Review

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The Use of Microtechnology and Nanotechnology in Fabricating Vascularized Tissues

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Tissue engineering (TE) is a multidisciplinary research area that combines medicine, biology, and material science. In recent decades, microtechnology and nanotechnology have also been gradually integrated into this field and have become essential components of TE research. Tissues and complex organs in the body depend on a branched blood vessel system. One of the main objectives for TE researchers is to replicate this vessel system and obtain functional vascularized structures within engineered tissues or organs. With the help of new nanotechnology and microtechnology, significant progress has been made. Achievements include the design of nanoscale-level scaffolds with new functionalities, development of integrated and rapid nanotechnology methods for biofabrication of vascular tissues, discovery of new composite materials to direct differentiation of stem and inducible pluripotent stem cells into the vascular phenotype. Although numerous challenges to replicating vascularized tissue for clinical uses remain, the combination of these new advances has yