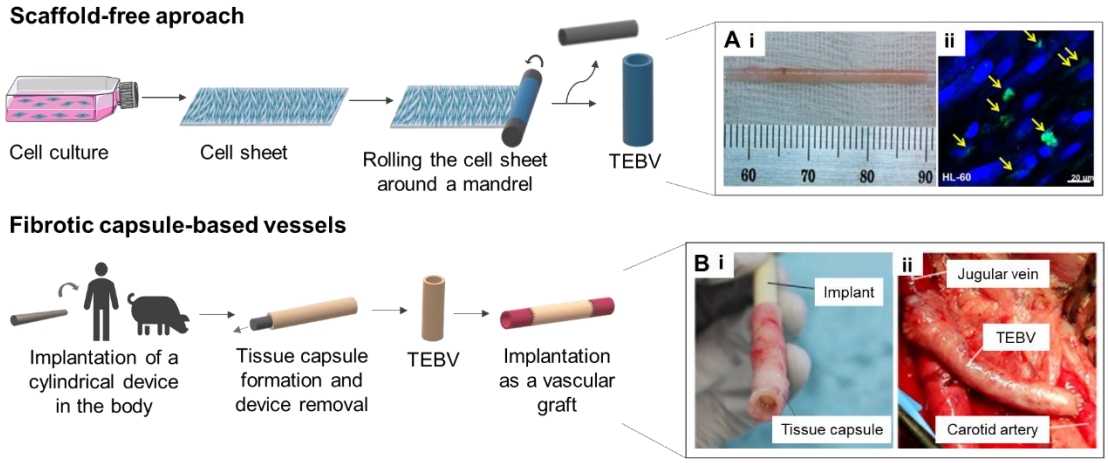

Figure 4. Scaffold-free approach and the *in vivo* bioreactor approach to produce tissue engineered blood vessels (TEBVs). The first one is based on the rolling of cell sheets around a mandrel, and the second one relies on the foreign body response to an implanted cylindrical device to form a tissue capsule around it. (A) A TEBV fabricated by rolling multiple human MSC sheets, produced on nanopatterned PDMS substrates, around a mandrel. (i) Macroscopic image of the human MSC sheets wrapped around a 1.3-mm diameter glass mandrel, and (ii) confocal microscopy image that revealed monocyte-like HL-60 cells (arrows) adhesion to endothelial cells after TNF-α activation. Blue: SMCs, green: HL-60.[108] (B) A tissue capsule produced after subcutaneous implantation of a cylindrical implant in the neck of goats. (i) Macroscopic image of the tissue capsule formed after 1 month, and (ii) implantation of the tissue capsule as an arteriovenous graft.[115]

4. Recreating the microvasculature

Various therapies directed at the enhancement of tissue perfusion and regeneration have been targeted to repair injured tissue. Those include the administration of angiogenic factors, genes and cells, and transplantation of engineered tissue constructs.[116] Although tissue-engineered constructs have been demonstrating some promising results *in vitro* and in pre-clinical models, the translation to clinical trials has only been successful for thin tissues and with low metabolic activity, such as skin, cartilage and bladder. When it comes to more complex and thicker tissues, such as heart, liver and kidney, the lack of an appropriate vasculature causes hypoxia and consequently short-term survival of the engineered construct.[117] Therefore, several strategies have been developed to promote the growth or integration of microvasculature within these constructs to provide gas, nutrient

and waste exchanges to all cells throughout the tissue, and thus ensure its survival and function.

4.1. Angiogenic approaches (scaffold-based)

Invasion of an a-vascular engineered tissue requires active migration of new vessels through the process of sprouting angiogenesis. This process is highly dynamic, involving cellular and cell-ECM interactions, as well as the coordination of distinct cytokines and growth factors.[40] Herein, we consider angiogenic approaches that rely on the presence of biochemical and/or biophysical cues to induce the growth of a vascular network – either through self-organization of previously seeded ECs, or in-growth of new blood vessels from the surrounding tissue. In this case, biomaterial scaffolds serve as guides for new tissue formation. The formation of microvasculature in tissue constructs through this process can be stimulated *in vitro* or *in vivo*, once a conducive environment is provided.

4.1.1. In vitro pre-vascularization and scaffold functionalization

The stimulation of *in vivo* angiogenesis can be achieved by creating *in vitro* artificial environments that provide either biochemical cues by different cell types and/or angiogenic factors, or biophysical and mechanical cues, to induce ECs to form blood vessel networks in engineered constructs (Figure 5).

Cellular co-cultures. The use of coculture systems plays essential roles in the establishment of cell−cell communications that emulate the ones occurring in native tissues. This approach aims at pre-forming a vascular network *in vitro* that could rapidly connect to the systemic circulation upon *in vivo* implantation, by a process named inosculation. Since the mechanism of angiogenesis relies on the interaction of ECs with other cell types under proper microenvironmental cues, co-culture systems are promising strategies for the development of vascularized tissue constructs. In fact, seminal studies from Kirkpatrick and colleagues focusing on the role of cells as primary fibroblasts and osteoblasts, epithelial cells, and macrophages have enlightened the importance of co-culture systems and cell-cell communication in modulating each cell type functionality and in promoting the formation of microvascular-like structures *in vitro*.[118–121] In addition, the angiogenic process also depends on cell-ECM interactions, and although classical 2D co-

culture models in polystyrene culture plates have already shown to promote the formation of stable capillary beds compared to the culture of ECs alone[116,121], the co-culture in 3D models where cells can be seeded or directly encapsulated mimics more closely the *in vivo* environment, as well as the native cells' behavior.

The majority of the performed studies that have reported the formation of capillaries *in vitro* were based on the co-culture of ECs or EPCs, with support cells such as SMCs, pericytes, fibroblasts and MSCs.[116] Those support cells have several important functions during the development, maturation and stabilization of the microvascular networks during angiogenesis.[122] While SMCs can control the vasoactivity of vessels through cellular contraction and relaxation, and the balance between ECM secretion and degradation[33], fibroblasts are responsible for ECM production and can be differentiated into SMCs under proper stimuli exerting contractile functions.[22] EPCs and multipotent MSCs have important roles in the regeneration of the endothelial layer and stabilization of vascular networks, respectively. MSCs have the ability to differentiate into multiple cell lineages including SMCs and pericytes and produce essential biomolecules, depending on the stimuli that they receive.[116] On the other hand, it has been shown that interactions between ECs and parenchymal or stem cells are beneficial for both, acting as inducive factors of angiogenic and tissue growth. For example, a synergistic effect occurs between human adipose stem cells (ASCs) and human adipose microvascular ECs, which promotes bone formation and angiogenesis via the secretion of growth factors (VEGF and bone morphogenic protein-2).[123] In addition, the co-culture of outgrowth ECs (OECs) – also called late EPCs - with bone marrow-derived MSCs, has also been shown to be essential for the formation of vascular structures in silk fibroin hydrogels.[124]

Furthermore, ECs and support cells have been co-cultured with tissue-specific cell types such as myofibroblasts, cardiomyocytes, keratinocytes or hepatocytes, in order to generate implantable skeletal muscle[125], myocardium[126], skin[127] and liver[128] vascularized tissue constructs, respectively. The culture of ECs with both tissue-specific cells and support cells such as fibroblasts allow increased formation of capillary-like structures with tubular morphology compared to ECs cultured alone or with one of the cell types (Figure 5, A).[128] In one study where vascularized muscle constructs were developed, the presence of fibroblasts increased the expression of VEGF, which enhanced endothelial lumens formation, and allowed the stabilization of the vessel structures which was confirmed by

the presence of SMA-positive cells (differentiation state of the fibroblasts) around endothelial like-vessels.[125] The presence of intraluminal red blood cells in the vessels of the construct confirmed the integration and anastomosis with the host vasculature after two weeks of *in vivo* implantation in animal pre-clinical models.[125]

Pro-angiogenic factors. The vascular microenvironment is not only composed by different cell types and ECM components, but also contains multiple cytokines, chemokines and growth factors that regulate vascular network formation and maintenance in tissues and organs.[129] Growth factors are signaling peptides that regulate cell behavior and function after binding to specific cell-surface receptors, through activation of various signaling pathways.[130] Many angiogenic growth factors have been combined with cells and scaffolds, including VEGFs, FGFs, transforming growth factors, platelet-derived growth factors and angiopoietins. These biomolecules can be soluble in the culture medium, immobilized on scaffolds through covalent or non-covalent interactions or simply be incorporated in scaffolds.[22] The immobilization and incorporation of growth factors in scaffolds brings the possibility of localized delivery of angiogenic compounds to ischemic tissues in a more spatially and temporally controlled manner, when compared to the exogenous administration of growth factors. Moreover, soluble growth factors are less stable and can be more easily degraded, which make them less effective as therapy.[22] Consequently, growth factor immobilization strategies have shown to be promising in the sense that the biochemical factors can promote angiogenesis for a prolonged period of time, reducing the amount of growth factor required.[131]

VEGF is one of the most widely used biomolecules incorporated or used to modify biomaterials in the scope of microvascular regeneration. This biomolecule exerts its effects after binding to tyrosine kinase receptors (VEGF-R1, -R2 and -R3) expressed in the surface of ECs membrane.[40] The covalent immobilization of scaffolds with VEGF through chemical reactions promoted EC proliferation and growth *in vitro* in fibrin hydrogels[132], and improved the *in vivo* angiogenesis and vascularization while incorporated in collagen[133] and poly(ethylene glycol) (PEG)[134] hydrogels. During the initial phase of sprouting angiogenesis, ECs are activated in response to a VEGF gradient and start to proliferate and migrate towards the angiogenic source in order to produce new blood vessels.[13] Indeed, a biomaterial-based bioinspired approach with gradients of immobilized VEGF on collagen scaffolds, promoted EC migration towards higher concentration sites.[135]

Moreover, VEGF has been combined with other angiogenic factors, such as PDGF-BB, bFGF or Ang1 in order to synergistically induce the multiple steps of angiogenesis and lead to the formation of more stable capillary networks. For example, Ang1 is produced by pericytes and promotes vascular remodeling and stabilization by binding to EC-specific tyrosine kinase receptor Tie-2 and also facilitating further recruitment of pericytes to the vessel wall.[40] The main regulator of pericyte recruitment to newly formed vessels is PDGF-BB, produced by activated endothelium.[136] Recruited pericytes then regulate endothelial function through both paracrine and cell contact-dependent signals, including the TGF-β, Angiopoietins and Ephrin pathways.[34] Therefore, it is expected that when combined with VEGF, their complementary effects will induce the formation of new capillary vessels (via VEGF) and promote their stabilization (via Ang1 or PDGF-BB), thus allowing the formation of mature blood vessels. Chiu *et al.* immobilized VEGF and Ang1 in porous collagen sponges via the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride chemistry, and observed that the immobilization of both cytokines significantly increased EC proliferation and improved tube formation compared to the controls (soluble VEGF and Ang1).[137] In addition, the authors performed the chick embryo chorioallantoic membrane (CAM) assay, to evaluate the angiogenic potential of their sponges *ex vivo*, and demonstrated that indeed the co-immobilization promoted neovascularization and increased the number of blood vessels connected to the host's vasculature, compared to scaffolds only modified with VEGF or Ang1.[137] On the other hand, the co-delivery of VEGF and PDGF-BB has been investigated to promote pericyte recruitment and the coordinated activation of their complex regulatory crosstalk with endothelium. Early work by Eli Keshet described a differential kinetics between endothelial and pericyte contributions to vessel formation, with pericyte recruitment lagging several days behind endothelial plexus formation.[138] This observation stimulated translational approaches aimed at ensuring that VEGF and PDGF-BB could be co-delivered in tissues with distinct release kinetics, e.g. by dual polymeric systems.[139] However, subsequent work showed no need for temporally independent delivery of the two factors, but rather that only the relative dose bears important therapeutic implications. In fact, simultaneous co-expression of VEGF and PDGF-BB at a fixed and balanced relative ratio has been shown to prevent aberrant angiogenesis by excessive VEGF and ensure the

induction of normal, stable and functional vascular networks regardless of VEGF dose, improving both therapeutic efficacy and safety compared to VEGF alone.[140,141]

Another way to promote vascularization through angiogenic biochemical cues include the incorporation or encapsulation (without chemical reactions/immobilization) of growth factors on scaffolds that degrade over time, ensuring a spatial and temporal controlled release of the incorporated biomolecules. For example, Losi and colleagues incorporated VEGF and bFGF in a polymeric scaffold composed of poly(ether)urethane-polydimethylsiloxane (PEtU-PDMS) and fibrin.[142] *In vitro* studies revealed that the scaffold provided a controlled release of both growth factors and that they remained bioactive promoting ECs proliferation up to 7 days. Subsequently, the angiogenic potential of the scaffold was evaluated *in vivo* in rat models, and, in fact, the presence of VEGF and bFGF enhanced angiogenesis which was suggested by the significant increased number capillaries compared to scaffolds without growth factors (Figure 5, B).

Growth factor delivery has been also inspired by the natural signaling roles of proteoglycans and GAGs present in the ECM and on the cell surface.[143] These molecules have the ability to bind to specific proteins including growth factors and its receptors resulting in modulation of signaling activities.[144] Specifically proteoglycans that have heparan sulfate (a GAG) side chains can bind to various growth factors involved in angiogenesis and regulate physiological and pathological processes.[144,145] As such, various biomaterials have been functionalized with heparan sulfate or heparin (a more sulphated variant of heparan sulfate found in connective tissue of mast cells) to control the delivery of heparin-binding growth factors such as VEGFs, FGFs and PDGF.[146–149] Additionally, scientists have found interest in the development of innovative biomaterials containing GAG derivatives with defined sulphation degree to study their influence in modulating growth factor recognition and consequently EC biological activity and angiogenesis.[150] Perlecan is the major heparan sulfate proteoglycan present in the vascular basement membrane, and its regulatory function has been associated to both pro- and anti-angiogenic effects.[144] Studies using naturally-derived perlecan have been made; however, its isolation results in low yields, so advances in recombinant expression and metabolic engineering have allowed the production of recombinant fragments of perlecan. These recombinant molecules have shown to promote EC sprouting and enhance angiogenesis both *in vitro* and *in vivo* via GAG-dependent growth factor signaling.[151,152] Moreover, when

immobilized in a silk fibroin-based scaffold and implanted subcutaneously in mice, it promotes blood vessel ingrowth and integration with the host tissue.[152]

The importance of VEGF interaction with ECM for the regulation of its functions is at the bases of another class of strategies, aiming at generating physiological distributions of VEGF within engineered matrices.[153] For example, rather than modifying biomaterials to increase their affinity for growth factors, the factors themselves can be engineered to endow them with super-affinity for a natural biomaterial matrix such as fibrin, collagen, and endogenous ECM, without covalent coupling. This has been achieved by engineering growth factors with a short peptide derived from Placenta-derived Growth Factor-2, named PlGF-$2_{123\text{-}144}$, and featuring broad binding properties to matrix proteins.[154] Presentation of factors within their physiological matrix-bound state was shown to significantly increase their therapeutic efficacy at very low doses.[154] Another approach consists in directing the progenitors themselves to over-express VEGF through genetic modification, independently from intervening hypoxia, so as to direct vascular growth towards the cells needing it. When using this approach to attract vascularization inside a cell-seeded scaffold, scaffold materials with different affinity for the produced VEGF can be used to tune its binding and determines its distribution within the scaffold[155] or its release into surrounding tissue to induce extrinsic angiogenesis.[156] Genetically modified cell lines can also be used to produce an ECM rich in specific growth factors: their subsequent removal by programmed cell death yields engineered decellularized matrices of tunable composition that present therapeutic factor in their physiological context.[157]

Biophysical cues: architectural control. In addition to the cell-cell and cell-scaffold interactions, the physical properties and surface topography of scaffolding materials seem to play an important role in general biological response[130] and, in particular, in promoting an adequate vascularization within engineered tissue constructs. During angiogenesis, ECs proliferate and migrate by interacting with ECM components through integrin binding, forming focal adhesion complexes, that influence the organization of actin filaments within the intracellular matrix of ECs, allowing them to sprout and form new blood vessels.[40] ECM proteins play an important role in this mechanism, especially collagen, since it is present as 3D organized fibrils that provide contact guidance for cells' polarization and migration.[40,158] It has been shown that topographical features and geometrical patterns on scaffolds play important roles in guiding the orientation of microvessels and directing

vascular morphogenesis *in vitro*.[159] This could be important since several tissues including skin, skeletal muscle and heart, have a highly organized and aligned microvasculature, and thus guiding the organization of the ingrowth vessels in engineered tissues may be determinant to mimic more closely native tissues.[159] Moreover, the presence of such features on scaffolds can mimic the vascular microenvironment since it has been shown that vascular basement membranes present surface topographies, including pores and fibers, with dimensions at the nano (1-100 nm) and submicron (100-1000 nm) scale, which are important to regulate several cell behaviors.[160]

The presence of pores in scaffolding biomaterials seems to be fundamental for cell infiltration and formation of capillary vessels, and the presence of fibers in scaffolds can be beneficial to orient cell migration and proliferation during angiogenesis.[122,159] For example, Chen *et al.* investigated the use of porous methacrylated gelatin (GelMA) hydrogels to generate vascular networks by co-culture of endothelial colony-forming cells and bone marrow-derived MSCs, and examine the influence of different degrees of methacrylation, and consequent different pore sizes, in capillary formation.[161] Softer hydrogels with higher pore size (≈ 50 μm) promoted the formation of more robust and interconnected endothelial networks, that exhibited a mature-like phenotype presenting hollow endothelialized lumens with abluminal support cells resultant from the differentiation of MSCs.[161]

Recently, Brown *et al*. established several design principles for synthetic hydrogels in order to understand how ECM properties can influence endothelial morphogenesis into microvascular networks.[162] EC-encapsulated PEG-based hydrogels were modified with cell adhesive sequences (RDG motif) and protease-sensitive peptide crosslinkers (for matrix degradation and remodeling). Lower polymer and crosslinking densities (softer hydrogels) favored the formation of an endothelial network (Figure 5, C). In addition, increased matrix degradation susceptibility as well as hydrogel neutral-swelling properties, were also proved as fundamental factors to promote endothelial morphogenesis during angiogenesis, since the process requires local ECM degradation and subsequent migration of encapsulated ECs to form vascular networks. The relevance of hydrogel matrices with controlled mechanical features as adjuvants of the formation of microvasculature was also evidenced by Forget *et al.*, in a study that addressed the influence of biomaterials' stiffness and growth factor supplementation in the stabilization of newly formed microvessels.[163] RGD-functionalized carboxylated agarose hydrogels with different stiffness were

implanted in the gastrocnemius muscle of immunodeficient mice. After 7 weeks, softer hydrogels with shear modulus similar to the fibrin matrix of blood clots, promoted improved vascular density and vessel stabilization (associated with the presence of pericytes) than stiffer gels, regardless of the addition of growth factors. The long-term angiogenic ability of this scaffold was attributed to its mechanical properties, which was shown to be related to the presence of a new population of immune cells (CD11b+/CD115+ monocytes) expressing the mechanosensitive ion channel protein Piezo-1, that hypothetically helped in vessel stabilization and maturation.

The creation of specific nano and micro-patterns in the surface of scaffolds has also been exploited to promote the formation of more organized vascular networks by regulating the cell behavior, morphology and function. Soft lithography, photolithography, laser photolithography, micro-molding and micro-contacting are examples of techniques used to form different nano and micro-patterned surfaces on tissue constructs (more details elsewhere [22,159]). In order to investigate the influence of submicron and nano-scale topographic features on several essential EC behaviors including orientation, alignment, proliferation and migration, Liliensiek and colleagues used patterned polyurethane surfaces fabricated by soft lithography.[164] The patterned polyurethane scaffolds were seeded with human ECs from both large and small arteries and veins. It was observed that indeed the topographical cues impact EC behavior, but differential behavioral responses depended also on the different anatomic origin of the cells. In that work it was only studied the influence of surface topography on EC behavior, but other studies have demonstrated also it impact on co-culture systems. For example, Soucy *et al.* verified that micropatterned fibronectin substrates cultured with fibroblasts, allowed the formation of a fibroblast-derived matrix, that oriented the formation of endothelial tubes when HUVECs were seeded onto this matrix.[165] Moreover, in another study, it was observed that the provision of nanopatterned PDMS substrates coated with collagen and the tri-culture of HUVECs, human dermal fibroblast and human pericytes derived from placenta, have induced angiogenesis, by promoting cellular alignment and the formation of stable capillary-like structures. The nano-topographical features and the presence of pericytes were crucial for maturation and stabilization of the vessels.[166] Alternatively, micropatterned fibrous mats produced by pouring a polymeric solution on a micropatterned collector through electrospinning have been developed to allow the formation of vascularized tissue-specific

constructs using parenchymal and vascular cells. For instance, Li *et al*. developed micropatterned fibrous mats that mimic the native structure of hepatic lobules[128] and the native anisotropic structure of myocardium[167], with patterns that allowed enhanced EC spreading and capillary-like networks formation.

Regulation of environmental cues. Strategies capable of inducing angiogenesis based on other factors than the administration of biochemical or topography-induced cues, and mostly relying on the control of the mechanical environment have been suggested. Such strategies have mostly been focused on the development of artificial environments taking advantage of *ex vivo* bioreactor cultures capable of combining biochemical and/or biophysical cues. For example, flow-induced mechanical stimuli have been used to mimic wall shear stress caused by blood flow, which affect EC's invasion ability.[168] Using microfluidic devices seeded with ECs, Galie and colleagues established a shear stress threshold (10 dyn/cm^2) above which endothelial sprouting is induced regardless on whether the shear is applied on the apical surface of ECs (through luminal flow), or on cell-cell junctions (through transmural flow). Fluid flow was crucial not only to stimulate sprouting, but also to sustain the sprouts and prevent their regression.[169] In another study, shear stress caused by fluid flow induced the reorganization of F-actin filaments of ECs parallelly to the direction of the flow, and concomitantly enhanced nitric oxide synthesis when compared to ECs cultured in static conditions (Figure 5, D).[170] The results from both studies emphasize the importance of creating dynamic and complex environments *in vitro* to recapitulate *in vivo* mechanical and physical stimuli, with the potential to regulate vascular morphogenesis and angiogenic potential of ECs.

The production of cellular pro-angiogenic factors has also been successfully achieved by reducing oxygen levels and creating a hypoxic environment. Several studies associate the expression of hypoxia-inducible factors to the regulation of many angiogenic factors including VEGF and its receptors, angiopoietins, FGF and others involved in the angiogenic process.[171] As such, hypoxic treatment has been used as a biochemical trigger to induce angiogenesis and the formation functional vasculature in the tissue engineering and regenerative fields.[172] However, this approach requires high control over conditioning time, since long-term and sustained hypoxia have been related to expression of antiangiogenic factors and cytotoxicity in cell culture systems.[173]

The induction of the formation of microvasculature in scaffolds *in vitro* has mostly been attained through the provision of different biochemical and biophysical cues (or combinations thereof). However, the major challenge in this field remains to ensure the stability and non-leakiness of the formed microvessels. The presence of support cells, including MSCs and fibroblasts, seems to be crucial for stabilization and maturation of microvessels. Moreover, the combination of different growth factors that act as mediators for the recruitment of important cells during the various steps of the angiogenic process seems also to favor the formation of functional microvasculature. In the sought for functional and safe therapies, it has been reported that it is extremely important to independently control the dose and duration of expression of angiogenic factors in the scaffold for transplantation purposes. For example, it is known that uncontrolled doses of VEGF can cause aberrant vascular growth leading to angiomas, and that VEGF stimulation must be sustained for at least 4 weeks to prevent vasculature regress and maintain newly formed vessels indefinitely.[13] Moreover, other important aspects that have been shown as crucial to control microvascularization consist on the physical and mechanical properties of scaffolds, so a biomimetic vascular environment for angiogenesis be provided.

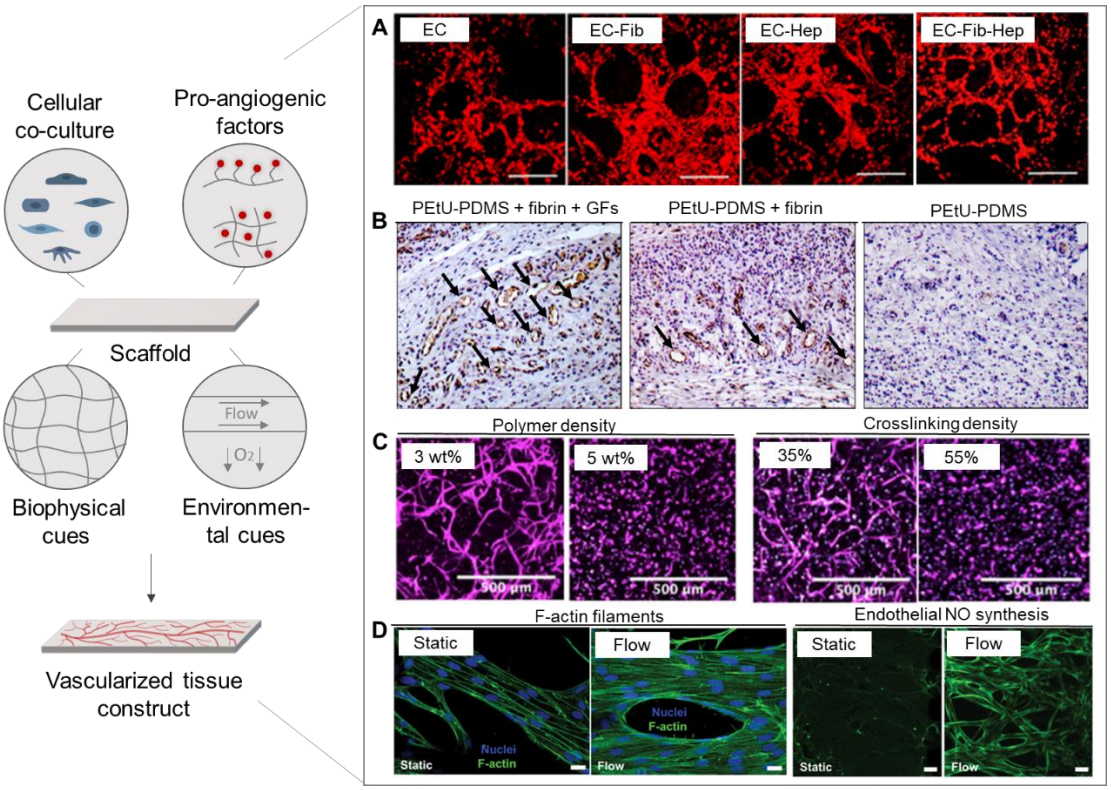

Figure 5. Strategies to induce the formation of microvessels and vascular networks on scaffolds through angiogenesis for *in vitro* pre-vascularization with cell co-cultures (cells from BioRender.com) and/or through scaffold functionalization with biochemical (pro-angiogenic

factors) or biophysical (surface topography and patterning) cues. (A) Confocal laser scanning microscope images of Matrigel-induced capillary morphogenesis after CM-DiI staining on ECs cultured alone or after coculture. Endothelial cells (ECs) formed more capillary-like structures with branching networks when cultured with fibroblasts (Fib) and hepatocytes (Hep) compared to ECs alone, or cultured with either Fib or Hep. Scale bar: 50 μm.[128] (B) Immunohistochemical staining of PECAM-1 positive vessels showed numerous well-defined vessels with lumen in PEtU-PDMS/fibrin scaffolds and more in scaffolds functionalized with growth factors (GFs) - VEGF and bFGF -, compared to PEtU-PDMS scaffolds only, when implanted subcutaneously in a rat model.[142] (ii) F-actin-stained hydrogels after 3 days in culture illustrate that lower polymer and crosslinking densities favored endothelial network formation.[162] (D) Endothelial cell responses to luminal fluid flow: F-actin filaments of ECs aligned in parallel of the direction of fluid flow after 2h of exposure, and upregulation of nitric oxide (NO) synthesis (low level of fluorescence signal of NO-reactive fluorescent dye DAF-FM DA under static conditions compared to flow/dynamic conditions. Scale bars: 20 μm for the F-actin images and 50 μm for the NO synthesis images.[170]

4.1.2. In vivo vascular supply

Taking advantage of the complex *in vivo* environment as a bioreactor to stimulate angiogenesis and induce the ingrowth of microvasculature in scaffolds, it is possible to produce autologous tissue constructs ready to be implanted. This strategy may include the implantation of a scaffold in a well-vascularized tissue of the body and waiting for the ingrowth of new microvessels from the surrounding host vasculature (Figure 6). However, this procedure is time-consuming, as it requires long periods for the complete vascularization of scaffolds, which additionally may lead to excessive formation of fibrous tissue as a response of the body to the implanted material.[116] Furthermore, after the excision of the vascularized scaffold, its anastomosis with the host vasculature when implanted at a tissue defect occurs through inosculation, which therefore depends on the casual connection between the formed microvessels and the host ones. Hence, since tissue reperfusion does not occur immediately, additional time is required for host-scaffold integration. Those reasons have hampered the application of this strategy to the clinic, and therefore scientists started to investigate more clinically translatable methods, namely the flap technique and the arteriovenous (AV)-loop technique, aiming to facilitate the perfusion within tissue constructs thereupon implantation through direct surgical anastomosis.[174]

Flap technique. In this technique, an intrinsically vascularized tissue flap (mostly muscle flap) is used to allow the ingrowth of microvessels in implanted scaffolds (Figure 6). Afterwards, both tissue flap with vessels (pedicles) and the scaffold with proper vascularization, are transferred to a defect site, where the flap pedicles are surgically anastomosed with the host vasculature, allowing immediate perfusion.[116] This technique

has been used to produce vascularized constructs for the replacement and regeneration of large bone defects, in order to circumvent the increased donor-site morbidity associated with standard clinical practice that uses autologous bone grafts from different locations such as fibula and iliac crest.[174] Warnke and colleagues reported the first clinical application of a bone-muscle flap technique for the reconstruction of a large mandibular defect on a patient who underwent previous cancer surgery.[175] The patient's right latissimus dorsi muscle was used as bioreactor to produce vascularized bone tissue around a titanium mesh cage specifically designed to fit properly on the mandible defect. To induce the formation of bone, the titanium mesh was filled with bone mineral blocks coated with an osteo-inductive factor, and patient's own bone marrow. Seven weeks after implantation, both the formed vascularized bone tissue and muscle with pedicles (thoracodorsal artery and vein), were transplanted into the defect site. The vessel pedicle was surgically anastomosed with host vessels, which allowed blood perfusion to the flap, contributing to bone remodeling and mineralization resultant from osteoblast activity. Although this clinical application clearly improved the patient's life quality, problems associated with titanium mesh fracture and the amount of bone induction factors were identified.[176] However, in general, the major problem associated with the flap technique is tissue loss at the donor site where muscle flap is harvested, with an associated risk of local donor-site morbidity.

AV-loop technique. In this technique a surgically generated AV fistula with a loop shape is incorporated into a scaffold allowing the spontaneous angiogenesis and microvessels' outgrowth along the loop (Figure 6). Subsequently, the vascularized tissue constructs can be directly anastomosed with host's blood vessels trough the fistula.[116] In contrast to the flap technique, this method normally uses an isolation chamber were the scaffold with the incorporated AV fistula are placed for vascular network formation, therefore no host tissue needs to be harvested, reducing donor-site morbidity.[174] Since 2000, different vascularized tissue constructs have been produced by axial vascularization in scaffolds using this method in pre-clinical models, including skeletal muscle[177], cardiac tissue[178,179] and bone[180]. Only in 2014, Horch *et al.* reported the first successful clinical application of this technique on the reconstruction of bone defects in the radius and tibia of two patients.[181]

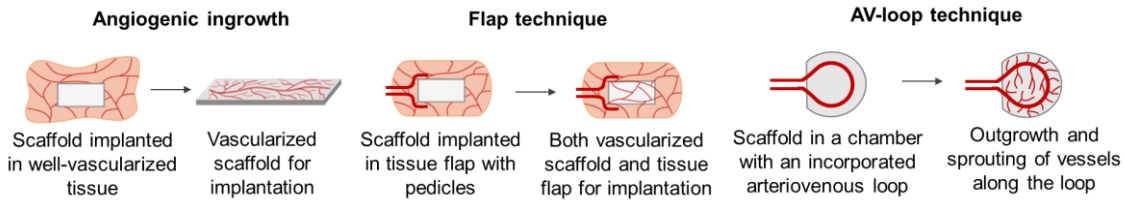

Figure 6. Strategies to induce a vascular supply *in vivo*, by the implantation of scaffolds on well-vascularized tissues (angiogenic ingrowth), on tissue flaps (flap technique) or in isolation chambers with an incorporated arteriovenous (AV)-loop (AV-loop technique). After angiogenesis, the vascularized scaffolds are used for implantation in damage tissues.

4.2. Cell-based approaches (scaffold-free)

Cell-based approaches rely on the ability of cells to self-aggregate into small microtissues with specific shapes, without the presence of scaffolds or using temporary scaffolds. To allow the formation of pre-vascularized tissue constructs, ECs are co-cultured with parenchymal or stem cells, and (i) spontaneously organize into capillary-like networks and/or (ii) promote the formation of new microvessels through angiogenesis.

3D cell aggregation. This approach traditionally involves the formation of spherical-shaped cell clusters with a 3D organization, named spheroids, formed by cellular self-assembly. Multicellular aggregates with spherical shape are widely used as tumor models for drug screening purposes due to their characteristic necrotic nuclei that mimics the hypoxic core present in various solid human tumors.[182] However, during the last years, numerous studies have been focused on the angiogenic and vascularization potential of spheroids for vascular tissue engineering applications. This approach normally combines ECs or their progenitor cells, with tissue-specific cells or MSCs (Figure 7). The formation of pre-vascularized microtissues with capillary-like structures in such culture conditions has been verified.[174] Furthermore, the implantation of pre-vascularized microaggregates *in vivo* has proved to improve vascularization and promote the integration with the host vasculature.[183] Additionally, the co-culture of OECs isolated from human umbilical cord blood with human bone marrow-derived MSCs, and their assembly into 3D spheroids showed angiogenic potential both *in vitro* and *in vivo*.[184] Aiming at the development of an injectable cell delivery system to promote vascularization in ischemic tissues, these co-cultures were incorporated as microtissues within round-shaped degradable alginate microgels that acted as temporary supports.[185] Such microgels showed low stiffness to allow cell mobility, partial oxidation for progressive network degradation, and RGD

domains for cell adhesion. Within 14 days, OECs organized into primitive vascular beds (Figure 7, A), and gene expression analysis demonstrated a phenotypic switching of the cells from a sprouting/angiogenic state to a vessel maturation state related with ECM deposition. Analysis of the secretome of entrapped cells, showed that cells secreted metalloproteinases as well as pro- and anti-angiogenic factors after 14 days of culture. The angiogenic potential of the microtissues was confirmed using an *ex vivo* CAM assay.

As an alternative to spheroids, recently Sousa *et al*. developed cell aggregates with a fiber-shaped configuration, called "fiberoids".[186] These scaffold-free structures were fabricated in a rapid and single step method induced by gravity, utilizing superhydrophobic surfaces patterned with wettable elongated channels. By dispensing a cell suspension in the channels, the fiberoids were produced in less than 24 hours. Human adipose derived stem cells (hASCs)-based fiberoids improved the secretion of VEGF and heterotypic hASCs+HUVECs fiberoids were able to integrate native tissues and promote angiogenesis *ex vivo*, demonstrated in a CAM assay. In addition, they presented injectable and fit-to-shape properties, which is advantageous for regenerative medicine approaches.

The cell accumulation technique was also suggested to effectively aggregate individual cells and generate vascularized microtissues. Structures with 50 μm thickness were obtained by the bottom-up assembly of fibronectin-gelatin individually coated cells.[187] The presence of nanofilms on cells' surface allowed the formation of 8-layered microtissues within 1 day. With this technique, microtissues with highly dense capillary networks were constructed within 1 week, through the seeding of HUVECs between four layers of human dermal fibroblasts.

Cell sheet stacking. Cell sheet technology has also been used for the fabrication of tissue constructs with microvasculature, without the need for supporting scaffold materials. For this purpose, cells are typically cultured in thermo-responsive culture substrates that are normally created by covalently grafting the temperature-responsive polymer poly-(N-isopropylacrylamide) (PIPAAm).[188] The physical properties of this polymer depend on the temperature of the environment: for temperatures below its lower critical solution temperature (LCST) (32 ºC), the polymer is hydrophilic and above the LCST, it shows a predominantly hydrophobic behavior. In this way, when cells are cultured at the normal culture temperature (37 ºC) they attach to the substrate and proliferate and, after forming a confluent cell sheet, lowering the temperature bellow 32 ºC will allow the contiguous cell

sheet to detach, since PIPAAm rapidly absorbs water and swells.[189] This non-invasive method ensures the preservation of cell-cell junctions and ECM produced by cells, in contrast with the enzymatic treatment (trypsin).[188,189] Additionally, due to the presence of endogenous ECM produced by the cells during *in vitro* culture, the harvested cell sheet can be easily re-attached to other culture dishes, cell sheets as well as host tissues.[189] Moreover, this technology can also allow the control of cell density and orientation by creating micropatterned thermo-responsive surfaces using lithographic techniques.[190]

Stacking or assembling multiple cell sheets composed of tissue-specific cells or MSCs, may lead to the formation of thick tissue constructs, but the lack of sufficient vascularization in constructs with more than 100-200 μm of thickness may induce hypoxia and tissue necrosis.[191] To overcome this limitation, ECs can be co-cultured on multilayered tissue constructs in the form of cell sheets or simply by seeding, to form capillary-like networks (Figure 7).[189] For example, in one study, vascularized cell-dense tissue constructs were fabricated by sandwiching alternatively HUVECs between five myoblast sheets. Three days after culture *in vitro*, the ECs formed partial capillary-like networks (Figure 7, B), and one week after subcutaneous transplantation in nude rats, the formed microvessels on the tissue construct inosculate with the host vasculature (confirmed by the presence of red blood cells), unlike five-layered constructs without ECs.[192] This strategy of stacking of individual cell sheets generated in thermo-responsive dishes has been already applied to repair and regenerate specific tissues, for example the myocardium, liver and cornea.[189,193] In fact, the implantation of pre-vascularized cardiac tissues engineered by the cell sheet technology was demonstrated to enhance neovascularization and improve cardiac function of ischemic rat hearts after implantation.[194]

Although the cell sheet technology based on thermo-responsive dishes has shown promising clinical results, limitations such as high cost, difficult manipulation of the cell sheets, and difficult spatial control of the position of the cells, stimulated the search for an alternative method that allow the formation of cell sheets and stratified tissue constructs based on magnetic forces.[195,196] Recently, Silva *et al.* reported the construction of thick tissues with three layers of adipose-derived stromal cells (ASCs) and HUVECs (ASCs/HUVECs/ASCs) in a fast and cost-effective manner, by incorporating magnetic nanoparticles in the cells.[195] The magnetic force (applied at the bottom of the cell culture plates) allowed cell-cell interaction and the development of cohesive and layered tissues.

The endothelial layer induced osteogenesis and osteogenic differentiation of ASCs and allowed the formation of tubular-like structures in the matrix deposited by cells after 21 days of culture, emphasizing the potential of this tissue for bone regeneration purposes. The *in vivo* angiogenic potential was assessed through a CAM assay, showing that it could stimulate blood vessels recruitment and integration with the host vasculature.

The absence of scaffolds in cell-based approaches favors proximity of the cells in a 3D organization that is fundamental to promote cell-cell interactions, allowing them to produce endogenous ECM and assemble into microtissues that can be used as cell delivery systems for tissue regeneration.[197] Indeed, for this purpose, transplantation of cell sheets into injured tissues have been widely used, but handling fragile cell sheets during surgery and the generation of thick tissues similar to the native ones remain the major challenges of this technology. To improve sheet handling and reduce invasiveness of cell sheet transplantation in damaged lungs, Maeda and colleagues designed a device for endoscopic surgery that allowed transplantation of fibroblast sheets through a small incision.[198] Additionally, the incorporation of ECs in cell sheet engineering seems to be essential for the creation of thicker tissues with improved survival and functionality after transplantation. Transplanted cell aggregates have also demonstrated functional integration with the host tissue and vasculature, and their pre-vascularization proved important to prevent necrosis in the tissue core and promote angiogenesis after implantation. Nonetheless, the full clinical potential of microtissues (mostly developed as multicellular spheroids) still needs further validation.

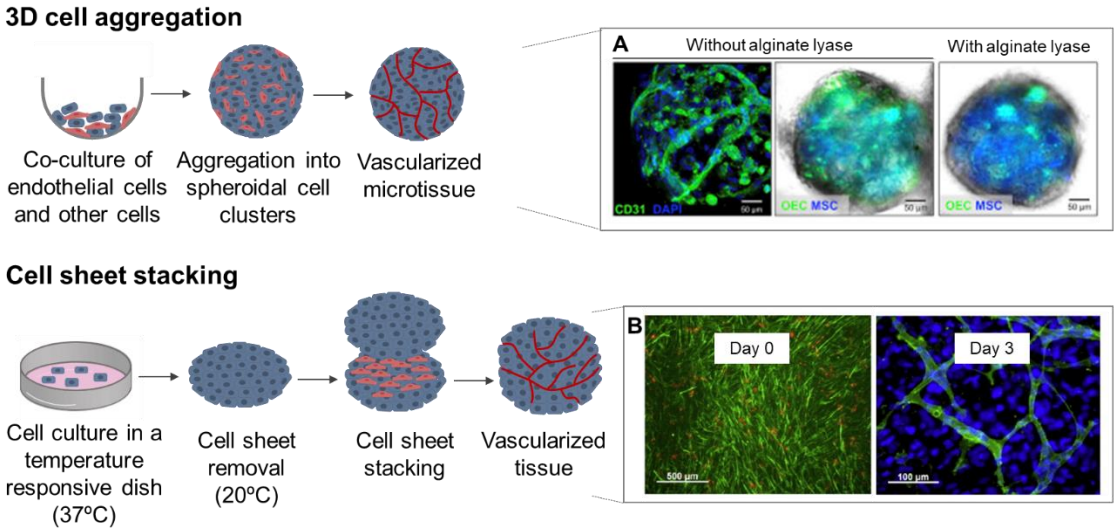

Figure 7. Traditional engineering strategies based on the self-assembly of cells into round-shape 3D microtissues and stacking of cell sheets produced in temperature responsive dishes (cells from

BioRender.com). (A) Cell aggregates of MSCs (blue) and OECs (green) formed in alginate microgels after 14 days, showed that OECs organized into primitive vascular beds forming vascularized microtissues, that remained cohesive upon alginate microgels degradation with alginate lyase. Scale bars: 50 μm.[185] (B) HUVECs sandwiched between two myoblast sheets formed vascular networks after 3 day culture. Day 0 – HUVECs (red) and myoblasts (green), Day 3 - networked ECs stained with anti-human CD31 (green) and nuclei with Hoechst 33342 (blue). Scale bars: 500 μm and 100 μm for day 0 and 3, respectively.[192]

4.3. Perfused channels-based approaches

Here, perfused channels-based approaches are defined as those that rely on the generation of perfusable hollow channels in scaffolds in order to develop engineered vascularized tissue constructs for implantation. Contrary to angiogenic approaches, ECs are not expected to self-organize into tubular structures and form a vascular bed through angiogenesis, since the scaffold itself already has a pre-incorporated tubular network where the ECs can be seeded. There are a large variety of techniques that have been used to generate pre-vascularized tissue constructs with perfusable channels including electrospinning, micro-molding, 3D bioprinting and microfluidics (reviewed elsewhere[116,199,200]). Here, the most commonly used strategies to fabricate hollow perfusable microchannels within scaffolds will be addressed, including templating, photoabsorption-based techniques and layer-by-layer stacking, which are schematically represented in Figure 8.

Templating techniques. Templating techniques can be approached from two different viewpoints. The first one includes the insertion of templates such as needles, rods or other cylindrical structures in a pre-polymer solution followed by mechanical removal of the template after crosslinking the gel.[201] This method leads to the generation of single linear microchannel *in vitro* models, which albeit not mimicking native capillary networks, can be useful to study several vascular functions.[22] Afterwards, ECs may be seeded in the lumen of the created hollow channels. However, the achievement of a continuous monolayer of ECs is challenging. Fukuda's group suggested an alternative method to fabricate endothelialized channels using a templating technique that avoids cell seeding, that is, by pre-assembly a monolayer of HUVECs on template gold rods through the adsorption of an oligopeptide containing an RGD domain. The EC-coated gold rods were embedded in a GelMA solution and, after cross-linking, an electrical potential was applied allowing cells to detach from the rod and be transferred to the hydrogel. Further on, they

created a double layer of cells composed of ECs and fibroblasts, around the gold rods, and proceeded with the same method, creating perfusable channels after rod removal. This method enabled the rapid creation of confluently cell-coated hollow channels inside a hydrogel.[202]

Sacrificial templates initially embedded within scaffold matrices may also be used to generate hollow tubes inside hydrogel molds. Despite being physically removed, those are converted into a liquid or are dissolved in a solvent and flushed out, resulting in the formation of hollow channels. Many materials have been used as sacrificial templates including alginate, gelatin, agarose and carbohydrate glass, in order to form vascular networks through electrospinning, 3D printing or molding techniques.[116,201] Hence, these methods open the possibility to produce branched and interconnected channels that recapitulate more faithfully the complex tree-like pattern of vascular network in tissues, in contrast to the straight channels produced by mechanically removed materials. Beyond the risk of channel collapsing, cytotoxic effects caused by dissolving agents and processes are the major concerns related to the use of sacrificial materials. In an attempt to avoid such problem, Matsusaki *et al.* recently used gellan gum (GG) fibers as sacrificial templates.[203] GG is a natural polysaccharide that form gels in the presence of cationic ions and cooling processes. In this paper, the authors found that GG gels can be completely dissolved with a buffer that is commonly used for cell culture experiments – Tris-HCl (pH=7.4) - and thus, this dissolving process does not show cytotoxicity. Straight vascular tubes were constructed in gelatin gels by adding a gelatin solution around a prefabricated GG fiber gel with 500 μm of diameter, and subsequent immersion in Tris-HCl buffer. Afterwards, fibronectin and HUVECs were injected in the lumen of the tube, and after 24h on rotating culture, cells adhered and homogenously covered the lumen (Figure 8, A). Moreover, the resulting endothelialized channel showed blood compatibility (Figure 8, A) and diffusion properties for small molecules.

Photoabsorption-based techniques. The fabrication of microchannels has been achieved utilizing pulsed laser beams to degrade predetermined regions of photocrosslinked hydrogels. When the hydrogel is exposed to pulsed laser irradiation, their electrons absorb photons and became excited leading to hydrogel degradation through various mechanisms (reviewed elsewhere[204]). The degradation mechanism depends on laser properties including frequency (pulse duration) and intensity, and on hydrogel

composition.[204] The hollow channels can be then seeded with ECs by microfluidic perfusion allowing the formation of an endothelium layer in the lumen.[205] High-resolution, spatiotemporal control, speed and possibility to program the formation of more complex and biomimetic channel networks based on a digital template of a native hierarchic microvasculature (Figure 8, B), are the major advantages of this method.[116,204,205] Additionally, it opens the possibility to produce channels with various intraluminal architectures that influences cellular behavior and morphology (Figure 8, B).[206]

A recent innovative strategy based on biocompatible photo-absorbers was described to produce perfusable microvascular networks with complex architectures within photo-crosslinked hydrogels.[207] A natural food dye additive – tartrazine – was used as a photo-absorber for a custom-designed projection stereolithography apparatus. This technology enabled the fabrication of poly(ethylene glycol) diacrylate hydrogels containing channels with intraluminal topologies, including functional valves that mimic the ones found in native venous vessels. Moreover, a bioinspired alveolar model with efficient intervascular oxygen transport was created, as well as a hepatic vascularized tissue construct with a microfluidic vascular compartment seeded with HUVECs. Once this liver tissue was implanted in a rat model with chronic liver injury for 14 days, histological analysis revealed it integration with host vasculature.

Layer-by-layer stacking. In this technique, microfluidic channels are created through the assembly of layers (hydrogels or polymeric scaffolds) prefabricated by lithographic or micromolding processes. The layers have specific micropatterns that, when aligned together and sealed mechanically (pressure) or through cross-linking, allow the formation of 3D interconnected perfusable channels with pre-defined structures and geometries.[22] Zhang and co-workers reported in 2016 the development of a microfluidic scaffold that they referred to as “AngioChip”, composed by a perfusable 3D microchannel network produced by a stamping technique, and a matrix of parenchymal cells.[208,209] The stamping technique consisted in the alignment and assembly of thin micropatterned poly-(octamethylene maleate (anhydride) citrate) layers onto each other and their bonding by photocrosslinking. HUVECs were seeded in the developed hierarchical vascular network, and micro-holes (10/20 μm) and nano-pores were patterned and incorporated in the walls, allowing effective molecular exchange, and EC sprouting to the parenchymal space in response to angiogenic stimuli. The presence of pores also enhanced the permeability to

large and small molecules through the microchannel walls, allowing ECs to be the dominant transport resistance, as it happens *in vivo*. Additionally, the EC lining was shown to be functional in the presence of an inflammatory stimuli, allowing perfused monocytes to adhere and migrate through the vessel wall to the parenchymal space. Finally, vascularized hepatic and cardiac tissue models were developed, and the configuration of the "AngioChip" allowed for surgical anastomoses *in vivo* by the connection of the inlet and outlet with femoral vessels, showing immediate perfusion. The same team reported a versatile platform to culture microtissues by fabricating vascular micro-channels with 15 μm micro-holes using the 3D stamping technique (Figure 8, C).[210] The platform allowed the recapitulation of a metabolically active liver, a functional cardiac muscle, and a metastatic solid tumor by encapsulating human hepatocytes (HepG2), cardiomyocytes and breast cancer cells (MDA-MB-231) in fibrin gels in the parenchymal space around the channel network, respectively (Figure 8, C). This technique enabled the construction of 3D branching networks, and the use of a biodegradable polymer allowed to circumvent the major limitations of classical microfluidic organ-on-a-chip technology, normally comprising 2D cell culture configuration and the use of PDMS as scaffold.

Overall, the formation of perfusable channels in scaffolds is an interesting way to create stable vascular networks. However, the successful implementation of this method is dependent on the scaffolds' intrinsic properties. For example, those must show adequate mechanical features to sustain perfusion in large constructs, avoid channel collapse, and enable functional anastomosis with host vasculature. In addition, the origin and composition of the scaffolding material play a crucial role in promoting channel endothelization, since EC adhesion, proliferation and self-organization – necessary for the formation of a high-quality and functional endothelium - are dependent on the biophysical and chemical cues provided by the biomaterials.

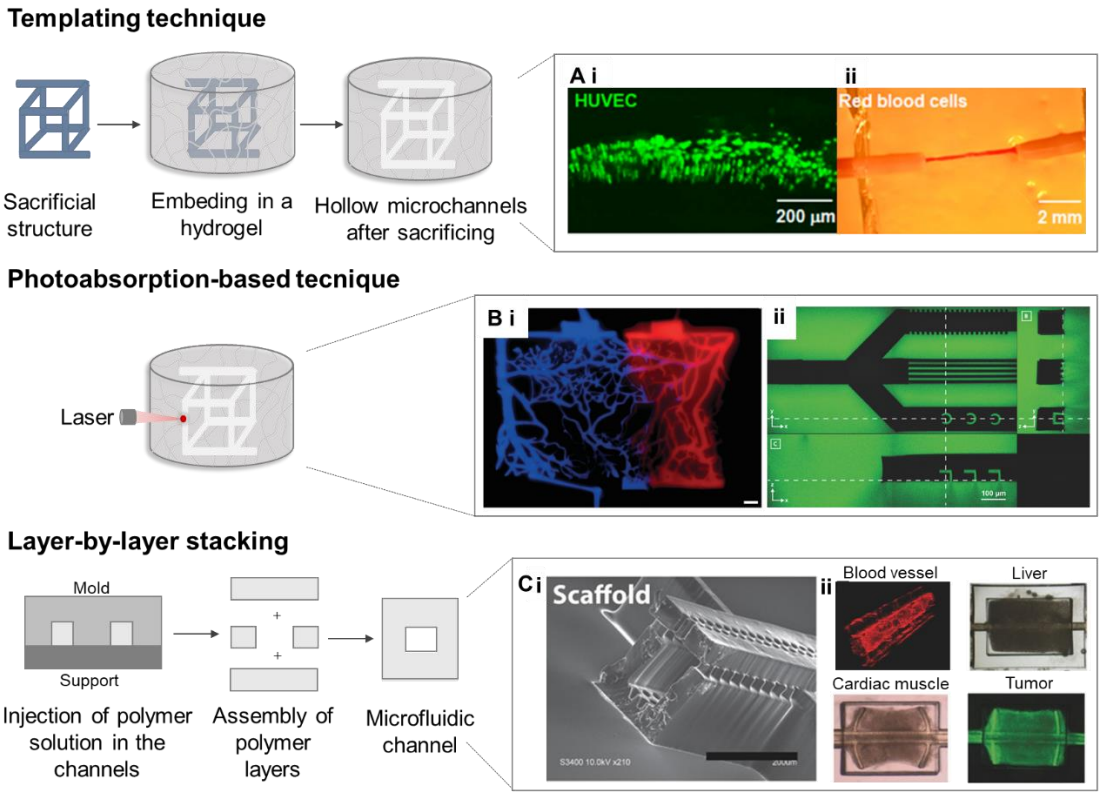

Figure 8. Strategies based on the creation of interconnected hollow channels in a scaffold and subsquent seeding of ECs to mimic vascular networks *in vitro*. Those may comprise templanting techniques using sacrificial materials, photoabsorption-based and layer-by-layer stacking techniques. The first one is based on the dissolution of a template materials within hydrogel matrices, the second on the Degradation of pre-determined regions of photo-crosslinked hydrogels using a laser beam, and the third one on the assembly of patterned scaffolds. (A) Perfusable channels produced after dissolving gelan gum in gelatin gels allowed HUVECs adherence after 24h of incubation (i), and perfusion with whole blood (ii).[203] (B) Channel networks produced using the ablative properties of focalized nano- or femto-pulsed lasers in poly(ethylene glycol) hydrogels. This technique allow the formation of channel networks with high complexity and biomimicry by using a capillary bed photograph (i). Scale bar: 100 µm.[205] (i) Microfabrication of intraluminal channel architectures within hydrogel biomaterials using laser beams. Scale bar: 100 µm.[206] (C) Method of stacking biomaterial layers with a predefined pattern. (C) A microfluidic channel scaffold with micro-holes on the side channel walls that represent the vascular space of tissue models (i). Scale bar: 200 µm. This microfluidic platform allowed the fabrication of blood vessel, liver, cardiac muscle and breast tumor models by incorporating parenchymal cells in fibrin gels that surrounded the vascular channels (ii).[210]

4.4. Versatile approaches to produce artificial free-standing vascular microtubes

Instead of constructing *in vitro* tissues with vascular networks, strategies focused on the development of tubular microstructures intend to recapitulate the properties of micro-sized blood vessels in self-sustained structures. For example, Savoji *et al.* used the FRESH technology to print different photocrosslinkable bioelastomer prepolymers within a

Carbopol hydrogel support bath, generating tubular structures. To attain a hollow core, a coaxial nozzle with a core flow of Carbopol gel was used. This allowed the formation of intact tubes by the removing of the soluble core gel along with the support bath with phosphate buffered saline solution, after the UV cross-linking of the bioelastomer. A porogen was added to induce porosity in the wall of the microtubes, conferring them with semi-permeability to oxygen and nutrient exchanges. The final porous microtubes with approximately 500 μm diameter and 100-200 μm wall thickness were assembled on a custom-made 96-well plate, developed for organ-on-a-chip applications, and were perfused with endothelial cells forming a vascular network that supported the formation of cardiac microtissues.[211] Another study reports the fabrication of PDMS tubes (inner diameter = 500 μm) using a templating technique. Those were used for modeling the sprouting angiogenic process.[212] For that purpose, tubes were perforated through laser ablation (pore size of 250 μm) and a suspension of HUVECs was injected in the fibronectin-coated lumen, forming a confluent layer. When two tubes were encapsulated in an ECM mimetic hydrogel, endothelial invasion through the pores was observed as well as microvascular network formation between the two tubes, under static and dynamic conditions. This platform also enabled the simulation of tumor angiogenesis using spheroids composed of MCF-7 breast cancer cells.

The dynamic conformation of proteins and their ability to interact with different molecules in self-assembly processes has been used to fabricate tubular materials. Recombinantly produced elastin-like polypeptides (ELRs), with temperature-dependent molecular conformation, were combined with peptide amphiphiles (PAs)[213] and graphene oxide (GO)[214] to assemble into multilayered membranes through a diffusion-reaction mechanism. Those membranes could be arranged into tubular structures in a spatiotemporal controlled manner, by their adherence to a surface and subsequent opening when a droplet of an aqueous solution of a PA was immersed in a large volume of an ELR aqueous solution. These tubes could be branched by simply touching and displacing the membrane, and could support endothelial cell growth.[213] More recently, in order to produce membranes and tubes with enhanced mechanical properties, GO was applied, instead of PAs. Similarly to ELR-PA assembly, the system could be modulated to grow into tubular structures, with an internal diameter of approximately 50 μm. Using this system, fluidic devices were generated using an extrusion-based printer, with varying

dimensions and bifurcations, capable of withstanding flow of up to 12.5 mL/min. In addition, it was shown to be a cytocompatible and bioactive system that allowed HUVECs growth within the membrane and in the lumen, forming an intact endothelial monolayer.[214]

Free-standing microtubular structures that can support EC growth and proliferation as well as fluid flow may be used in organ-on-a-chip applications which are becoming promising approaches for drug testing and implantation purposes. Organs-on-a-chip are microfluidic cell culture systems with controlled and dynamic environments that recapitulate physiological properties of human tissues.[215] The inclusion of these vascular tubular structures in such systems may allow direct anastomosis with the host vasculature after implantation through microsurgical procedures, by connecting the inlet and outlet with host's blood vessels, as Zhang and co-workers had demonstrated.[208] This method allows immediate perfusion of the tissue construct; however, biomimetic engineered microvessels must be sufficiently mechanically robust to allow handling and suturing during surgery. Modeling angiogenesis can be another application for these self-sustained tubular structures. These platforms may target studies in molecular biology or in screening of anti- or pro-angiogenic drugs for the treatment of vascular diseases or cancer.[216]

5. Clinical outlook and key challenges

To address early graft failure associated to thrombotic events, one major clinical advance relied on the coating synthetic grafts' surface with biological components, with the goal of reducing platelet adhesion and subsequent obstruction. Several heparin-coated synthetic grafts have been tested in clinical trials[217,218], for example the product FUSION BIOLINE (MAQUET Cardiovascular LLC, NJ, U.S.A.) was implanted in patients with peripheral artery disease, showing higher patency rates than uncoated standard ePTFE grafts.[219] Other 'of-the-shelf' vascular grafts produced by the company Humacyte Inc. are currently undergoing clinical trials, with promising results in terms of patency rates.[220] The latter TEBVs are derived from seeding autologous vascular cells onto biodegradable synthetic PGA scaffolds, maturation in perfusion bioreactors and decellularization prior to implantation. Table 1 showcases some recent clinical studies using TEBVs.

Although significant advances towards the clinical application of tissue engineered vascular grafts have been made over the past few decades, there are still some aspects that need to be addressed to prevent graft failure and improve clinical translation. The selection

of materials and cells is of extreme importance and may dictate the performance of the vascular graft after implantation. Development of non-immunogenic materials with appropriate biochemical and mechanical properties that resemble native ECM is of great interest for improved remodeling and lumen endothelization to prevent thrombosis. Moreover, advances in cell culture systems can bring opportunities for the development of patient-specific grafts. For example, hiPSCs are becoming promising clinical alternatives to autologous vascular cells, due to their differentiation ability into vascular lineages. Therefore, attention from the scientific community has been devoted to the development of high-yield differentiation protocols. Recently developed hiPSCs-TEBVs demonstrated promising functionality after implantation in pre-clinical models.[221] On the other hand, biomaterial-based technological advances may also have an important role in addressing major complications in graft implantation. For example, 3D printing and imaging technologies may enable the fabrication of personalized vessel substitutes with dimensional resemblance at the anastomotic site, which could prevent intimal hyperplasia.[109] However, the requirement of specialized equipment, cost and regulatory hurdles may compromise the clinical translation of such technologies, making the idea of having readily available vascular grafts from simple decellularization or coating procedures more clinically attainable in the short term.

At the microvascular level, clinical applications for the treatment of ischemic diseases unable to be treated by replacing or bypassing macrovessels most comprise cell delivery[222], the direct administration of growth factors[223], and gene therapy[224]. However, despite their potential, there are still some challenges to overcome. One major limitation reported for the direct administration of angiogenic molecules via injection consist on the insufficient stabilization of the formed vessels, leading to the formation of leaky and transient structures.[13] On the other hand, intravenous stem and progenitor cells injection may lead to their recruitment to other neoangiogenic sites, possibly causing adverse side effects that may include tumorigenesis and atherosclerosis.[12] Innovative approaches comprising the transplantation of microvascular structures derived from the digestion and filtration of adipose tissue, have been recently suggested as promising strategies to induce rapid and effective blood perfusion of ischemic cardiac tissue, upon co-transplantation with hiPSC-derived cardiomyocytes in infarcted rat hearts.[225] The use of easy-to-retrieve and easily accessible tissues, such as adipose tissue, may enable the high-yield and patient-

compliant isolation of autologous structures. In fact, the use of autologous biological material in cell- and/or tissue-delivery approaches enables transplantation without the need for further life-long intake of immunosuppressants, needed to attenuate adaptive immune response-mediated graft rejection observed in allogeneic and xenogeneic transplantation procedures, and correlated with patients' susceptibility to infection, and development of comorbidities.[226] Nonetheless, the transplantation of organ-like structures has been reported to activate the innate immune system due to metabolic and thermal changes that occur during and after transplantation procedures.[227] Such phenomena may pose further challenges in the effective clinical translation of these therapies, requiring surgical optimization. Regarding gene therapy, the efficiency of the transfection and transfer methods and the achievement of long-term gene expression remain major challenges.[7,224] Thus, despite pre-clinical and clinical trials showing the partial effectiveness of these approaches for the treatment of ischemic diseases, further research is still needed to define clear clinical procedures.[10]

Few tissue engineering strategies that use biomaterials, either combined or not with cells and/or growth factors, or scaffold-free structures have been tested in clinical trials. For example, the delivery of an hydrogel derived from porcine decellularized myocardial ECM - VentriGel (Ventrix, Inc., San Diego, California) – was recently shown to be a new potential and safety treatment for patients with post-myocardial infarction through transendocardial injections during 3 to 35.5 months.[228] Table 1 comprises recent clinical studies for the treatment of ischemic tissues based on tissue engineering approaches. Nevertheless, clinical translation of engineered constructs is still in its infancy. Development of biomimetic tissue-specific constructs with functional complexity and vascular structures is important to improve integration with the host's tissue after implantation. While some metabolic active tissues do not present characteristic vascular geometries, highly organized and aligned vascular structures are found in heart and skeletal muscle tissues, so mimicking such geometries with topographical features on scaffolds may be important. Additionally, technological advances in high-resolution techniques may help in the development of vascular architectures based on patient-derived imaging digital templates, which represents an important step towards personalized medicine. However, scale-up difficulties and cost constitute the major translational barriers of these technologies. On the other hand, advances in microfluidic technologies had allowed the

fabrication of biomimetic free-standing microvessels which when combined with a fibrin gel and cardiac cells, promoted both cardiac function and neovascularization, and also suppressed inflammation in large animal myocardial infarction models, representing an innovative potential therapy.[229,230]

The choice of cells may also influence the performance after implantation. Different EC phenotypes are present in different tissues, and this should be considered so as the generated endothelium had proper barrier functions according to tissue-specific functions. Aiming at the generation of tissue-specific ECs, the development of cell culture differentiation protocols from hiPSCs has been explored.[231,232] However, a major challenge of using specific cells differentiated from hiPSCs is that they are usually immature in their gene expression profiles and functional phenotype, and therefore their ability to integrate functional vessels requires further assessement.[215]

Finally, since the general objective of engineering vascularized tissues as well macrovessel substitutes for implantation is to integrate with the host and remodel, it would be interesting to apply lessons from each other approaches. For example, in scaffolds with pre-incorporated vascular microchannels, besides seeding with ECs, coating or functionalizing the surface of channels with biological cues would allow prevention of thrombosis. For its part, taking the knowledge that topographical features directly influence cell behavior, it would be interesting to add patterns that resemble native orientation of vascular cells in large blood vessels on vascular grafts in order to improve vascular remodeling.[233] This can allow for example SMCs proper aligning and functionality, improving contractile and dilation properties of the vascular graft after implantation.[233]

Table 1. Recently completed and ongoing clinical studies using TEBVs and tissue engineering clinical studies on the treatment of ischemic diseases.

Approach	Tissue engineered construct	Condition being treated	Number of subjects	Duration	Related patency rates/outcomes	Major complications	Refs
Macrovasculature							
Scaffold based	FUSION BIOLINE (Maquet Cardiovascular)	Peripheral artery disease	207	Start: 2010 End: 2013	6-month primary patency of 86.4% compared to 70% of standard ePTFE grafts	Graft occlusion due to thrombosis	219
	Human acellular vascular graft (Humacyte Inc.)	End-stage renal disease	60	Start: 2012 Estimated to end: 2026	12-month primary patency of 86.4% compared with 79.9% of standard ePTFE grafts	Graft occlusions and stenosis	220
	ePTFE graft coated with autologous adipose-derived stem cells	Lower limb ischemia	60	Start: 2011 End: 2020	-	-	-
	Decellularized allogeneic veins with autologous blood components (Verigraft AB)	Chronic venous insufficiency	15	Start: 2020 Estimated to end: 2021	-	-	-
Microvasculature							
Scaffold-based	VentriGel (Ventrix Inc.)	Early and late myocardial infarction	15	Start: 2014 End: 2019	Improvements in exercise capacity and left ventricular remodeling	Cardiogenic shock and intracardiac thrombus	228
	Human umbilical cord–derived mesenchymal stromal cells in collagen scaffolds	Chronic ischemic cardiomyopathy	45	Start: 2015 End: 2019	Improved cardiac function and no signs of immune rejection	Heart failure	234
	Human embryonic stem cell-derived progenitors in a fibrin patch	Ischemic heart disease	10	Start: 2013 End: 2018	Increased systolic motion	Heart failure and silent alloimmunization	235
	Bone marrow-derived autologous hematopoietic stem cells concentrate and platelet-rich plasma gel	Critical limb ischemia; Peripheral artery disease	18	Start: 2011 End: 2016	-	-	-
Scaffold-free	Autologous skeletal stem-cell sheets	Ischemic and dilated cardiomyopathy	27	Start: 2010 End: 2015	Improvements in exercise capacity and recovery of patients symptoms	Arrythmia and heart failure	236

6. Conclusions and Future Perspectives

For the last three decades, efforts to generate both large- and small-diameter macrovascular substitutes have been made in the tissue engineering field to replace or bypass injured blood vessels, in order to circumvent the limitations of the current medical procedures that normally use autogenous or synthetic vascular grafts. Biodegradable and biomimetic materials have been used as scaffolds for this purpose, and even scaffold-free approaches are considered promising strategies; however, those require prolonged culture periods to form easy-to-handle sheets, which hamper their immediate clinical use. Additionally, a more clinically translatable method uses the body environment to produce vascular grafts; however, this approach is highly invasive due to the number of surgeries needed. On the other hand, vascular tissue engineering strategies have been also focused on the development of vascularized tissue constructs, envisioning the treatment of ischemic or damaged tissues. To generate them, stimulation of angiogenesis in scaffolds has been shown to be a promising strategy to promote the formation of capillary-like networks, through biochemical and biophysical cues, or by implanting the scaffold *in vivo*; nonetheless, mature blood vessels may take long periods to be formed, and their stability is not always ideal. As a solution to this problem, perfusable hollow channels can be formed in scaffolds using different techniques, allowing the formation of interconnected vascular networks in a more spatio-temporal controlled manner. The major limitation of most common vascular channels is the lack of dimensional biomimicry of the typical diameter of capillaries (5-10 μm). As such, it is critical to generate appropriate vascular structures at that scale, and photodegradation methods may offer that possibility. However, those are limited by the dependency on photopolymerizable materials, 3D spatial penetration of the laser in thicker materials, and possible cytotoxic effects of high laser intensity and long exposure times. Table 2 summarizes a critical discussion targeted on the methods to produce vascular grafts and vascularized *in vitro* tissues.

From an applicational standpoint, many clinical studies have been conducted using engineered macro-scale vascular grafts. However, *in vitro* tissue vascularization is still mostly limited to an academic perspective due to technical, functional and commercialization difficulties. In fact, the transplantation of pre-vascularized engineered tissues in animal models has given strong evidence of their therapeutic potential to treat damaged and ischemic tissues, and the presence of vascular networks is undoubtedly

essential for their survival and integration with the host tissue. The potential of the pre-vascularization of tissues has not only been explored for the treatment of ischemia, but has also shown promising results in the field of pancreatic islets transplantation.[237] Sefton and coworkers established a pre-vascularized islet model, based on HUVECs, to enable their transplantation and effective function, survival and integration into the animals' vasculature in the low vascularized subcutaneous region. On the other hand, technological advances have offered great opportunities for organ-on-a-chip applications to recreate tissues/organs and their functions in a physiological context, or to model vascular pathologies or other diseases to study fundamental molecular mechanisms resulting from the disease or to discover new therapeutics. Moreover, over the last decade, the introduction of new simplistic yet complex human organ analogs - organoids - formed by the self-organization of stem or adult progenitor cells, have gained the attention of many scientists. Organoids have offered hope as a new strategy for several biomedical applications, including drug discovery, regenerative medicine, and especially personalized medicine. The major limitation of these models is related to the formation of a necrotic core upon the achievement of organoids with relevant sizes, as a consequence of limited oxygen and nutrient exchanges in the inner core. This phenomenon, associated with the lack of vascularization, has hampered the long-term application of these structures, leading to the search for strategies to create a new generation of organoids with proper perfusion.[238] Incorporation of ECs into organoids have shown to be essential for microvascular development and anastomosis with animal host tissues.[239] However, achieving functional organoid complexity and vascularization *in vitro* is still a major challenge, since it would require a balanced environment able to support both tissue/organoid growth and angiogenesis.[238] Additional challenges in the development of effectively vascularized organoids have been raised, including the ability of such in vitro-induced structures to effectively adapt and respond to environmental changes (e.g., oxygen consumption patterns, tissue growth), and to effectively mimic the physiological active vasculature.[238] The degree of complexity necessary in organoids to achieve close-to-native tissues is unclear. While these structures should mimic the function of native healthy (or diseased) vascularized tissues, a balance must be met between processing feasibility (controlled by the complexity of coculture systems and fabrication techniques) and the achievement of physiological-like functions.

In summary, significant advances have been made in order to find new solutions for emerging problems in the vascular tissue engineering field, overall envisioning the improvement of human health and clinical practice. The development of more functional and biomimetic vascular engineered conduits and tissue constructs is dependent on a good understanding on the biology and physiology of native blood vessels, and all the environmental cues and interactions needed for their formation and maturation. Thus, efforts in the development of more effective biomaterials and fabrication techniques, as well as understanding of the most promising cell culture systems must continue in order to develop high-quality vasculature at its different hierarchical levels.

Table 2. The major advantages and disadvantages of the mentioned approaches for the fabrication of vascular grafts (macrovasculature) and vascularized tissues (microvasculature).

	Approach	Advantages	Disadvantages
Macrovasculature	Scaffold-based		
	(Biodegradable) Synthetic origin	- Good mechanical properties - Tissue remodeling capacity	- Lack of bioactivity and cell binding sites - Thrombogenic potential
	Natural origin	- Tissue remodeling capacity - Promotion of cell adhesion and proliferation	- Poor mechanical properties - Difficult manipulation for anastomotic suture
	Biological origin (decellularized)	- Preservation of native vessel's ECM architecture - Biomimetic mechanical properties - Reduced immunogenicity and antigenicity	- Limited availability - Glutaraldehyde treatment of animal blood vessels (toxic)
	Scaffold-free	- Possibility to fabricate autologous grafts - Structural similarity to native vascular tissue - Completely biological ECM	- Difficult manipulation of cell sheets - Prolonged maturation time - Not readily available (autologous)
	Fibrotic capsule-based	- Completely autologous grafts - Non-immunogenic - Non-toxic	- Prolonged incubation time in the body - High invasive method
Microvasculat ---	Angiogenesis-based	- Allows cell-cell and cell-ECM interactions - Formation of mimetic capillary networks	- Prolonged fabrication time - Difficult spatial organization of blood vessels throughout thicker tissues - Possible generation of leaky/immature microvasculature

Cell-based		
3D cell aggregation	- Cell-cell interactions that mimic better *in vivo* conditions - Cellular interactions favor growth factor secretion - The presence of ECs can prevent necrotic core formation	- Prolonged culture time - Leaky microvasculature
Cell sheet stacking	- Completely biological ECM - Preserved cell-cell and cell-ECM interactions	- Prolonged fabrication time - Difficult manipulation of the cell sheets
Perfused channels-based		
Sacrificial templating	- Easy to perfuse - Channel dimensions control	- Possible channel collapse - Toxicity of dissolving/chelating agents - Multi-step method
Photoabsorbation-based	- Complex and biomimetic designs - High-resolution - Spatio-temporal control	- Dependent on photocross-linkable polymers - 3D spatial limitation of laser penetration - Possible cytotoxicity
Layer-by-layer stacking	- Easy to perfuse - Customizable design - Tailored dimensions	- Specialized fabrication techniques - Multi-step method

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I.3. Understanding the Potential and Current Limitations of Aqueous Two-Phase Systems as Systems for Cell-based Strategies

Subchapter I.3.

This subchapter is based on the Review paper:
"Understanding the Potential and Current Limitations of Aqueous Two-Phase Systems as Systems for Cell-Based Strategies"
(Manuscript in preparation)

Understanding the Potential and Current Limitations of Aqueous Two-Phase Systems as Systems for Cell-Based Strategies

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1. Introduction to aqueous two-phase systems

Aqueous two-phase systems (ATPSs), also known as aqueous biphasic systems, are characterized by the presence of two water-rich immiscible phases that are formed when two incompatible components (e.g. polymers and salts) are mixed above critical concentrations, resulting in liquid-liquid phase separation.[1] At equilibrium, each of the phases, frequently represented as top and bottom phases, is enriched in one of the components (segregative system), or both components are present in one of the phases (attractive system).[2] In a thermodynamic perspective, the phenomenon of phase separation in ATPSs occurs when the enthalpic contribution of the system overcomes the entropic driving force for mixing.[1,3,4]

Many combinations of hydrophilic components have been used to generate ATPSs, including most commonly polymer/polymer and polymer/salt. Dextran and polyethylene glycol (PEG) are examples of incompatible polymers, that when mixed in water-based solvents above critical concentrations, spontaneously separates into a dextran-rich phase (bottom) and PEG-rich phase (top). These systems as well as polymer/salt systems have been extensively studied for more than six decades since the Albertsson's first works in 1950's[5], having demonstrating high potential in areas of bioseparation and biotechnological processing, as well as, more recently, in the biomedicine and tissue engineering fields. Nevertheless, in order to extend the range of applications of ATPSs, several alternative biphasic systems containing ionic liquids, alcohols, polysaccharides or surfactants have been proposed.[1]

To determine the potential range of concentrations of phase-forming components necessary to generate an ATPS, a phase diagram must be created for a set of conditions such as temperature, pH, and salt concentration. In general, ATPSs are ternary systems composed of water and two components; however, phase diagrams are typically denoted in more simplistic two-axis orthogonal representations where the concentration of water is absent (Figure 1).[1] The concentration of the bottom phase component is represented on the horizontal axis (abscissa), while the top phase concentration is plotted on the ordinate. All the mixtures above the binodal curve undergo liquid-liquid phase separation resulting in a two-phase system, while those at or below the curve give a homogenous monophasic system.[2,6] When applying ATPSs it is important to understand that systems near critical conditions, such as the critical point - where the composition and volume of the two phases are identical - and points along the binodal curve, are more unstable and sensitive to environmental variations.[4] The lines that connect two points on the binodal named tie-lines are determined to give the total composition and volume ratio of the phases in equilibrium conditions, and the end points (or nodes) of the tie-lines represent the final composition of the two phases after complete separation.[1,2,6]

The determination of the binodal curve has been achieved using traditional titration methods such as turbidimetric and the cloud-point methods, or by determining the tie-line's nodes for several systems. Titration methods rely on the visual measurement of the turbidity of the mixture, which is indicative of phases immiscibility. After phase separation has occurred, two clear and transparent bulk phases separated by a visible and well-defined interface are formed.[6]

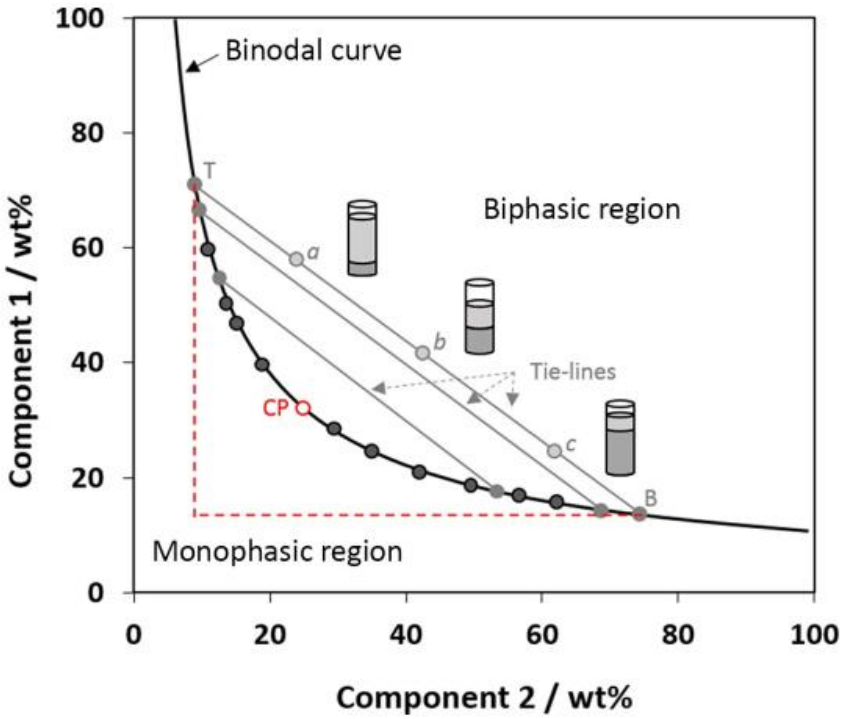

Figure 1. Orthogonal phase diagram for a hypothetical system composed of component 1, component 2 and water (in weight/weight %), with the respective binodal curve, tie-lines and critical point (CP). Above the binodal curve the formed system has two phases, and below it one-phase systems are generated. On the same tie-line with the end points T and B that represent the final composition of the top and bottom phases, respectively, three mixtures (*a*, *b*, and *c*) with different total composition and volume ratios can be observed.[1]

2. Factors influencing ATPS formation and applications

The phase separation process and thermodynamic equilibrium of ATPS are strongly influenced not only by temperature and pH but also by osmolarity and other physicochemical properties of the phases that depend on the chemical nature of the phase-forming components such as viscosity and density. In the biomedical field, various studies have taken advantage of this dependence on external stimuli and physicochemical properties of the phases in order to control the formation of all-aqueous structures. These structures are formed when one phase is dispersed in the other, typically in a jetting or dripping mode[7], allowing the formation of emulsion-like jets or droplets delineated by the interface of the ATPS. Those may then serve as templates for advanced materials fabrication such as hydrogels, capsules or particles, as bioinspired cellular models, as micro-bioreactors for (bio)chemical reactions, or may be used for cell patterning and encapsulation purposes.[4,7,8]

Here, the effect of temperature, pH, osmolarity, viscosity and density on ATPS formation and stabilization will be addressed, and examples of how those factors directly or indirectly influence the applicability of the system will be given.

Temperature. The generation of two-phase systems is strongly influenced by temperature. It has been noticed that, in general, lower temperatures favors phase separation of PEG/dextran systems, which can be visually observed through a shift of the binodal curve to lower polymer concentrations, thus increasing the biphasic region.[1,9] This effect has been exploited as a means to control micro-compartmentalization inside cell inspired structures.[10,11] In the interior space of living cells, small membraneless compartments consisting of droplets of biomolecule condensates, are believed to be generated from a phase separation phenomena, similar to that of ATPSs.[12] In a way to mimic compartmentalization of living cells, Long *et al.* encapsulated PEG/dextran ATPSs in giant lipid bilayer vesicles[11], and were able to control protein compartmentalization by inducing phase separation through a cooling process.[10] At higher temperatures (50ºC) protein was uniformly distributed within the lipidic vesicle due to single phase composition of the system. With decreasing temperature, two phases were generated leading to protein encapsulation in a dextran-rich compartment.

Control over temperature can also be explored as a solidification strategy for the fabrication of microparticles and capsules using polymers with a sol-gel transition behavior, such as collagen and agarose. For example, by loading collagen in the dextran phase of a PEG/dextran emulsion droplet system, collagen microparticles can be successfully fabricated through thermal gelation by heating at 37ºC for 12 hours.[13] On the other hand, temperature also strongly affects phase behavior in systems composed of PEG and temperature-responsive polymer gels such as gelatin. When gelatin is mixed with a PEG solution at 60ºC, the homogenous mixture separates into two distinct phases with a gradual decrease in temperature to 15ºC. During the cooling time, the mixture become opaque indicating phase immiscibility and, within few minutes, phase separation occurs at both above and below the gelation temperature of gelatin.[14] This behavior is dependent on the concentration of PEG and gelatin, molecular weight of PEG, and also on the gelatin type (A or B).[14,15] The use of PEG with higher molecular weight decreased the concentration of gelatin required for phase separation, and ATPSs made of gelatin type B

have a larger biphasic region than of gelatin type A.[15] The mechanism of phase separation is believed to be driven by excluded volume effect caused by macromolecular crowding, resulting in a denser bottom phase enriched with gelatin molecules and a PEG-rich top phase, when concentrations of both components achieves critical conditions.[15] These systems have been used for the fabrication of gelatin microparticles by rapid cooling and crosslinking of dispersed gelatin droplets in a continuous PEG phase, with potential applications as delivery and drug release systems.[8,15] Moreover, gelatin capsules have been generated in all-aqueous double emulsions generated from a quaternary system composed of water/PEG/gelatin/alginate.[16] By controlling the temperature, mononuclear droplets with a PEG core and a gelatin shell were formed in a continuous PEG solution, upon heating above the gelation temperature of gelatin. This approach allowed the formation of liquified hollow capsules that could uptake payloads showing potential in areas of synthetic biology as protocells or bioreactors, as well as microencapsulation and drug delivery.[16] Also, ATPSs composed of photo-crosslinkable methacrylated gelatin (GelMA) and PEG or poly(ethylene oxide) (PEO) have been proposed for the fabrication of porous hydrogel sponges.[17,18] After mixing GelMA and PEO aqueous solutions, phase separation occurs after 30 min, leading to the formation of PEO emulsion droplets dispersed within a GelMA continuous phase. The continuous phase is photocrosslinked under UV light, and after immersion in PBS for 24h, the PEO droplets are removed forming a highly porous GelMA hydrogel, whose uniformity and pore size can be easily controlled by changing the phases' volume ratio.[17]

Another type of ATPS whose formation depends on temperature is composed of one polymer only in water. Those are typically random copolymers of ethylene oxide (EO) and propylene oxide (PO) that have temperature-responsive properties. Above their lower critical solution temperature (LCST), polymer conformation changes and it solubility decreases leading to phase separation into a polymer-rich bottom phase and an upper phase composed mostly of water.[19,20] Among the EO-PO-based thermo-separating polymers, Pluronic F127 (LCST around 37ºC) has been widely used in biomedical applications. It can be combined with dextran to produce biphasic systems that may serve as templates for the fabrication of temperature-responsive microparticles at mild conditions.[21] Phase diagram of Pluronic F127/dextran systems is influenced by the temperature in a way that

lower polymer concentrations are required for two-phase formation at higher temperatures, contrary to PEG/dextran systems.

pH. The effect of the pH on phase diagrams of salt-based systems has been well stablished demonstrating that an increase of the pH increases biphasic region, therefore allowing phase separation to occur at lower salt concentrations. In polymer/polymer systems the study of this effect has not been greatly reported.[1] However, several studies have been identifying a great influence of pH in the partition behavior of solutes including proteins, monoclonal antibodies, nucleic acids, and polyelectrolytes in the polymer-rich phases.[22] Partitioning of solutes between the phases depends not only on the properties of the ATPS but also on the physicochemical properties of the solutes including their electrochemical charge, size, biospecific affinity, conformation and/or surface hydrophobicity.[23] The charge and surface properties of the solutes may change with the ATPS's pH.[22] In the case of polyelectrolytes, since they are polymers with charged ionic groups that dissociate in aqueous solutions, their electrochemical charge can be easily altered through the system pH. They are classified as strong polyelectrolytes if they remain fully charged in solution independently of pH and ionic strength, or weak polyelectrolytes if their degree of ionization is affected by those chemical factors.[24] Recently, Ma *et al.* have controlled the partitioning of oppositely charged strong polyelectrolytes (poly(allylamine hydrochloride) (positive) and poly(sodium-4- styrene sulfonate) (negative)) by changing the pH of a PEG/dextran system.[25] They observed that increasing pH values induced preference of the polyelectrolytes towards the dextran-rich phase explained by an increase in hydrogen bonds between them and the hydroxyl groups of dextran, while decreasing from pH 7 to 5, increase their partitioning to the PEG-rich phase. Due to this pH-induced partitioning behavior, they were able to generate microgel particles and microcapsules through electrospray, by increasing or decreasing the system's pH, respectively. Moreover, the speed of formation of the structures as well as the wall thickness of capsules were also found to be pH dependent, in a way that capsules are formed more rapidly, and their thickness increases for lower pH values.

Osmolarity. Another property that influences ATPS formation is the osmotic pressure, which can be modulated through the concentration of phase-forming components

or by adding salts or sugars to the system.[4] At equilibrium, the two aqueous phases have the same osmotic pressure. In contrast, if the solutes are not equilibrated in terms of concentration in the ATPS, an osmotic gradient is temporarily created across the aqueous interface, driving the movement of water from one phase to the other.[8] Water movement across the interface driven by osmotic gradients has been used as a strategy for the creation of multi-compartmentalized structures with higher degree of complexity[26,27], for the formation of microparticles[28], and also to mimic the early stage of cell division using the previously mentioned cell inspired lipidic vesicles with incorporated ATPSs[29].

Osmolarity differences between two aqueous solutions can induce phase separation inside all-aqueous emulsion droplets by the removal of water from the droplet to the continuous phase, allowing the formation of complex double emulsions. Song and Shum observed this phenomenon when a solution composed of both PEG and dextran with concentrations below the binodal curve (single-phase) was used as emulsion droplet phase and a more concentrated PEG solution as the continuous phase.[26] The higher osmolarity of the continuous phase compared to the droplet phase, drove water removal from the droplets, increasing the concentrations of both PEG and dextran inside of them. Hence, phase separation occurred as soon as critical concentrations were reached inside the main droplet. During phase separation, small PEG-rich sub-droplets are formed which eventually coalesce as water is continuously removed, forming a PEG-rich core.[26] Based on this principle, core-shell microcapsules have been produced, composed of gelled collagen shells, due to collagen partition to the dextran-rich phase, and PEG-rich cores.[13]

Osmosis-driven phase separation in ATPS not only enables dynamic transformation of single emulsions to double but also to triple and even quadruple emulsions. In a tissue engineering and biomedical perspective, these structures may serve as templates for the fabrication of complex biomaterials, or as bioinspired multi-compartmentalized structures for synthetic biology approaches.[30] By dispersing a salt-rich phase in a continuous PEG-rich phase (from different equilibrated PEG/salt systems), multiple-phase emulsion droplets were spontaneously formed. As the water moves continuously across the interface from the droplet to the continuous phase due to osmolarity differences, phase separation occurred repeatedly inside the main droplet. Even in small amounts, PEG is also present in the salt-rich emulsion phase, which explained the occurrence of multiple phase separation processes throughout the time.[27] In addition to osmolarity, the extent of phase separation

and consequently the complexity of the resultant structures, can also be controlled by the initial concentrations of components in the emulsion phase. In this example, it was observed that droplets with initial compositions close to the vertex of the binodal curve are able to form more complex structures than compositions far from that zone.[27]

On the other hand, the method of extracting water of emulsion droplets through osmotic gradients has also been utilized as a solidification strategy for the fabrication of microparticles for proteins encapsulation without compromising their bioactivity. By dispersing aqueous starch solution as droplets in highly concentrated PEG solutions, starch microparticles could be produced. The removal of water from the droplets resulted in an increase in starch concentration which led droplets to solidify forming the microparticles. Using this methodology, the formation time and volume of microparticles could be tuned by changing the osmotic pressure gradient as well as PEG molecular weight.[28]

Viscosity and density. The viscosity of the phases affects the settling time of the bottom phase during the phase separation process.[4] In general, in polymer/polymer systems, the viscosity of the phases can be manipulated by varying the polymer concentration and molecular weight in a directly proportional relation – higher polymer content or molecular weight increases phase viscosity.[31,32] However, the manipulation of these two parameters should be careful since they strongly influence two-phase formation. It is not possible to work with two distinct phases if polymer's concentrations are too low as can be easily observed in phase diagrams, whereas phase separation can be more easily induced at lower concentrations with high molecular weight polymers since they have a stronger phase-forming ability.[23]

Most of biomedical applications of ATPSs require the generation of micro-scaled all-aqueous emulsion droplets or jets, which can be controllably formed using microfluidic systems. The viscosity of the phases is an important factor that may determine the fluid dynamics in the microfluidic device, thus allowing the control of the resultant structures.[4,30] In addition, the viscosity of phases also influences the mass transport of biological materials in the ATPS, which might compromise their partitioning behavior if an highly viscous environment is present.[23] By contrast, high viscosity degrees may be fundamental to enhance the stability of engineered biomaterial-based structures. For example, in order to increase stability and impart ideal shear-thinning properties for 3D

printing fiber-shaped structures, Luo *et al.* increased the viscosity of the phases, allowing to preserve the printed microstructures for more than 10 days.[33]

Like viscosity, phase density is also an essential factor in ATPS formation. In general, the larger the density and viscosity difference between phases, the faster phase separation occurs.[34] The density of the phases can be manipulated by changing the concentration of phase-forming components, or by adding water-miscible co-solutes (e.g. D_2O).[35] In terms of applications, density-driven phase separation has been purposed as a strategy to produce multi-phase aqueous systems, with more than one aqueous interface, by mixing different polymers, surfactants and salts, which can be used to drive the partition and separation of solutes with different densities.[35,36]

3. Interface properties and dynamics

ATPSs behave like emulsions of water and oil presenting an interface between the two bulk phases with an associated interfacial tension (γ); however, compared to water/oil (w/o) interfaces (γ = 1-40 mN/m), the interfacial tension of ATPSs is very low, within the range of 10^{-4} to 10^{-1} mN/m.[37] ATPS's interfacial tension depends on several factors including salt concentration, polymer concentration and molecular weight, in the way that the larger the ability of phases to separate and the far above the binodal curve, the higher is the interfacial tension.[4] This important feature of ATPSs has been explored in micro-scale biomedical applications involving the formation of jets and droplets in microfluidic systems. In fact, the ultralow interfacial tension of water/water (w/w) emulsions favors jet formation rather than droplets, due to the slow growth of the Rayleigh-Plateau instability associated to the breakup of liquid jets into multiple droplets.[38] The growth rate (ω) of the instability of an aqueous jet dispersed in a continuous outer fluid can be expressed by equation (1) which depends on the interfacial tension of two phases (γ), viscosity (μ), size of the channel (R), perturbation wavenumber (k), radius of the jet (x), and the viscosity ratio (λ).

$$\omega = \frac{\gamma}{16\mu_0 R}\frac{F(x,\lambda)(k^2-k^4)}{x^9(1-\lambda^{-1})-x^5}$$

$$F(x,\lambda) = x^4(4-\lambda^{-1}+4lnx) + x^6(-8+4\lambda^{-1}) + x^8[4-3\lambda^{-1}-(4-4\lambda^{-1})lnx]$$

$$\lambda = \frac{\mu_0}{\mu_i}, \quad x = \frac{r_0}{R}, \tag{1}$$

where r_0, μ_0, μ_i, corresponds to the radius of the inner jet, the viscosity of the outer fluid and the viscosity of the inner jet, respectively.[39]

According to this equation, the lower interfacial tension induces slower growth rates. Thus, in w/w jets, the growth rate of the instability is much smaller compared to w/o systems that have higher interfacial tensions. This results in a significantly lower breakup into droplets and consequent formation of long liquid jets. Indeed, droplets are eventually generated from the Rayleigh-Plateau instability; however, it is very challenging to control the efficiency of their formation as well as their size and uniformity. For this reason, when the goal is to create uniform droplets, external forces such as pulse, mechanical vibrations, piezo-electric or electrohydrodynamic perturbations are frequently introduced to the jetting process, in order to accelerate the breakup into droplets and precisely control their formation.[7,8] Moreover, another possibility to enhance the Rayleigh-Plateau instability of liquid jets and generate ATPS droplets would be to increase the interfacial tension of the all-aqueous system. For example, Mastiani *et al.* observed that using a PEG/salt system having a relatively higher range of interfacial tension (0.1–1 mN/m) compared with a PEG/dextran system, uniform droplets could be easily generated without applying external perturbations. Even if the interfacial tension of the PEG/salt system is still very low compared to that of w/o systems, the faster growth rate of interfacial instability and jet breakup, prevented the formation of long jets and non-uniform droplets as was observed in the PEG/dextran system.[40]

The ultralow interfacial tension makes the ATPS very sensitive to external fluctuations derived for example from mechanical vibrations or electrical fields, resulting in periodic structures with unique morphologies at the w/w interface.[30,41,42] These structures have potential use as templates to fabricate complex and highly structured aqueous devices and biomaterials.[7] On the other hand, a problem may reside in the maintenance of all-aqueous structures due to the ultralow tension of ATPS's interfaces since the droplets tend to coalesce with each other and liquid jets tend to eventually breakup into droplets. As such, strategies focused on the interfacial assembly of molecules and particles have been proposed in order to stabilize those structures. This stabilization has been achieved by the adsorption of amphiphilic copolymers, liposomes and colloidal particles at the interface, as well as by the interfacial assembly and complexation of oppositely charged species including polyelectrolytes (most commonly), particles and polyssacharides.[7,8] Regarding this last strategy, interfacial complexation spontaneously occurs when two aqueous phases from an ATPS containing either positively or negatively

charged polyelectrolytes, are in contact. Polyelectrolyte complexes are formed at the w/w interface which consist of coacervate-like materials or membranes that may irreversibly stabilize all-aqueous droplets and liquid jets and produce sophisticated biomaterials including microcapsules[25,43–47] and hollow fibers[48] with potential use in encapsulation and sustained release of molecules.

4. Bio- and cytocompatibility

In contrast to w/o systems that use organic solvents harmful for cells, ATPSs are often considered to be biocompatible systems. In fact, the large water content of the phases (generally over 80% by weight) seems to provide a mild surrounding environment to preserve the activity of biomolecules and viability of cells.[49] For this reason, in the past few years, there has been a growing interest in the use of ATPS, for cell applications.[4] Those applications inserted in the biomedical and tissue engineering fields have been including cell micropatterning for controlled spatial organization of cell cultures[4,50], microtissue formation[4,51–53] and cell encapsulation in microparticles[54,55], core-shell microcapsules[56] and hydrogels[17]. However, in *in vitro* culture of mammalian cells, restricted experimental conditions are needed in order to ensure cell viability and long-term culture periods. The cellular environment should be aqueous and controlled conditions of temperature (37ºC), pH (7.4), gas concentration (5% CO2 and atmospheric O2 levels) and osmolarity (~300 mOsM) must be taken into account.[57] In addition, culture medium is required to provide essential nutrients, growth factors and hormones for cell growth and proliferation, and also to regulate pH and osmotic pressure of the culture. As such, when envisioning cell applications, the applied ATPS should be compatible with the required physicochemical conditions for cell culture.

The most commonly used polymers (PEG/dextran) generate stable biphasic systems in a wide range of temperatures, allowing the maintenance of the system during incubation at 37°C needed for cell growth and proliferation. In addition, a wide range of osmotic pressures is compatible with these systems, enabling the dissolution of polymers in physiological buffers or culture medium appropriate for cell culture.[17,58] Therefore, most of performed studies use especially polymer/polymer-based systems. The cytocompatibility of polymer/salt systems is negatively affected by the salt concentration needed for the formation of stable ATPSs, compromising the viability of cells.[40] Other ATPSs composed of PEG/gelatin may offer great potential in cell applications since gelatin possesses

functional groups that promotes cell adhesion[59]; however, processing of devices in such systems may demand temperatures not compatible with cell culture.[16] One major challenge of utilizing ATPSs with cells consists in the ability to balance controlled conditions for both all-aqueous system stability and cell viability. This may have had an impact in the exploration of ATPS for long-term cell culture systems, which is an important aspect to consider for the establishment of highly functional cell encapsulating devices.

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I.4. Statement of purpose

The main aim of this thesis is the production of vessel-like structures in the form of freestanding tubular fibers in a fast and cost-effective way, by taking advantage of the physicochemical properties of aqueous two-phase systems (ATPSs). The fiber-shape will be retained by the formation of a coacervate complex resulting from the interaction between two oppositely charge polyelectrolytes, namely alginate and ε-poly-L-lysine, at the interface of the ATPS. Since electrostatic complexation occurs at the interface level, these structures are expected to be hollow, and able to withstand the perfusion of liquid solutions. The ability to produce structures with complex architecture with interconnected branched configurations will be addressed. Also, control over physicochemical properties of the obtained fiber-shaped materials, including their stability, mechanical properties, and swelling will be explored. Considering the emerging use of ATPSs in cell applications due to their well-known aqueous and biocompatible properties, the hollow fibers will be tested as cell encapsulation systems. The cytocompatibility of the system will be assessed using mesenchymal stem cell (homotypic fibers), via image analysis and cell metabolic activity evaluation. Furthermore, the ability to produce pre-vascularized structures in co-culture models of stem and endothelial cells (heterotypic fibers) will also be assessed. This strategy is expected to thus provide a new perspective for the development of cell-laden and biologically functional devices for tissue engineering and regeneration purposes.

Chapter II
Materials and Methods

1. Materials

Poly(ethylene glycol) (PEG, average MW 8 kDa), dextran (from *Leuconostoc spp.*, MW 450 – 650 kDa), sodium alginate from brown algae (MW 120,000-190,000 g/moL), and phosphate buffered saline (PBS) pellets were purchased from Sigma-Aldrich. ε-Poly-$_L$-lysine (EPL, Epolyly®, MW ~ 4700 g/mol) derived from fermentation of *Streptomyces albulus* PD-1 was purchased from Handary S.A. (Brussels, Belgium). For cell assays, it was used sodium alginate NOVATACH™ MVG GRGDSP (GRGDSP-coupled high MW alginate) which was purchased from NovaMatrix (Sandvika, Norway). To adjust the pH of solutions, sodium hydroxide (NaOH) was purchased from Nouryon and hydrochloric acid (HCl) from Sigma-Aldrich.

For cell culture media and supplementation, Medium 199 (M199) (with Earle′s salts, L-glutamine and 25 mM HEPES), Endothelial Cell Growth Supplement (ECGS), and sodium bicarbonate suitable for cell culture, were purchased from Sigma-Aldrich. Alpha Modified Eagle's Medium (α-MEM), Fetal Bovine Serum (FBS), antibiotic/antimitotic solution containing penicillin/streptomycin (ATB), Gibco® GlutaMAX™ Supplement, and trypsin were purchased from Thermo Fisher Scientific. Heparin sodium salt was purchased from BioChemia, PanReac AppliChem. For cell viability assays, Calcein-AM, Propidium Iodide (PI) and AlamarBlue™ reagent were purchased from Thermo Fisher Scientific. For the immunocytochemistry protocol and cell nuclei and F-actin staining, primary antibody CD31 anti-human purified and Flash Phalloidin™ Red 594 were purchased from Biolegend, and the secondary antibody Alexa Fluor 488 anti-mouse (goat) and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) from Thermo Fisher Scientific. Calcium chloride ($CaCl_2$) anhydrous was purchased from Sigma-Aldrich.

2. Fabrication of fibers using an ATPS

Polymer/polymer ATPSs based on PEG and dextran are the best characterized and most widely used in both bioengineering and biomedical applications.[1] Dextran is a non-toxic hydrophilic polysaccharide composed of a linear backbone of α-linked glucopyranosyl monomers and glucose branches, isolated from *Leuconostoc* species, while PEG is a synthetic hydrophilic polymer composed of ethylene oxide repeating units.[2]

ATPSs have been applied in microfluidic systems involving the dispersion of a jet or droplet of one phase in a continuous outer phase.[1,3–5] To allow the formation of fiber-

shaped materials in a PEG/dextran ATPS, a stabilizing membrane was created at the water-water emulsion interface by the complexation of two oppositely charged polyelectrolytes (PEs). PEs are polymers with charged ionic groups on its repeating units that dissociate in polar solvents like water, forming a charged polymer while releasing the counter-ions in solution.[6] Since the early 90s, PE interactions have been extensively studied in the production of very thin multilayered membranes, by the alternate deposition of oppositely charged PEs using a layer-by-layer process, for biomedical applications.[7,8] Recently, the complexation between oppositely charged PEs has been used as an alternative method to stabilize water-in-water emulsions and produce interesting biomaterials such as PE microcapsules[9,10] and fibers[11].

Alginate is a natural anionic polysaccharide consisting of β-D-mannuronic acid and α-L-guluronic acid monomers (Figure 1, A), and represents the most studied and characterized polymer used in the fabrication of biomaterials for cell encapsulation, due to it abundance, easy gelling and innocuous properties.[12] In fact, classical microencapsulation systems consist of core-shell capsules made of alginate cores surrounded by a polycation layer and an external layer of alginate. Poly-$_L$-lysine and chitosan are the most frequently used polycations for this purpose[13]; however, chitosan solubility in aqueous solutions is limited to acidic conditions, precipitating at pH above 6.[14] Polylysine is a positively charged homopolymer of repeating units of the aminoacid lysine that can be divided into two classes: α-polylysine and ε-polylysine. While α-poly-$_L$-lysine is synthetically synthesized with limited biomedical applications due to increased toxicity, ε-poly-$_L$-lysine is naturally produced by microbial synthesis with a broad range of applications mainly in food preservation due to its antimicrobial properties.[15] The latter consists of 25–35 residues of L-lysine with ε-amino group linked to α-carboxyl groups (Figure 1, B), and is biodegradable, water soluble and with relatively low toxicity.[16] Here, alginate and ε-poly-$_L$-lysine (EPL) were used as polyanion and polycation, respectively, to produce membrane-bounded fibers through electrostatic complexation between amine and carboxylic groups from the respective PEs.

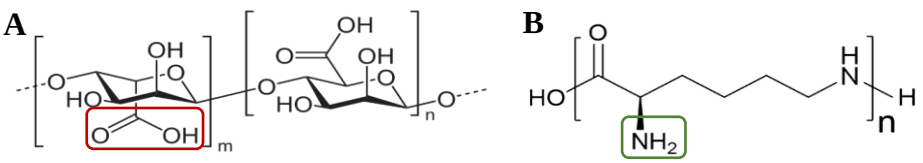

Figure 1. Chemical structure of alginate (A) and ε-Poly-$_L$-lysine (B) containing carboxylic and amine groups, respectively, able to interact electrostatically.

Complexation of alginate and EPL was performed in an ATPS composed of dextran 500kDa 15% (w/w) and PEG 8kDa 17% (w/w) for the fabrication of fiber-shaped structures. They were produced by dispersing a solution of dextran and alginate (phase I) in a thread-like configuration, in a solution of PEG and EPL (phase II), allowing the spontaneous formation of a coacervate-like membrane at the interface between the aqueous solutions. These solutions were prepared by dissolving the phase-forming polymers in PBS under stirring and subsequent addition of PE reagents, for complete dissolution. Phase's pH was adjusted and measured using a pH meter (Consort) to achieve physiological pH (7.2-7.4).

After solution preparation, fibers could be prepared manually using a syringe, but in order to control the flow of the dispersed phase, a syringe pump (Harvard Apparatus) was used. For that, 10 mL of the continuous phase II was added to a petri dish which was placed on a Troemner™ Talboys™ Aluminum Lab Lift in order to stablish a distance of 3 mm between the site of extrusion and the bottom of the petri dish. A 1 mL plastic syringe was filled with phase I and a microfluidic fluorinated ethylene-propylene tubing with 0.5 mm of inner diameter (Dolomite) was placed in front of the syringe so that the phase was extruded perpendicular to the petri dish (schematically represented in Figure 3). A flow rate of 0.2 mL/min was used, and the petri dish was manually moved to allow the design of threads and spontaneous formation of PE fiber-shaped structures. The ends of the fibers were further removed still within the complexation bath by cutting with a spatula, in order to remove irregularities derived from the processing method and produce straight structures with a specific length if required (approximately 1.5 cm). Since polyelectrolytes were still able to interact inside the complexation bath, the ends spontaneously closed after cutting. Afterwards, to prevent the structures from sinking to the bottom of the petri dish due to higher density of phase I compared to the continuous phase II and low viscosity of phase II, they were placed in an orbital shaker at 30 rpm during the complexation time. This step allowed PE complexation to occur uniformly throughout the entire segment, providing more stability to the structures.

2.1. 3D printing of fiber-shaped materials

To explore the versatility of the system to produce free-form structures, an extrusion 3D printer (Inkredible+, Cellink) was use. A blue food dye was added into aqueous solution of dextran and alginate to make the printing procedure visible. For fabricating the different shapes using computer aided design models, a 22 gauge nozzle was used to extrude the dyed solution under 90 kPa pressure at a speed of 10 mm/s. The structures were produced after immersing the printing nozzle in a petri dish containing the aqueous solution of PEG and EPL (10 mL) using $z = 11$.

3. Characterization of the fiber-shaped material

3.1. Stability determination according to polyelectrolyte's concentration, complexation time and pH

The stability and robustness of the fibers was shown to be strongly dependent on several factors including the polyelectrolyte concentration, complexation time and the system's pH. In order to analyze that influence it was stablished a qualitative parameter that determines the ability of fibers to resist to specific conditions after processing. For that, different alginate concentrations (1.5 wt%, 2 wt% and 2.5 wt%) and EPL concentrations (0.5 wt%, 0.75% and 1 wt%) were tested. After solutions' preparation, fibers were made using the syringe pump method, and after a specific complexation time, namely 2, 5, 10 or 15 min, the continuous phase was immediately removed, and PBS was added. This washing step was repeated two more times. With this procedure, a stability yield was determined for the fibers that could remain stable without opening after the washing steps and an overnight incubation period at 37°C, using a representative number of 10 fibers for each analyzed condition:

$$Stability\ yield\ (\%) = \frac{Number\ of\ stable\ fibers}{Total\ of\ produced\ fibers} \times 100$$

The formation and structural integrity of the PE hollow fibers were also analyzed for different system pH, namely, 5, 7 and 9, and imagens of the fibers were acquired in a microscope (Zeiss, Primostar), all in the same light conditions. To assess darkening of the membranes and infer fiber robustness, the mean gray value was determined using ImageJ. Moreover, the yield-related stability was also determined as previously described in systems with varying phase I and II pH values.

3.2. Scanning Electron Microscopy

Scanning electron microscopy (SEM) analysis was performed in order to analyze the hollow lumen of the structures. Fibers with different complexation times were produced with the syringe pump method and were dehydrated with ethanol solutions following a concentration gradient of: 30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), 96% (v/v) and 100% (v/v), by immersing them in these solutions for 15 minutes. To visualize the lumen, transversal cross sections were made using a scalpel blade prior to dehydration. In order to make them conductive, samples underwent gold sputtering for 3 minutes, and afterwards imaged via SEM using Ultra-high Resolution Analytical Scanning Electron Microscope HR-FESEM Hitachi SU-70 (Hitachi, Tokyo, Japan).

3.3. Mechanical properties

To characterize the stiffness of the fibers, a mechanical tensile test was performed in a Universal Mechanical Testing Machine (Instron) with a 50 N load cell. Fibers (with alginate 2 wt% and EPL 0.75 wt%) were prepared using the previously described syringe pump method, and washed (3x) with PBS after completing the desired complexation time. A pre-load of 0.01 N was applied to all samples before the beginning of each tensile test, and samples were stretched until breakage at a deformation rate of 1 mm/min.

3.4. Swelling ratio and external diameter range

When the fibers were against PBS, volumetric expansion occurred. The expansion of the fibers was monitored as a function of time. For that, fibers were produced as previously described, and after a certain complexation period (2, 5, 10 and 15 min) they were washed three times with PBS and images were acquired using a microscope (Ziess, Primo Star) to determine their diameter. The swelling ratio (ε) was determined by the change in size as follows:

$$\varepsilon = \frac{d_t}{d_0},$$

where d_0 represents the original diameter of the fibers in the phase II solution, and d_t represents the diameter of the fibers against PBS.

In order to determine the range of diameters possible for the fabrication of fibers, different needles with varying diameters were used. Images of the fibers were acquired using a Stereo Microscope (Zeiss, Stemi 508) and external diameters were determined

before and after washing with PBS. For both the experiences, ImageJ software was used to determine the diameter by converting the acquired pixels, considering the amplification used, into millimeters, and a representative number of 6 fibers was used for each condition.

4. Cell functionality

4.1. Cell culture

In order to test the cytocompatibility of the reported fiber system, human adipose-derived stem cells (hASCs, purchased from LGC Standards, American Type Culture Collection (ATCC)) and human umbilical vein endothelial cells (HUVECs, derived from human umbilical cord isolation procedures performed in the group in accordance with the ethical agreement established with Centro Hospitalar do Baixo Vouga) were used. Mesenchymal stem cells, including the ones derived from the adipose tissue, have been widely used in tissue engineering and regeneration fields, due to their ability to differentiate into multiple cell lineages, immunomodulatory properties, self-renewability and release of trophic factors.[17] HUVECs in turn remain the gold standard endothelial cell lineage used in vascular tissue engineering applications, due to low cost, easy isolation and high angiogenic potential.[18] hASCs were cultured (Figure 2, A) in α-MEM with sodium bicarbonate, supplemented with 10% (v/v) FBS and 1% ATB, and HUVECs were cultured and expanded (Figure 2, B) in M199 with sodium bicarbonate, supplemented with 20% (v/v) FBS, 1% (v/v) ATB and 1% (v/v) GlutaMAX^{TM}. Additionally, the endothelial medium was complemented with 50µg/mL of heparin and 5µg/mL of ECGS. Prior to passage or use in experiments, both cell types were detached from culture flasks using trypsin, upon reaching approximately 80-90% of confluence. HUVECs and hASCs were used for experiments at passages 5-8 and 6-9, respectively. All cells were manipulated in aseptic conditions and maintained in cell culture flasks in humidified and temperature-controlled incubators at 37 ºC and 5% CO_2.

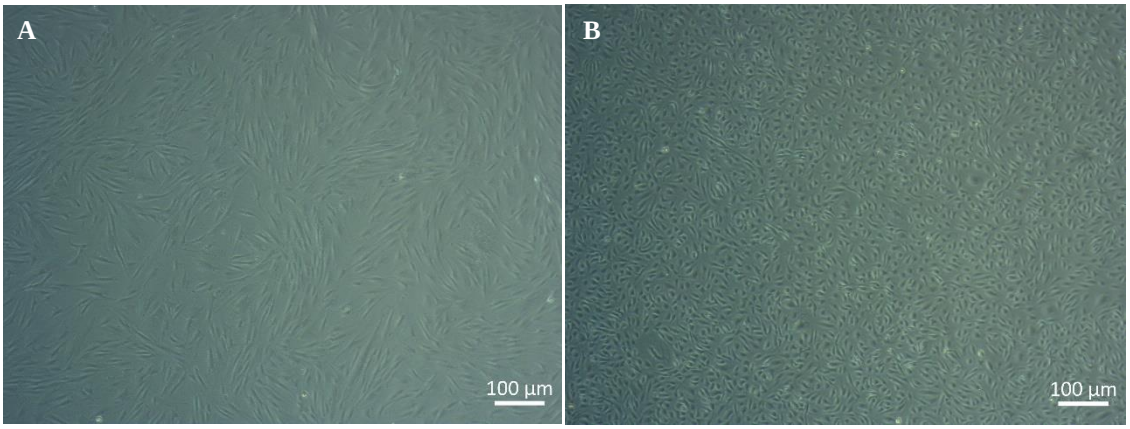

Figure 2. Microscopy images showing the morphology of 2D cultured hASCs (A) and HUVECs (B).

4.2. Cell encapsulation

To assess cell viability inside the fibers, cells were encapsulated in phase I, for the formation of both homotypic (hASCs) and heterotypic (hASCs and HUVECs) fibers. In this case, phase I was composed of dextran 15 wt%, alginate 2% and alginate functionalized with the cell adhesion arginine-glycine-aspartate (RGD) domain (alginate-GRGDSP) in a concentration of 0.5 wt%[19], to enhance cell adhesion in the fibers.

For cell encapsulation, confluent cells were tripsinized, resuspended in the right cell culture medium and counted using a hematocytometer. Then, in order to obtain a cell density of $5x10^6$ cells per mL of phase I, the adequate amount of the cell suspension was centrifuged for 5 min at 1249 rpm, and the pellet was resuspended the phase solution. For the heterotypic fibers, hASC and HUVEC suspensions were mixed in a 1:2 ratio before centrifugation, presenting a final concentration of $15x10^6$ cells/mL. This cell density was optimized after several encapsulations, in order to achieve the adequate cell amount that could fill all the fiber and promote a better cellular contact necessary for cell viability.

Prior to encapsulation, both phase I and II were sterilized. For that, ATPS phases containing the dissolved polymers were filtered using sterile Millipore Express® Membrane Filters and Whatman® Puradisc 30 Syringe Filters with 0.2 µm pore size. Alginate, alginate-GRGDSP and EPL reagents were exposed to UV light during 40 min. The sterile polyelectrolytes were added to the filtered solutions to dissolution using autoclaved stirring magnets.

After cell encapsulation in phase I, fibers were made using simply sterile 1 mL syringes and 25 gauge needles to facilitate the processing method. After a complexation

time of 2 minutes, they were washed three times with DPBS and placed in a DPBS bath (30 mL) where were vigorously shaken. After washing, homotypic fibers were maintained in α-MEM cell culture medium and heterotypic in a mixture of complemented M199 and α-MEM in a 1:1 ratio, and incubated at 37°C and 5% CO_2, for cell viability and immunocytochemistry evaluation.

4.2.1. Cell viability assays

The cell viability of homotypic and heterotypic fibers was analyzed by live/dead assay (Invitrogen, USA) and Alamar Blue® Cell Viability assay at different time points. Both assays were performed in accordance with manufacturer instructions.

For the live/dead assay, propidium iodide (PI) and Calcein-AM, were used to distinguish between live and dead cells through fluorescence microscopy. Non-viable cells with compromised cell membranes could be identified by red fluorescence due to binding of PI to their nuclei, while viable cells present green fluorescence due to conversion of the membrane permeable Calcein-AM to fluorescent calcein by intracellular esterases. At specific time points, the cell encapsulated fibers were incubated with PI and Calcein-AM at concentration of 1 μL/mL and 2 μL/mL, respectively, during 10 min at 37°C. After incubation, fibers were washed with culture medium, and fluorescence micrographs were acquired in an upright widefield fluorescence microscope (Axio Imager M2, Carl Zeiss, Germany). Image analysis and processing were performed in Zeiss Zen 2.3 Blue software.

AlamarBlue assay was used to access metabolic activity of encapsulated cells. Resazurin, the active ingredient of AlamarBlue reagent, is a non-toxic and cell-permeable compound that is blue in color and non-fluorescent. Upon entering living cells, resazurin is continuously reduced to resorufin, which is highly fluorescent and produces colorimetric changes in the medium, allowing the quantitative measurement of the cells' metabolic activity. It was used a ratio of 10μL AlamarBlue™ reagent per 100μL of culture medium, with an incubation time of 8.5 hours. Fibers produced in the same conditions but without encapsulated cells were used as control, and a representative numbers of 5 fibers was used. Fluorescent measurements ($\lambda_{excitation}$: 540 nm, $\lambda_{emission}$: 600 nm) were performed in a Synergy HTX microplate reader using a 96-well black-clear bottom plate containing 100μL of the reaction medium per well.

4.2.2. Immunocytochemistry

CD31 is a transmembrane glycoprotein expressed on hematopoietic cells and fully differentiated endothelial cells such as HUVECs. Besides that, endothelial cells express other molecular markers, including VE-cadherin and vWF.[20] As such, to evaluate the presence of this cell type in the heterotypic fibers, an immunocytochemistry was performed. This technique allows the detection of a specific protein or antigen in cells using a specific antibody that binds to it. In brief, cell encapsulated fibers were fixed at specific timepoints with cold 100% methanol (solution was left at -20 ºC for at least 2h prior to use) for 10 minutes and washed twice with a $CaCl_2$ 0.1M solution. Although paraformaldehyde (PFA) is the most common used fixative, here it was necessary to choose another fixation method since the cell fibers rapidly disintegrate in PFA solutions. Methanol have both fixation and permeabilization properties, acting by precipitating the proteins while dehydrating cells; however, it can damage cell membrane and organelles.[21] Nevertheless, cold methanol solution was used since it allowed preservation of the fiber's membrane and structure. Normally the washing step is performed using PBS solutions; however, here, taking advantage of crosslinking properties of alginate in the presence of divalent cations[22], $CaCl_2$ 0.1M was used, in order to maintain the structure of the PE membrane by allowing the interaction between alginate carboxyl groups and Ca^{2+} ions. Afterwards, the cell fibers were open and fragmented with up and down movements using a micropipette, to allow access of the antibody to the cells inside the fibers, and blocked with 5% FBS for 30 minutes. For the immunostaining, fibers were incubated with anti-human purified CD31 antibody (diluted 1:50 in 1% FBS) for 2 hours, washed 5 times with $CaCl_2$ 0.1M, followed by an incubation period of 1 hour with fluorescently labeled Alexa Fluor 488 goat anti-mouse secondary antibody (diluted 1:300 in 1% FBS), at room temperature. The fibers were submitted to more three washing steps and counterstained with Flash Phalloidin™ Red 594 (diluted 1:50 in $CaCl_2$ 0.1M) and DAPI (5 μg/mL solution diluted 1:1000 in $CaCl_2$ 0.1M), with a 30- and 10-minutes incubation period, respectively. For the negative control, fibers were not incubated with primary anti-CD31 antibody.

5. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 8 Software, and results are expressed as mean ± standard deviation. Statistical significance between the groups

was determined by one-way and two-way analysis of variance (ANOVA) using Tuckey's multiple comparisons test, considering a statistically significant difference if $p < 0.05$.

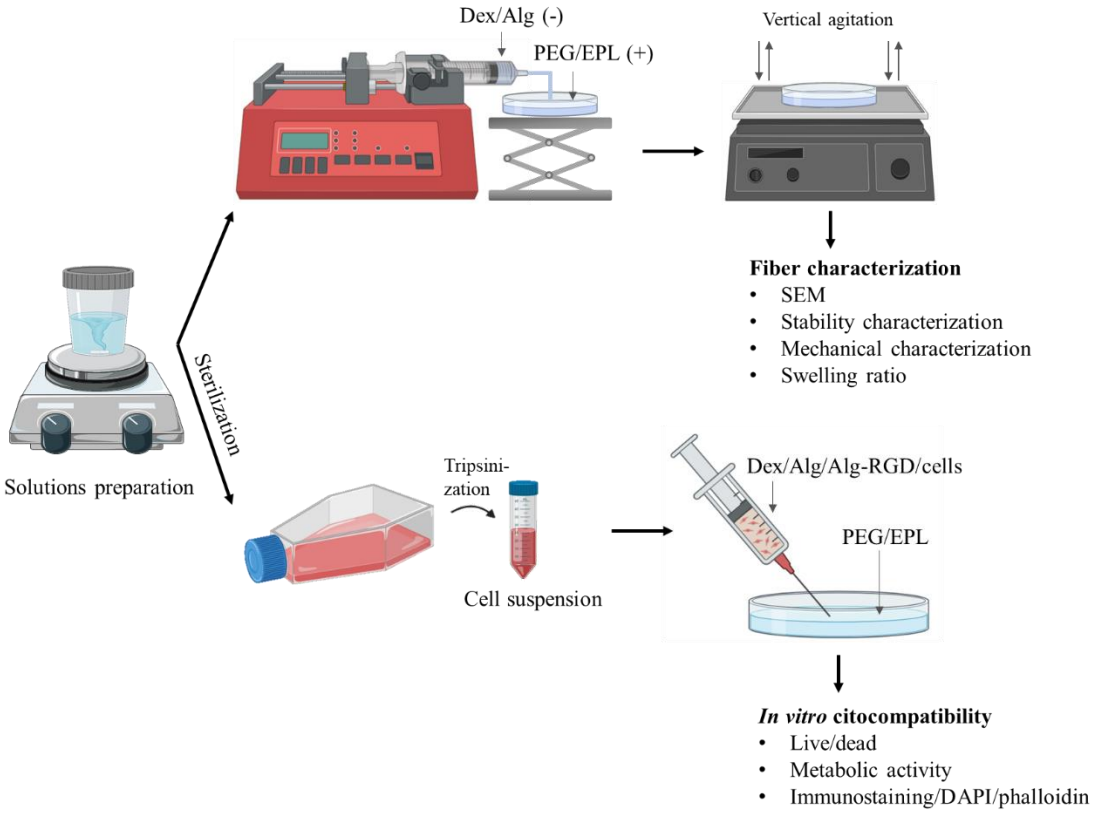

Figure 3. Schematic representation of the methods.

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Chapter III
Results and Discussion

III.1. Cytocompatible all-aqueous fabrication of freestanding hollow-core fibers

Subchapter III.1.

This subchapter is based on the article entitled:
"Cytocompatible all-aqueous fabrication of freestanding hollow-core fibers"
(Manuscript in preparation)

Cytocompatible all-aqueous fabrication of freestanding hollow-core fibers

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Abstract

The ability to fabricate hollow structures that aim to recapitulate architectures and features of biological tissues such as blood vessels or nerves may represent great potential in areas of tissue engineering and regenerative medicine. Here, resorting to the inherent properties of aqueous two-phase systems and electrostatic complexation between nature-derived polyelectrolytes, we report a strategy to rapidly generate stable and size-controllable fibers with customizable shapes (straight and branched structures) in an all-aqueous environment by the formation of a robust coacervate-like membrane. We demonstrate the system's capacity to withstand liquid fluid flow, and to deform into versatile shapes by free-form deposition. Furthermore, mechanical stability in *in vitro* cell culture conditions was confirmed for the application of such structures as cell encapsulation systems or as hybrid implantable tubular constructs. Using human adipose-derived stem cells (hASCs) we demonstrate the cytocompatibility of the system, as well as its ability to promote cell adhesion and proliferation. Medium-term cell culture conditions (up to 14 days) in heterotypic fibers containing both hASCs and human umbilical vein endothelial cells (HUVECs) proved the beneficial effect of co-culturing systems in the maintenance of endothelial cell survival, with potential to be used as pre-vascularized structures in future studies. Overall, the produced fibers with encapsulated cells have potential to be integrated in tissue engineered constructs and produce therapeutic materials for tissue regeneration.

Introduction

Strategies with high relevance in the biotechnology and bioengineering fields, including drug/protein delivery and enzyme immobilization, have relied on compartmentalization approaches compatible with mild processing.[1] Such encapsulation methods have been adapted and extended to withstand cell encapsulation important for tissue engineering and regenerative medicine, and to recent technological developments on disease modelling.[2] Most emphasis has been given to spherical-shaped cell encapsulation systems due to ease of processing, as well as to the architecture-enabled achievement of controlled molecular release profiles. More recently, though, fibrillar geometries have gained momentum, mainly owing to their ability to mimic complex and hierarchical architectures of naturally occurring fiber-shaped and tubular tissues including vasculature.[2,3] Also, flexible fiber-shaped materials may be deposited with high three-dimensional (3D) freedom, enabling the bottom-up fabrication of complex geometries, much needed in tissue regeneration based on defect-filling strategies.[4]

Several biofabrication technologies have been explored for the generation of self-sustained tubular materials, mostly with the objective of recapitulating blood vessel functionalities. Those may include template molding[5], sheet rolling[6] and 3D stamping[7]. However, these techniques are often non-straightforward to implement, requiring specialized equipment, and/or relying on time-consuming and multistep procedures.[8] The direct design of arbitrary geometry and mechanical properties is another considered important challenge of these technologies.[9] Moreover, none of these strategies allows to easily fabricate tubes of controlled and virtually unlimited length. Therefore, strategies aiming at the direct fabrication of perfusable tubular structures with free-form distributions, mostly relying on coaxial extrusion-based 3D printing, have emerged as promising solutions to address those constrains.[9–12]

Aqueous two-phase systems (ATPS) may represent a versatile platform to produce free-form fiber-shaped structures in all-aqueous environments. ATPSs are formed when two aqueous solutions containing incompatible polymers, salts or others, separate into two immiscible phases.[13] Poly(ethylene glycol) (PEG) and dextran solutions are a classical combination of polymers used to form ATPSs compatible with biomedical-related applications. Its use has enabled the fabrication of advanced biomaterials such as

hydrogels, microcapsules, microparticles and microfibers.[14,15] Typically in such strategies, the biomaterials are fabricated trough thermal gelation or chemical crosslinking, using gel-forming polymers such as collagen, gelatin and alginate, or through UV radiation when phase-forming polymers are chemically modified with photocrosslinkable groups.[15] Furthermore, dispersing oppositely charged polyelectrolytes (PEs) in the different phases of the ATPS has been explored as a way to produce biomaterials through a one-step PE complexation. This method has been mainly used for the generation of PE capsules and particles with potential applications in encapsulation and controlled release of active agents[16–20] and, more recently, for the fabrication of disease models.[21] However, the processing of such structures in tubular form are still poorly explored [22], and the ability of ATPS-based systems to generate cell-laden materials with architectural complexity has never been reported.

In this work, we hypothesize that interfacial PE complexation in a PEG/dextran-based ATPS could support the direct and rapid generation of fibers with hollow features and complex geometries in a single step. To achieve this goal, a phase containing alginate (polyanion) was dispersed, using a jetting flow system, in another phase containing ε-poly-$_{L}$-lysine (polycation), enabling the formation of a coacervate stabilizing membrane at the interface. The ability to form stable (after washing) and perfusable fiber-shaped structures in ranges of pH, PEs concentration, and times of complexation was assessed. Human mesenchymal stem/stromal cells derived from the adipose tissue (hASCs) were successfully encapsulated in an optimized fiber formulation, also modified with cell adhesive moieties, and the cytocompatibility of the method was proven by their survival and proliferation for 7 days of culture. To target the development of pre-vascular structures, human umbilical vein endothelial cells were co-encapsulated with hASCs, demonstrating their permanence and survival up to 14 days. We believe that our system presents relevant advances on the fabrication of biomaterial-based structures with the ability to resemble native tubular tissues, with potential application in tissue regenerative applications.

Materials and Methods

1. Formation of fibers using ATPS

Solutions of 17 wt% poly(ethylene glycol) (PEG, average MW 8 kDa, Sigma-Aldrich) and 15 wt% dextran (MW 450 – 650 kDa, Sigma-Aldrich) were prepared in PBS (Sigma-Aldrich). Sodium alginate (Sigma-Aldrich) and ε-poly-l-lysine (EPL, Handary S.A.), were dissolved separately in the dextran 15 wt% and PEG 17 wt% solutions, respectively. Fibers could be produced using a simple manual injection method, where a solution composed of dextran and alginate is dispersed through a syringe in a bath solution composed of PEG and EPL, by moving the syringe in thread-like configurations. To improve the control over the injection flow rate and shape of the structures, a syringe pump (Harvard Apparatus) with assembled syringes with 25 gauges was used. The flow rate used was 0.2 mL/min. After formation, fibers were kept in agitation in an orbital shaker at 30 rpm during a pre-determined complexation time. To test the ability of fibers to be perfused, complexation was interrupted at pre-determined times by removing the bath phase and then washing the fibers with PBS (Sigma-Aldrich). Afterwards, the closed ends were easily removed using a spatula to leave the fibers with open ends and allow the liquid-core solution to exit. A blue dye consisting of diluted trypan blue (Thermo Fisher Scientific) solution (1:2) was injected through one end using a syringe and a small 34 gauge needle.

1.1. 3D printing of fiber-shaped materials

To explore the versatility of the system to produce free-form structures, an extrusion 3D printer (Inkredible+, Cellink) was used. A blue food dye was added into aqueous solution of dextran and alginate (phase I) to make the printing procedure visible. For fabricating the different shapes using computer aided design models, a 22G nozzle was used to extrude the dyed solution under 90 kPa pressure at a speed of 10 mm/s. The structures were produced after immersing the printing nozzle in a petri dish containing the aqueous solution of PEG and EPL (10 mL) using $z = 11$.

2. Fibers characterization

2*.1. Stability characterization*

To analyze the stability of the fibers according to the concentration of PE, complexation time and system's pH, the ability of the fibers to resist specific conditions

after processing was assessed. Different alginate concentrations (1.5 wt%, 2 wt% and 2.5 wt%) and EPL concentrations (0.5 wt%, 0.75% and 1 wt%) were tested as well as different complexation times, namely 2, 5, 10 or 15 min. The continuous phase was immediately removed after complexation, and PBS was added. This washing step was repeated two additional times. With this procedure, a stability yield was determined for the fibers that could remain stable without opening after the washing steps plus an overnight incubation period at 37°C (Eq. 1). Two independent experiments were performed with a total representative number of 10 fibers used for each condition. The formation and structural integrity/stability of the PE fibers were also analyzed for different system pH, namely, 5, 7 and 9, and imagens of the fibers were acquired in a microscope (Primostar, Zeiss), all in the same light conditions. To assess the darkening of the membranes, associated with higher extents of complexation, the mean gray value was determined using ImageJ.

$$Stability\ yield\ (\%) = \frac{Number\ of\ stable\ fibers}{Total\ of\ produced\ fibers} \times 100 \qquad \textbf{Eq. 1}$$

2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis was performed to analyze the morphology and the presence of a hollow lumen in the formed structures. Fibers were produced using the syringe pump method with different complexation times, and were dehydrated with ethanol solutions following a concentration gradient of : 30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), 96% (v/v) and 100% (v/v), by immersing them in these solutions for 15 minutes. To visualize the lumen, transversal cross sections were made using a scalpel blade prior to dehydration. In order to make them conductive, samples underwent gold sputtering for 3 minutes. Samples were then imaged using a Ultra-high Resolution Analytical Scanning Electron Microscope HR-FESEM Hitachi SU-70 (Hitachi, Tokyo, Japan).

2.3. Mechanical properties

To characterize the stiffness of the fibers, a mechanical tensile test was performed in a Universal Mechanical Testing Machine (Instron) with a 50 N load cell. Fibers (with alginate 2 wt% and EPL 0.75 wt%) were prepared using the previously described syringe pump method, and washed (3x) with PBS after completing the desired complexation time.

A pre-load of 0.01 N was applied to all samples before the beginning of each tensile test, and samples were stretched until breakage at a deformation rate of 1 mm/min.

2.4. Swelling ratio and external diameter range

The expansion of the fibers while immersed in PBS was monitored as a function of time. For that, fibers with different complexation times (2, 5, 10 and 15 min) were produced as previously described, and images were acquired using a microscope (Zeiss, Primo Star) to determine their diameter using ImageJ. The swelling ratio (ε) was determined by the change in size as follows (Eq. 2):

$$\varepsilon = \frac{d_t}{d_0}, \qquad \textbf{Eq. 2}$$

where d_0 represents the original diameter of the fibers in the phase II solution, and d_t the diameter of the fibers against PBS.

In order to determine the range of diameters possible for the fabrication of fibers, fibers were made using different needles with varying diameters. Images of the fibers were acquired using a Stereo Microscope (Zeiss, Stemi 508) and external diameters were determined before and after washing with PBS using ImageJ. For both the experiences, 6 fibers were used for each condition.

3. *In vitro* cytocompatibility

3.1. Cell culture and encapsulation

The cytocompatibility of the PE fibers was evaluated using human adipose-derived mesenchymal stem cells (hASCs) (purchased from LGC Standards, ATCC) and human umbilical vein endothelial cells (HUVECs) isolated from one umbilical cord, in accordance with the ethical agreement established between our research group and the Centro Hospitalar do Baixo Vouga. hASCs were cultured in α-MEM, supplemented with 10% (v/v) fetal bovine serum (FBS, ThermoScientific) and 1% antibiotic/antimycotic (ThernoScientific), and were used at passages 6-9. HUVECs were cultured and expanded in Medium 199, supplemented with 20% (v/v) FBS, 1% (v/v) antibiotic/antimycotic, and 1% (v/v) GlutaMAX^{TM} (ThermoScientific), as well as 50µg/mL of heparin (BioChemia, PanReac AppliChem) and 5µg/mL endothelial cell growth supplement (ECGS, Sigma-Aldrich). Cells were used in passages between 4 to 8. Cell suspensions were prepared after trypsinization and were encapsulated in the phase to be dispersed containing dextran and

alginate. To promote cell adhesion, alginate functionalized with the cell adhesion peptide arginine-glycine-aspartate (RGD) (NOVATACHTM MVG GRGDSP, NovaMatrix) was used. Both solutions containing the phase-forming polymers and PEs were sterilized. For that, ATPS phases containing the dissolved polymers were filtered using sterile 0.2 μm pore-sized filters, and alginate, alginate-GRGDSP and EPL reagents were exposed to UV light during 40 min. The sterile polyelectrolytes were added to the filtered solutions to dissolution using autoclaved stirring magnets. To assess the cytocompatibility of the structures, homotypic fibers containing hASCs were prepared ($5 \times 10^6\ cells/mL$) and, to evaluate the influence of co-culturing different cell types, heterotypic ones containing both hASCs and HUVECs in a 1:2 ratio ($15 \times 10^6\ cells/mL$), were produced. A syringe containing the phase with cells was immersed in the phase containing PEG and EPL. After 2 minutes of complexation, PBS was added, and fibers were transferred to a 30 mL PBS solution and then to the appropriate cell culture medium. Cell encapsulated fibers were maintained in incubators with controlled temperature (37ºC) and 5% CO_2 .

3.2. Live/Dead assay

To analyze live and dead cells at pre-determined timepoints, fibers were incubated with propidium iodide (PI) (Thermo Fisher Scientific) and Calcein-AM solution (Thermo Fisher Scientific) at concentration of 1 μL/mL and 2 μL/mL, respectively, during 10 min at 37°C. Fibers were washed with culture medium and examined in an upright widefield fluorescence microscope (Axio Imager M2, Carl Zeiss, Germany).

3.3. Cell metabolic activity assay

The AlamarBlue® assay (Thermo Fisher Scientific) was used to access metabolic activity of encapsulated cells. Cell encapsulated fibers with approximately 0.5 cm of diameter and 1 cm long were placed in a 48 well-plate with 1.2 cm of diameter per well. 10 μL of AlamarBlue™ reagent was added per 100 μL of culture medium, with an incubation period of 8.5 hours. Fluorescent measurements ($\lambda_{excitation}$: 540 nm, $\lambda_{emission}$: 600 nm) were performed in a Synergy HTX microplate reader using a 96-well black-clear bottom plate.

3.4. Immunocytochemistry

Immunocytochemistry was used to evaluate the presence of endothelial cell surface maker CD31 in the fibers comprising cellular heterotypic cultures. Cell-encapsulated fibers were fixed at specific timepoints with cold pure methanol (solution was left at -20 ºC for at least 2h prior to use) for 10 minutes and washed twice with a $CaCl_2$ 0.1 M solution. Afterwards, cell fibers were opened and fragmented with up and down movements using a micropipette, and blocked with 5% FBS for 30 minutes. For the immunostaining, fibers were incubated with anti-human purified CD31 antibody (Biolegend) (1:50, in 1% FBS) for 2 hours, washed 5 times with $CaCl_2$ 0.1M, followed by an incubation period of 1 hour with fluorescently labeled Alexa Fluor 488 goat anti-mouse secondary antibody (Thermo Fisher Scientific) (1:300, in 1% FBS), at room temperature. The fibers were submitted to three additional washing steps, and counterstained with Flash Phalloidin™ Red 594 (Biolegend) (1:50, in $CaCl_2$ 0.1M) for F-actin identification and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Thermo Fisher Scientific) (1:1000, in $CaCl_2$ 0.1M) for nuclei localization, with a 30- and 10-minutes incubation period, respectively. A negative control was prepared for all studied samples by skipping the incubation step with the primary anti-CD31 antibody.

4. Statistical analysis

GraphPad Prism 8 was applied for data statistical analysis, and results are presented as mean ± standard deviation. Statistical significance between the groups was determined by unpaired *t* tests using one-way and two-way analysis of variance (ANOVA), considering a statistically significant difference if $p < 0.05$.

Results and discussion

1. Formation of fiber-shaped structures

Aqueous solutions of PEG and dextran are typically used to form ATPSs which are characterized by the presence of water/water interfaces with a very low interfacial tension compared to water/oil systems.[13,20,23] The highly aqueous environment provided by these systems and the unique characteristics of their interfaces, have broadly opened the range of applications of such ATPSs, creating new opportunities in the tissue engineering and biomedical fields.[15] Here, PEG/dextran systems were used to support the fibers' formation

by exploiting the interfacial complexation of two oppositely charged PEs: alginate and EPL.

Here, alginate was dissolved in the dextran 15 wt% solution (phase I) and immersed in a larger volume solution made of EPL dissolved in PEG 17 wt% (phase II) (Figure 1, A). Although synthetic α-polylysine has been the most widely polycation used for the generation of membrane-bounded cell microencapsulation systems, an EPL/alginate-based approach consisting on the deposition on a sacrificial solid alginate template has been previously reported.[24] Here, when phase I was immersed in phase II using a jetting flow system, fiber-shaped structures were rapidly formed by adding a desired movement during the formation process (Figure 1, B and C). The rapid interaction of the PEs was crucial for the development of stable structures: when a PE-free phase I was immersed in PE-free phase II, long threads can be formed upon the application of movement. However, due to the well-reported low interfacing tension of the ATPS, the thread eventually brokeup into smaller droplets due to the Plateau-Rayleigh instability (Supplementary Figure 1A, Supporting Information).[15] When oppositely-charged PEs are in contact in solution, a condensed complex is formed, mainly driven by the electrostatic interaction between their charged groups and entropy gain upon the release of counter ions and solvating molecules in solution, in a well-known PE complexation phenomenon.[25,26] These PE complexes consist of coacervate-like materials or membranes that may irreversibly stabilize the liquid thread of the immersed phase and produce sophisticated biomaterials.[22,27] The importance of the ATPS system in ensuring the formation of stable coacervates with pre-defined architectural features was also assessed. By using the same experimental procedure with PEs dissolved in water, hard-to-handle and easily damaged structures were formed (Supplementary Figure 1B, Supporting Information).

In our approach, the presence of alginate (polyanion containing carboxylic negatively charged groups) and EPL (polycation containing amine positively charged groups) separately dissolved in the polymer phases allowed the formation of a stable membrane at the interface around the immersed phase within the first 30 seconds in contact, expectedly resulting from PE complexation (Figure 1, B). Due to the aqueous nature of the system, the dissolved PEs are hypothesized to slowly diffuse towards the interface of the ATPS. Overtime, more polyions are expected to interact, causing the darkening of the membrane, and the consequent formation of more robust fiber-shaped structures (Figure 1, B). After 2

minutes, PE complexation was interrupted by removing phase II bath and adding a high volume of PBS. Upon such disruption of the ATPS stability, the structure could remain intact without collapsing (Figure 1, D). Interestingly, the free-standing structure showed robustness and could be easily handled with tweezers both in a water-based solution, as well as while in contact with air (Figure 1, E and F). Since the membrane was formed at the interface of the aqueous system, ones would expect that, by cutting off the ends of the materials, the produced fibers would be hollow upon releasing their liquid content, producing tubular structures. The ability to perfuse liquids directly through bioengineered constructs is considered a key requirement for the generation of functional tubular tissues.[12] The ability of the tubes to be perfused with aqueous solutions was examined by injecting of a blue dye using a syringe. The entire length of the materials could be perfused, while keeping their structural integrity in a liquid environment (Figure 1, G).

The absence of a pre-formed solid template enabled the easy development of free-form configurations using an extrusion 3D printer (Figure 1, H). The deposition of phase I into phase II was achieved in multiple directions, conferring this method with the potential to be used in the fabrication of fit-to-shape materials to fill pre-determined shapes, such as tissue defects.[28]

The features of the produced structures may recapitulate architectural features of biological hollow tubular tissues, such as blood vessels. Blood vessels are spread in a hierarchical fashion throughout the body by branching from millimeter-sized vessels to micrometer-sized, ending in capillaries that form complex and dense networks in tissues for proper molecular exchanges.[8] In order to mimic tubular and branched geometries found in the human body, tubes could be ramified using a simple method based on the use of two syringe needles. A Y-shaped structure was fabricated by initiating the extrusion of phase I with two contacting syringes, which initially formed a single tube. Afterwards, by simultaneously separating the syringes during extrusion, well-connected branches could be formed (Figure 1, I). After washing, the obtained Y-shaped structures remained intact and could be entirely perfused (Figure 1, J). Altogether, these properties show the versatility of the developed system that enabled the flow of aqueous liquids, opening the possibility to produce sophisticated microfluidic systems where the ends of the tubular structure would constitute the inlet and outlet.

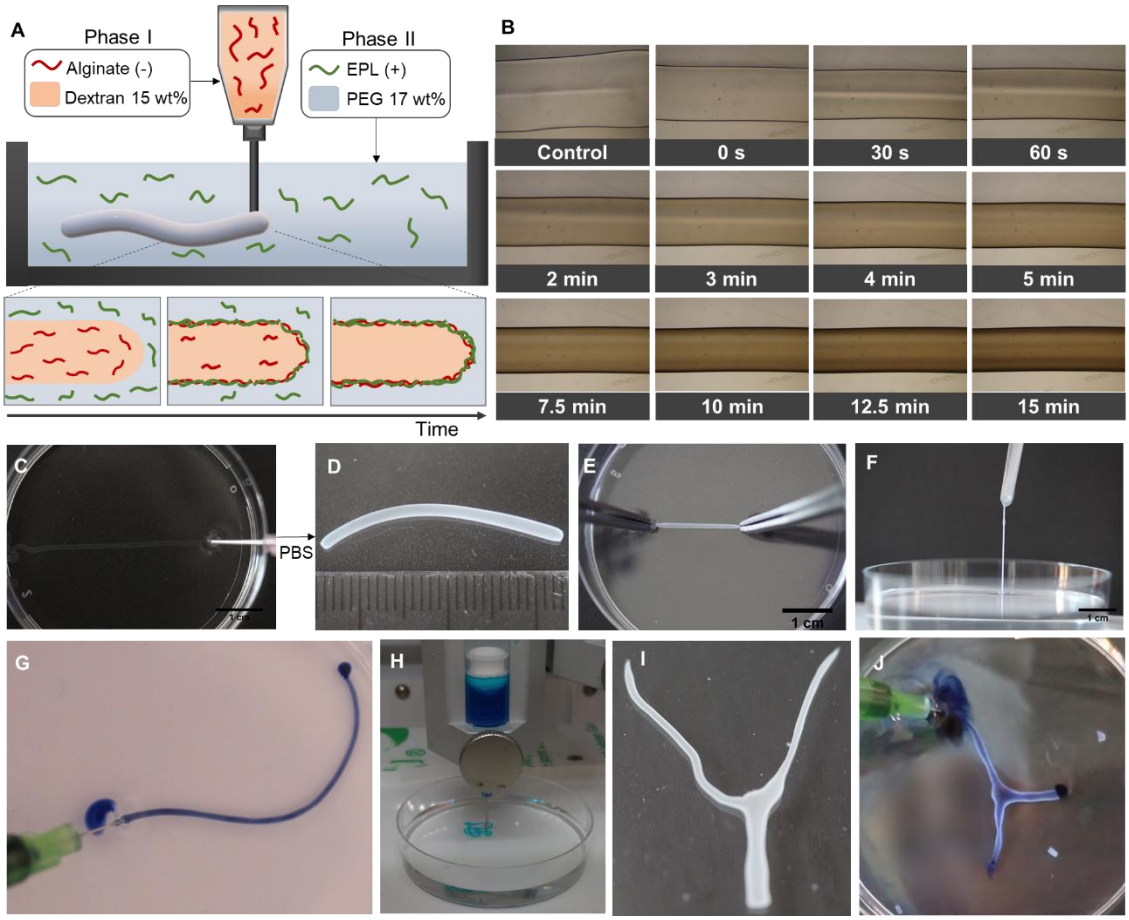

Figure 1. A) Schematic representation of fiber formation using polyelectrolyte complexation between alginate and ε-poly-$_L$-lysine (EPL) at the interface of an aqueous two-phase system composed of PEG and dextran. B) Microscopic images showing the darkening of the membrane over time, compared to the control consisting of dextran phase immersed in PEG phase without the polyelectrolytes. C) Macroscopic image of the fiber during processing using a syringe-needle method, which remain intact after 3x washing with PBS (D). The structure was able to be handled with tweezers both in PBS (E) and in air (F). Scale bars:1 cm. G) Perfusion of the hollow fibers with a blue dye throughout the entire segment. H) 3D printing of free-form shapes. I) Y-shaped structure that was able to be perfused with a blue dye (J).

2. Characterization of fibers produced by interfacial complexation

Previous works using PE complexation in ATPSs to produce biomaterials, have demonstrated that the phase in which the PEs are initially positioned influences the structure being formed and its stability.[22,29] This phenomenon has been explained by the different affinity of the PEs to partition to the different phases. Here, we empirically investigated the effect of inversing the PEs position in the phases, that is, the effect of

having alginate in the outer phase with PEG and EPL in the inner phase with dextran, or vice-versa. Macroscopic observation revealed the formation of stable hollow-core fibers when alginate was in the inner phase and EPL in the outer one, and open and disintegrated structures when alginate and EPL were in the outer and inner phases, respectively (Supplementary Figure 2, Supporting Information). As for the choice of dextran and PEG as the inner and outer phase, respectively, this was followed by the extensive reported literature using dextran as the inner phase in both biomaterial and cell applications.[13,15]

2.1. Stability characterization

Combination of different concentrations of the two PEs was assessed to find optimal conditions to obtain stable structures compatible with cell culture conditions. In a first stage, the yield of fibers that remained intact, without opening or collapsing after washing and incubation at 37ºC overnight, was evaluated for different PE concentrations. Moreover, given the increase in darkness over time, the structural stability of the materials was also evaluated for different complexation times. Fibers were prepared using a syringe pump system, that allowed to control the flow rate of phase I and increase reproducibility. In this study, solutions were prepared at physiological pH values (7.2-7.4). Figure 2, A summarizes in an intensity map the stability yield obtained for all tested conditions at pH ~7. The lack of stability associated to the opening of fibers may be related with the disturbance of the ATPS support and disruption of the interfacial tension-mediated barrier after the complete removal of phase II and addition of large amounts of PBS. Interestingly, a tendency for decreasing stability with increasing complexation time seems to occur, regardless of PE concentrations. Beyond the attractive electrostatic interactions, the entropy gain from the release of water is considered to be one of the driving forces for the formation of PE complexes or coacervates.[25] In fact, it has been previously described the presence of highly hydrophilic polymers in solutions with oppositely charged PEs enhance the dehydration of the PE constituents and favors entropically the formation of the coacervation condensate.[30] We therefore hypothesized that, with increasing time, the increasing PE interactions and consequent dehydration of the PE molecules and possible local accumulation of elastic stress[31] may have caused the tightening of the membrane mesh, inducing the opening of thermodynamically instable areas of the fiber. Lower PE concentrations (alginate 1.5 wt% and EPL 0.5 wt%) were not adequate for the generation

of sufficiently stable structures (Figure 2, A). The overall analysis of these conditions allowed stablishing alginate and EPL concentrations of 2 wt% and 0.75 wt%, respectively, for further characterizations and biological evaluation.

Both alginate and EPL are considered weak PEs, and therefore their charge density can be easily tuned by varying the pH of the solution.[32] The ability to vary the charge density of weak PEs enables the control over properties of the complexed material.[33] Therefore, we further evaluated the role of pH on the formation and stability of the fiber-shaped material. More robust fibers were formed when the pH of the global system decreased to 5, while increasing the pH to 9 seemed to even prevent the formation of fibers (Figure 2, B). This result was deemed to be mainly influenced by the degree of protonation and conformation of EPL, since the charge density of alginate (pKa ~ 3.2-3.6) was previously shown not to be significantly influenced by solutions' pH above 5.[34] At acidic pH, the repulsion of protonated amine groups of EPL might promote an electrostatically expanded conformation which favors the interaction with the carboxylic groups from alginate.[35] As the pH increases, EPL structural conformation starts to fold resulting in less exposed amine groups accessible to ionization, and thus the positive net charge decreases.[35] This may explain the slight decrease in the speed of complexation when increasing the pH from 5 to 7, examined by the degree of darkening (Figure 2, B). EPL has an isoelectric point around 9.0[36], meaning that at this pH the net charge of the molecule is electrochemically neutral, consequently, interfacial electrostatic interaction between the PEs is mostly absent, which may had prevented the formation of the membrane bounded fibers. This is confirmed by the higher transparency of the formed fibers at pH 9. Considering these findings, the stability yield of structures able to be preserved after washing and overnight incubation was evaluated for fibers obtained using phases at different pH (Figure 2, C). In general, a decrease in stability with increasing pH of phase II, where EPL was dissolved, was observed. At pH 5, the interfacial membrane formed more quickly yielding highly stable structures, while at pH 9, structures barely formed. For phase I solution (containing dextran and alginate) at pH 9, the yield values for pH 5 and 7 of phase II were lower compared to pH 5 and 7 of phase I (Figure 2, C). The assembly of PEs in an ATPS depends not only on the electrostatic interaction between the PE's charged groups but it is also critically influenced by the affinity and partitioning-induced distribution of the PEs in the distinct polymer phases.[37] Based on the theory purposed by

Ma *et al.* which states that an increase in pH may enhance hydrogen bonds between polyelectrolytes and the hydroxyl groups of dextran,[37] we hypothesize that higher pH values in our solutions could have increased the affinity of alginate towards the dissolving phase. Consequently, its availability at the interface would be decreased, culminating in the reduced stability of the produced fibers. In summary, it was possible to control the formation speed and stability of formed fibers structures, both in charge dependent and apparently independent manners, by tuning the pH of the system.

The processing of fiber-shaped biological materials requires aqueous conditions with controlled temperature, pH, and ionic strength to enable cell viability and bioactivity of some incorporated sensitive molecules.[3] Here, by controlling PEs' concentration, complexation time and pH, we demonstrate the ability to fabricate stable fibers able to persist in *in vitro* cell culture conditions.

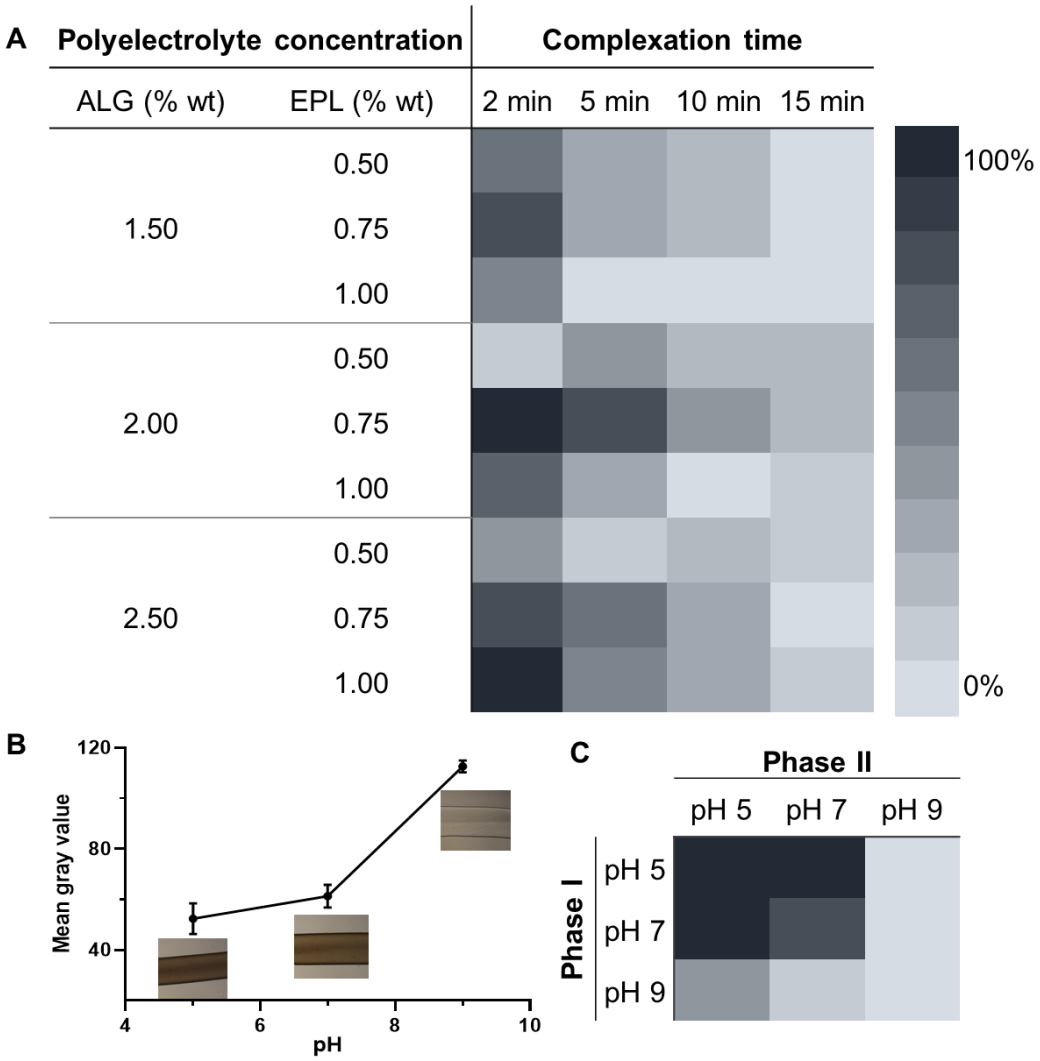

Figure 2. A) Stability yield measuring the ability of the fibers to remain intact after 3x PBS washing, and an overnight incubation period at 37ºC (n=10 fibers). B) Mean gray value determined for microscopic images of different fibers formed in a system with a global pH of 5, 7 and 9 (means that both phase I and II were adjusted to the related pH). C) Analysis of the yield-related stability and structural integrity of fibers formed in systems with varying phase I and II pH values (n=10 fibers).

2.2. Morphological and mechanical characterization

The effect of increasing complexation time on the properties of the materials was assessed by scanning electron microscopy (SEM) analysis, mechanical tensile tests and swelling studies. SEM micrographs of cross-sectioned and dehydrated fibers with different complexation times are shown in Figure 3, A. The tubular feature of the fabricated structures could be confirmed especially for increased complexation times (10 and 15 minutes), while for 2 and 5 minutes, the dehydration process led to the collapse of the structure due to the low thickness of the membrane, resulting in a lack of structural integrity. The internal lumen of the tubes formed after 10 and 15 minutes of complexation presented an interesting microtextured surface. These topographical features may be related with the generation of secondary structures consisting of microdroplets that could be visualized on microscopic images (Supplementary Figure 3, Supporting Information). Considering that the used ATPS's phases are not derived from an equilibrated system and that the components dissolved in the solutions may be able to diffuse from one phase to another (taking into account the molecular weight cut-off of the membrane that separates both phases and also the viscous nature of the inner phase), we hypothesize that these droplets may be associated with phase separation phenomena induced by the slowly diffusion of both polymers and PEs. In fact, microscopic images of two-phase formation show the formation of microdroplets indicative of liquid-liquid phase separation.[13,15]

In the field of tissue engineering it is important to assess the mechanical properties of biomaterials, not only to predict the performance *in vivo* but also due to their key role in regulating cell behaviors such as migration and differentiation.[38,39] We observe a tendency for an increasing of the (tensile) elastic modulus with the increase in the complexation time (Figure 3, B). A statistically significant difference of almost two-fold was detected between 5 min (15 ± 0.7 kPa) and 15 min (27 ± 1.9 kPa), meaning that stiffer fibers are produced for increased complexation times. Indeed, more PE molecules would interact

with each other with time, inducing the stiffening of the membranes formed at the interface of the system. It is worth to clarify that tensile stress measurements were performed using the fibers in a wet state (in PBS) in order to mechanically characterize the structures in conditions relevant in a clinical scenario. Therefore, softer fibers may have been obtained from lower times of complexation due to their ability to absorb higher amounts of water, as corroborated in swelling tests discussed later. Using this system, the mechanical performance of fibers could be easily tuned by the complexation time, presenting a range of stiffnesses resembling some native tissues such as cardiac and skeletal muscle[40] providing them with potential for cell support and tissue integration.

A volumetric expansion of the fibers was observed upon washing in PBS, which was attributed to the disruption of the interfacial tension barrier from the ATPS after complete removal of phase II during the washing. The disturbance of the ATPS may had stablished osmotic gradients that induced the movement of water molecules and consequent fiber expansion.[20] The expansion process was monitored over time by registering the changes in fibers' diameter. Figure 3, C summarizes the swelling ratio obtained over time for fibers prepared with different complexation times. The average swelling ratio increased with time and eventually reached equilibrium after 40 minutes for all the conditions. Moreover, fibers washed after a reduced time of complexation (2 and 5 min) could expand more than fibers with 10 and 15 min of complexation. Previously, we demonstrated that lower complexation times led to the formation of softer fibers, therefore, their membrane can more easily deform by swelling, and fibers expand more upon the entrance of water, while stiffer fibers resist more to expansion resulting in lower swelling ratios.

Finally, fibers' external diameter could be easily tuned by changing the needle used to deposit phase I into phase II (Figure 3, D). This approach allowed stablishing a range of low-dispersion external diameters comprising macrometer (1.50 ± 0.2 mm) to micrometer scales (0.17 ± 0.0 mm). Due to their water-swelling properties, the diameter significantly increased for all the tested needle sizes after the washing process.

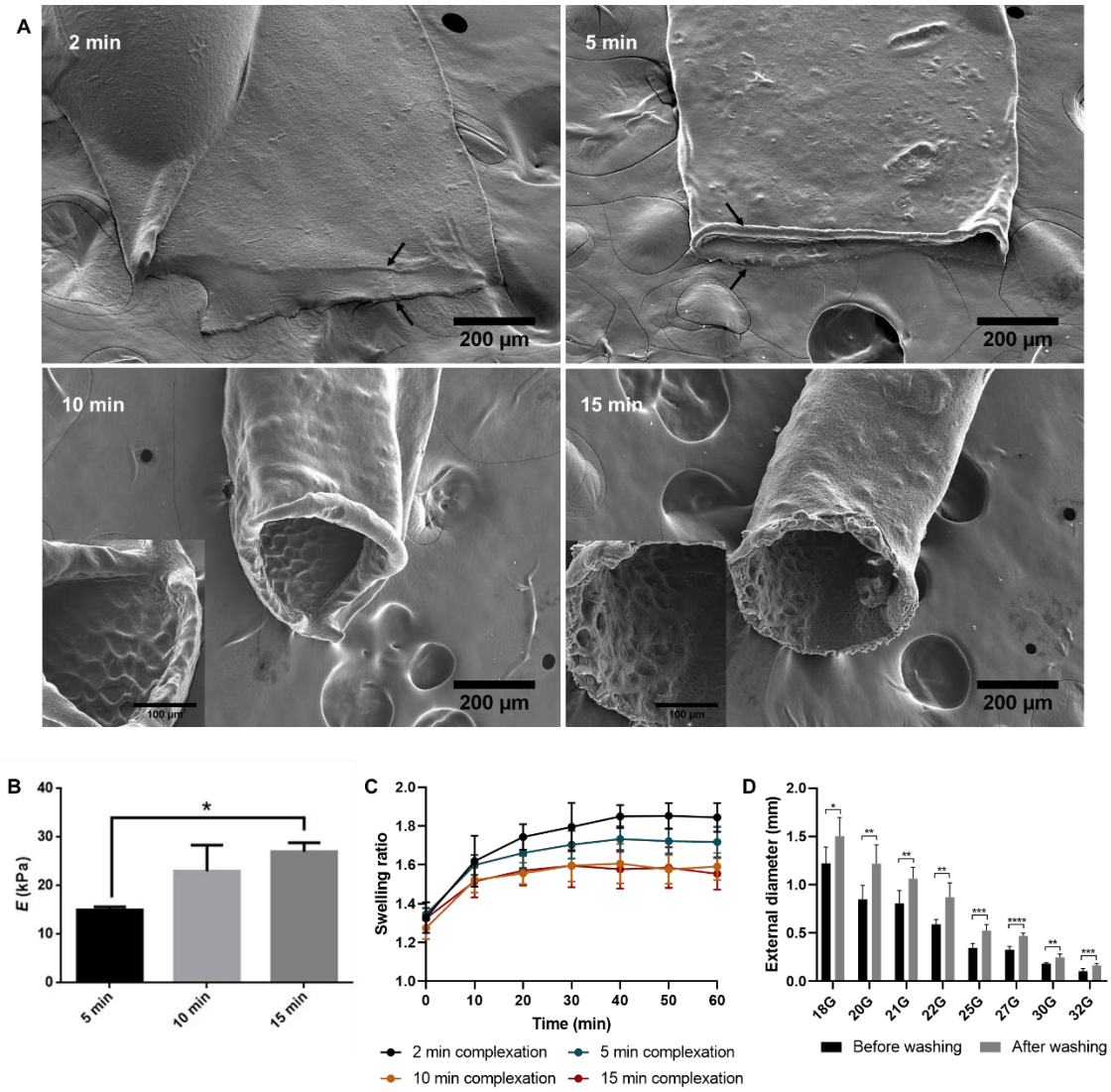

Figure 3. A) SEM micrographs of cross-sectioned fibers with different complexation times demonstrating hollow features. Arrows indicate upper and lower layers of collapsed structures with 2 and 5 minutes of complexation. B) Young's modulus obtained for 5, 10 and 15 minutes of complexation. No measurements were possible for fibers with 2 minutes of complexation due to increased fragility. C) Swelling ratio over time of fibers fabricated at different complexation times, when exposed to PBS. D) External diameter after processing and after removing the bath consisting of phase II and 3x washing with PBS. * indicates a statistically significant difference with $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, and **** $p < 0.0001$.

3. Liquid-core fibers as cell encapsulation systems (*in vitro* cytocompatibility)

The stability and robustness of the system in cell culture-like conditions offers an exciting possibility to develop biological materials relevant for cell colonization and encapsulation. This potential was assessed by mixing mesenchymal stem cells derived

from human adipose tissue (hASCs) in phase I, and producing homotypic fiber-shaped materials using the syringe-needle method in sterile conditions. Mesenchymal stem cells have been considered promising therapeutic cells for the regeneration of damaged tissues. This potential is mainly associated to their differentiation potential into many specific cell types, as well as to paracrine effects characterized by the release of a variety of trophic factors that have been related to their capacity to modulate the immune system, promote cell survival and proliferation, and enhance angiogenesis.[41]

During the optimization process, an EPL concentration-dependent as well as complexation time-dependent cytotoxicity was visible (Supplementary Figure 4, Supporting Information). Although EPL has a relatively low toxicity against mammalian cells[42] and studies on EPL-based materials, namely EPL-polycaprolactone copolymer nanoparticles, with EPL in its insoluble form, have shown no apparent cytotoxicity on mammalian cells at maximum concentrations of 1 mg/mL[43]; here, the conditions required for the formation of stable fibers seemed to considerably affect the viability of the cells. This way, further cell encapsulation procedures aimed to reduce as much as possible the contact of EPL with cells by reducing complexation time (also compatible with fiber stability) and wagering on the washing step. Another aspect that was taken into account was the anchorage-dependency of MSCs.[44] To potentiate cell adhesion, alginate functionalized with the RGD sequence was used for the assembly of the fibers. This peptide sequence is present in several extracellular matrix proteins, and is responsible for mediating cell adhesion through integrin-binding.[45] The purpose of including alginate-GRGDSP was to further promote cell adhesion throughout the structure while maintaining the processing requirements for the generation of the material enabling PE complexation with EPL molecules.

The viability of cells encapsulated in the fibers was monitored by fluorescence microscopy using a live/dead staining, and cellular metabolic activity was monitored up to 7 days of cell culture (Figure 4, A). hASCs adhered and showcased a spread morphology in fibers on day 4, and a similar behavior was observed for 7 days of culture, with the formation of an increasing number of interconnected networks with neighboring cells. These data highlight the biocompatibility and potential of the developed material to support cell survival and growth. Although a significant decrease in cell metabolic activity was observed after 4 days of culture, cellular activity recovered after 7 days (Figure 4, A).

Apart from few avascular tissues, almost all human body tissues depend on stable vascular networks for proper function and survival.[46,47] This key role of blood vessels has led to increasing exploitation of strategies to improve long-term survival and *in vivo* integration of bioengineered tissues based on angiogenesis stimulation and development of vascular structures, where the incorporation of endothelial cells has been shown to be crucial.[8] Having this in mind, human umbilical vein endothelial cells (HUVECs) were co-encapsulated with hASCs. The density of hASCs was maintained, and additional HUVECs were mixed.[48] The use of co-culture systems plays key roles in the establishment of cell−cell communications responsible for modulating each other cells functionality and biological activity.[49] One important role of mesenchymal stem cells in the regenerative process has been associated to angiogenesis promotion.[41] Previously reported literature has been shown that ASCs can support angiogenesis through paracrine secretion of proangiogenic factors such as VEGF, and through cell differentiation into vascular phenotypes.[50,51] Moreover, their paracrine functions have been also associated to the release of extracellular vesicles such as exosomes and microvesicles containing coding and non-coding RNAs, that modulate endothelial cells' behavior promoting their proliferation, invasion and tube formation.[52,53] Here, hASCs were hypothesized to help increasing the survival and proliferation of HUVECs through intercellular interactions, facilitating the pre-endothelization of the artificial tube wall. Of note, preliminary studies on HUVEC-only systems showed high cell death after 7 days of culture (Supplementary Figure 5, Supporting Information). Live/Dead analysis of heterotypic fibers (hASCs+HUVECs) revealed increased cell spreading and interconnected network formation at days 4 and 7 days (Figure 4, A). Moreover, when compared to hASCs-only fibers, initial increased cell death was observed, which was speculated to be mostly related to encapsulated HUVECs (Figure 4, A). The overall metabolic activity of the co-cultured system showed a statistically significant increase at day 7. However, to confirm that endothelial cells effectively survived in co-encapsulation systems, immunostaining of the CD31 endothelial surface marker was performed after 14 days of culture (Figure 4, B). CD31 is a specific adhesion molecule expressed on the membranes of endothelial cells, and not on MSCs.[54] The presence of CD31-expressing cells with spread morphology at day 14 allowed confirming endothelial cell survival in the encapsulation system. The cytocompatibility of

both mono- and co-culture systems highlights the potential of the reported system as an effective cell encapsulation device for efficient promotion of regenerative processes.

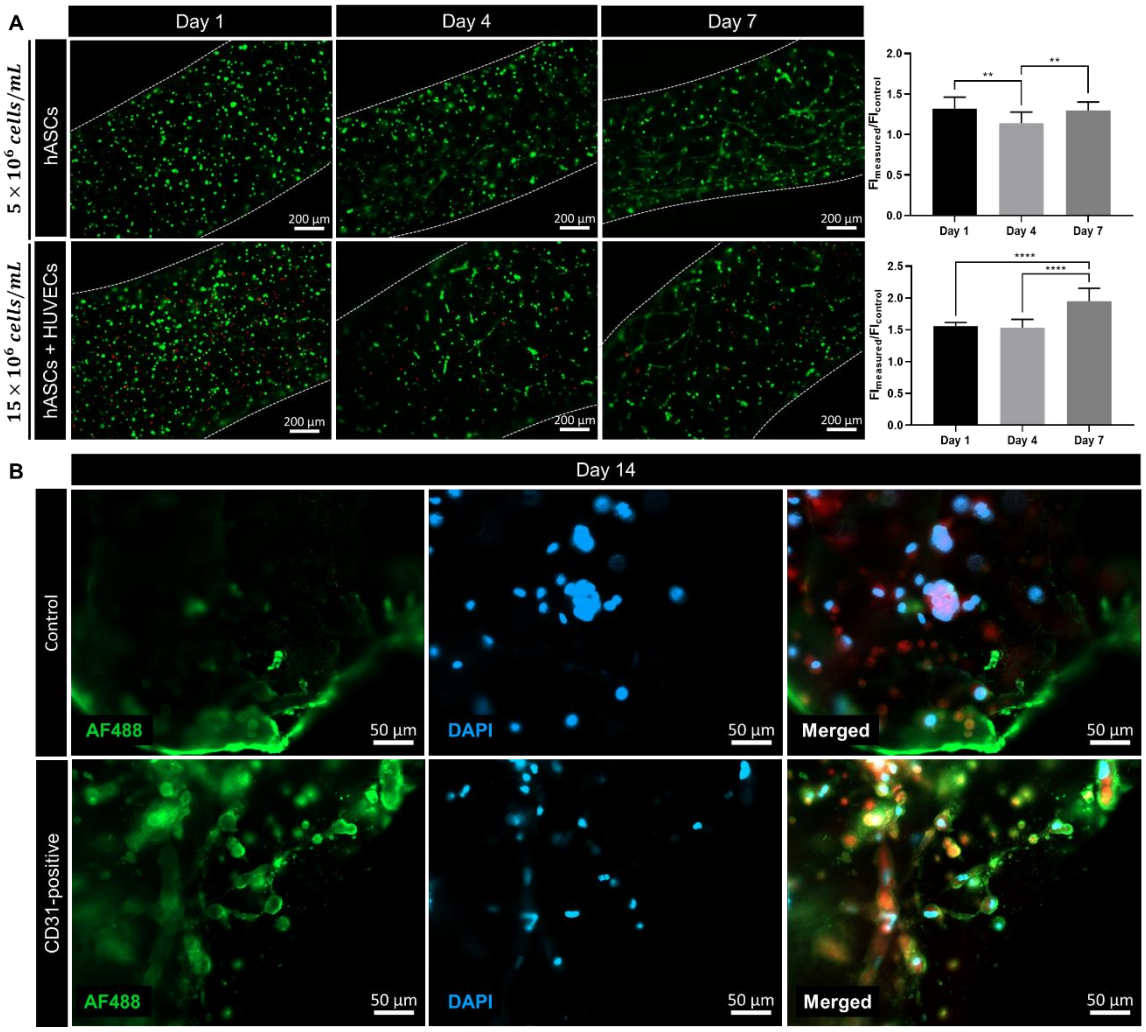

Figure 4. A) Cell viability at days 1, 4 and 7 of culture. In the left, Live/Dead micrographs of homotypic fibers with hASCs and heterotypic fibers containing both hASCs and HUVECs. Green: calcein-AM (live), red: PI (dead). Scale bars: 200 µm. In the right are the respective cell metabolic activity measured by the fluorescence intensity using AlamarBlue assay. Samples with size of approximately 0.5cm of diameter and 1cm long were used (n = 5 replicates). ** and **** indicates statistical significance with $p < 0.01$ and $p < 0.0001$, respectively. B) Immunostaining (CD31, green) of the heterotypic fibers at day 14, counterstained with DAPI (cell nuclei, blue) and phalloidin (F-actin filaments, red). Green fluorescence background was observed in the acquired micrographs for both control (without anti-CD31 incubation) and CD31-staining conditions, caused by the entrapment of the fluorescent-labeled secondary antibody (AF488) in the material.

Conclusions

In summary, we stablished a rapid and straightforward method for directly fabricate stable fiber-shaped materials via interfacial complexation of oppositely charged natural polyelectrolytes in an all-aqueous environment. Our system enables the generation in a single step of straight and geometrically complex fibers with a Y-shape, with features of tubular tissues after end removal, enabling the perfusion of liquid fluids. The diameter of the structures could vary from millimeter to micrometer sizes depending on the size of the needle, and their mechanical and swelling properties could be tuned by adjusting the time of complexation. Optimized polyelectrolyte concentrations, complexation time and pH in completely aqueous conditions led the production of devices with potential to encapsulate delicate cargos and maintain their bioactivity. Mesenchymal stem cells could be easily encapsulated within the fiber material, and could adhere and spread for up to 7 days, in tubes prepared with alginate containing cell adhesion peptide sequences. Encapsulated endothelial cells in co-culture with the stem cells survived for medium-term culture conditions.

The free-standing and tubule-like nature of the reported materials offer potential application in the tissue engineering field enabling its integration into three-dimensional matrices as well as be connected to perfusion sources. Moreover, the cytocompatibility allied with inherent free-form properties of these fibers may offer desired spatial control to fill tissue defects in the scope of regenerative medicine. Compared with mostly applied spherical-shaped cell encapsulation systems, which are individual and small elements that can be easily lost in the time of injection, the fiber can be positioned with more accuracy and stability in the damaged or defected tissue.

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Chapter IV
Conclusions and Future Perspectives

Conclusions and Future Perspectives

In the field of tissue engineering, scientists have been realizing over the years that increasing biomimicry in *in vitro* constructs is an important and key step towards the achievement of effective regenerative therapies, and also for generating more reliable tissue or disease models for fundamental studies or discovery of new drugs. Hence, there is a well-identified need for the fabrication or inclusion of vascular structures *in vitro*, since they are present in almost all tissues of the human body, performing fundamental functions for their development and survival, being also a key element in several processes such as tissue regeneration, wound healing and tumor invasion. Several strategies have been developed to vascularize bioengineered constructs, one of which include the fabrication of tubular structures, that allow the internal flow of liquids and enable the adhesion of endothelial cells, recapitulating the features of native blood vessels to a certain extent. However, fast and easy-to-implement methods, that allow the manufacture of sufficiently stable and robust structures in a single step and in mild conditions necessary for cell survival, are desirable.

Here, by combining inherent properties of aqueous two-phase systems and polyelectrolyte complexation, hollow-core materials could be produced in a rapid and straightforward manner. By dispersing a phase solution containing alginate into another containing EPL, a coacervate-like membrane formed at the interface of the solutions allowing the generation of hollow fiber-shaped materials in a single step, with complex geometries and unrestrictive free-form deposition. These structures could withstand the perfusion of fluid without disrupting their structural integrity, and may have a wide range of sizes varying from macro- to micrometer scales. Moreover, tunable mechanical and water-swelling properties were attained by simply adjusting the complexation time between the oppositely charged polyelectrolytes. More importantly, optimized conditions allowed the formation of stable fibers in cell culture conditions, enabling their application as cell encapsulation systems. Human adipose-derived mesenchymal stem cells could remain viable and proliferate up to 7 days, and seem to have positively impacted the survival and viability of endothelial cells when in co-culture.

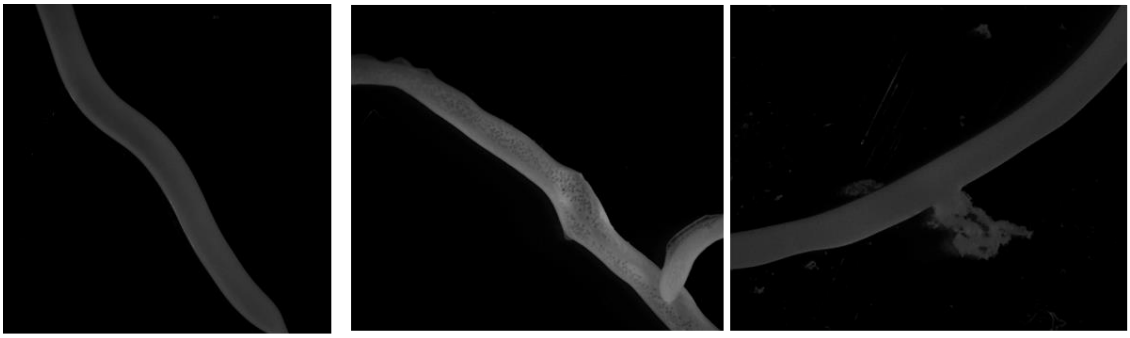
Despite the promising results in terms of cell viability, that is still room for improvement, as well as for understanding the potential of the reported system as a pro-regenerative device. Studying cell response in extended timepoints of 14 and 21 days

would be of great importance, in order to understand if the increase in cell proliferation continues for prolonged culture periods, since preliminary studies after 14 days of co-culture systems have shown promising results. Moreover, immunostaining on heterotypic fibers with other endothelial cell markers such as vWF may be used to corroborate results from CD31 immunocytochemistry. It would be also interesting to understand if cell encapsulated fibers can release important trophic factors such as bFGF and VEGF to the outside of the generated fibers, by using ELISA assays, and thus prove their ability to promote cell proliferation and angiogenesis.

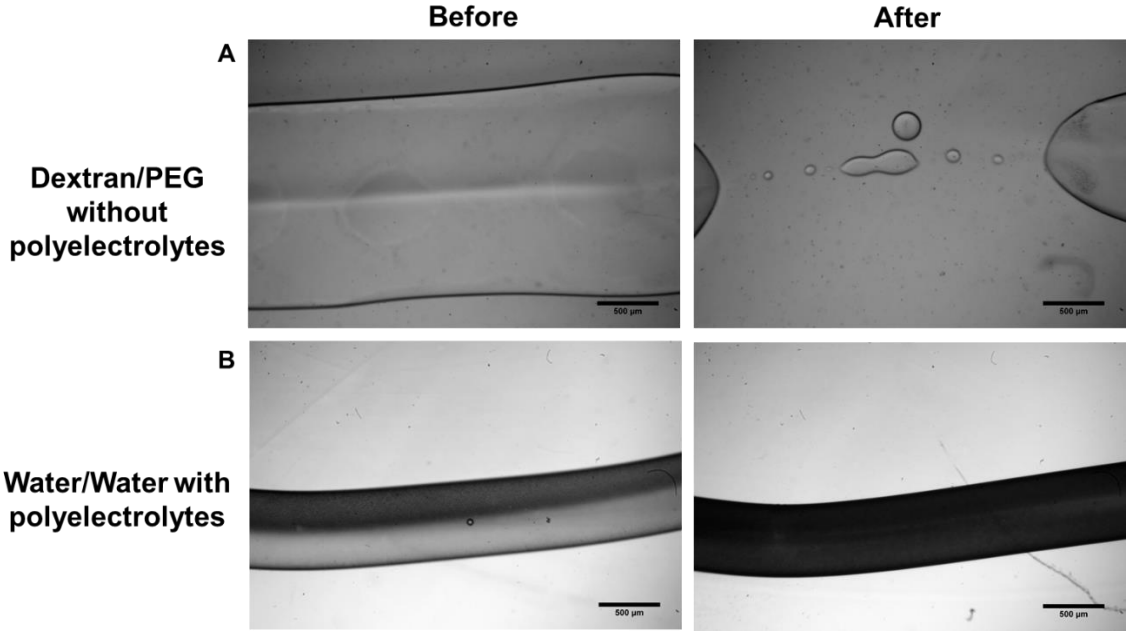
Supporting Information

Supplementary table 1. Summary of the optimization process regarding the production and stability of fiber-shaped material. Representative images of stable and not stable (open) structures are showcased below.

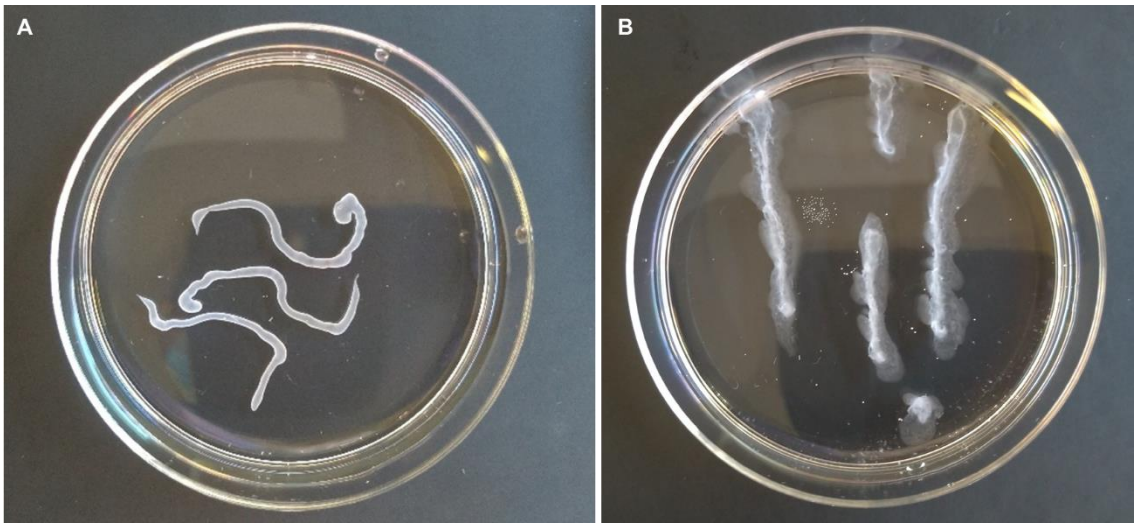
Optimization parameter	Helped increasing stability of the structures?
Using polymer phases derived from a phase-equilibrium system	✗
Increasing PEG molecular weight (20 kDa)	✗
Controlling the flow rate of phase I	✓
Agitation during complexation time	✓
Increasing complexation time	✗
Increasing polyelectrolytes concentration	?



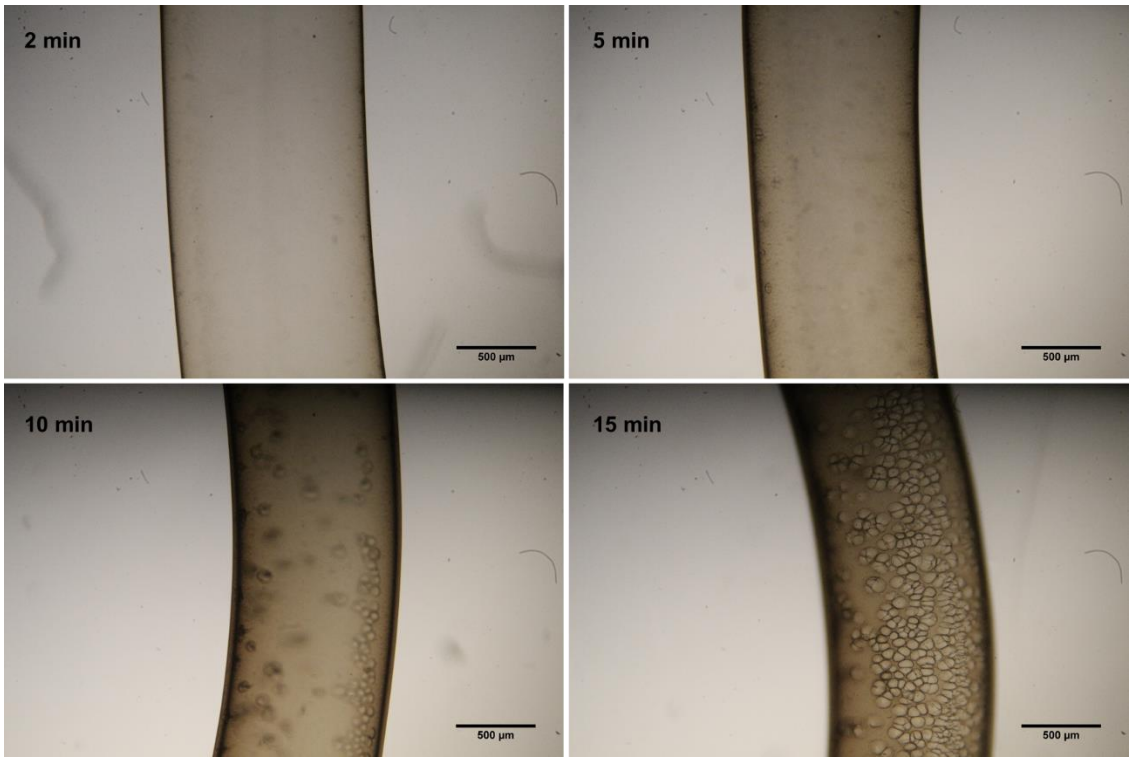
Stable structures | Open/not stable structures



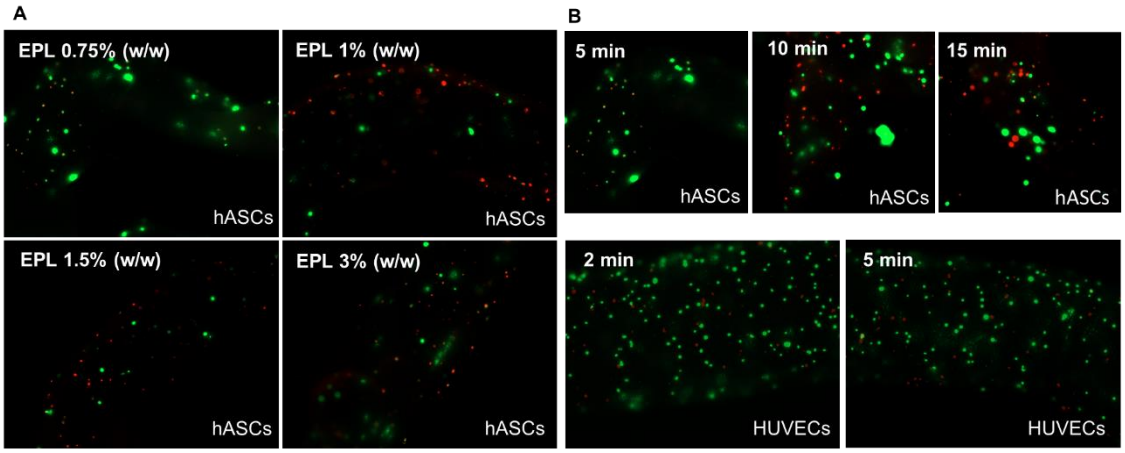
Supplementary Figure 1. Comparison of control conditions consisting of structures produced in the ATPS system without the polyelectrolytes (alginate and EPL) (A), and in aqueous solutions of the polyelectrolytes without the presence of the ATPS (B). Microscopic images on the left show the initial state of the structures and on the right images after few seconds (for the A condition) and after 5 minutes (for the B condition). Without the polyelectrolytes, the segment starts to breakup into droplets, while materials produced in the absence of the ATPS induced by polyelectrolyte complexation are formed very rapidly but are very sticky to substrates and to each other (hard-to-handle). It was used concentrations of 15 wt%, 17 wt%, 0.75 wt% and 2 wt% for dextran, PEG, EPL and alginate, respectively.



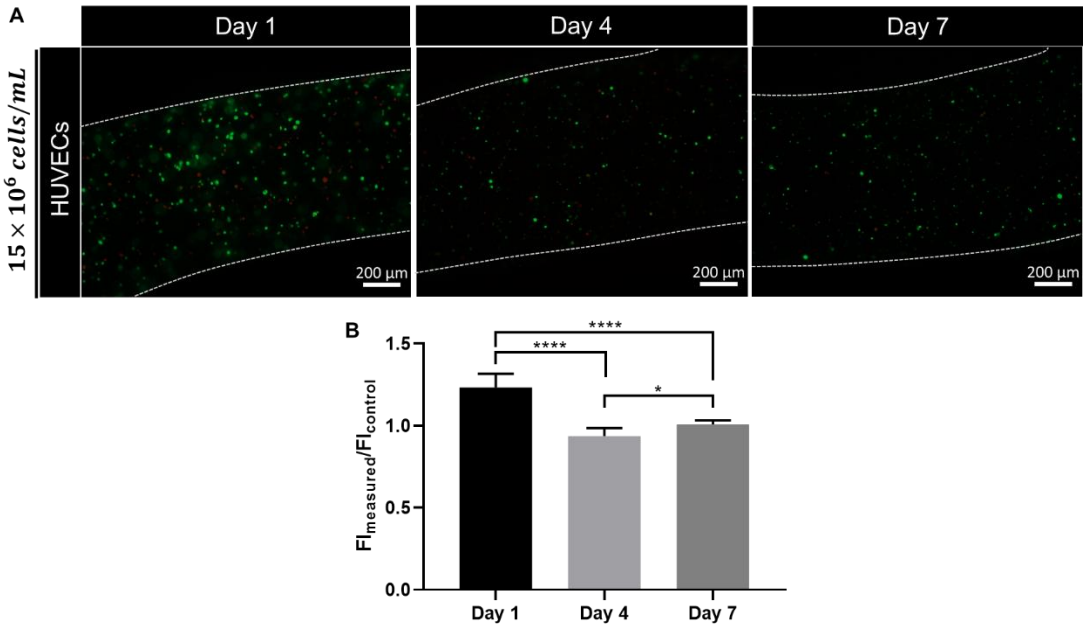
Supplementary Figure 2. The solution where the polyelectrolytes are dispersed affects structure formation. A) When the outer phase is composed of PEG and EPL and the inner phase of dextran and alginate, stable fiber-shaped materials can be fabricated. B) When the outer phase contains PEG and alginate, and the inner phase dextran and EPL, the structures open and fall apart. It was used concentrations of 15 wt%, 17 wt%, 0.75 wt% and 2 wt% for dextran, PEG, EPL and alginate, respectively.



Supplementary Figure 3. Increasing complexation time led to the formation of microdroplets inside the fiber structure. Microscopic images of fibers immersed in PBS after 2, 5, 10 and 15 minutes of complexation. It was used concentrations of 0.75 wt% and 2 wt% for EPL and alginate, respectively.



Supplementary Figure 4. Cytotoxic effect of EPL concentration and complexation time at day 1. A) Increasing concentration of EPL decreases cell viability in a representative model of fibers encapsulated with human adipose-derived stem cells (hASCs). B) Lower complexation times favors cell viability both in hASCs and human umbilical vein endothelial cells (HUVECs).



Supplementary Figure 5. Cell viability of HUVEC-encapsulated fibers analyzed by live/dead (A) and AlamarBlue (B) assays. The results showed a significant decrease in cell viability within 4 days and up to 7 days, indicating the poor ability of the system to promote endothelial cell survival and growth.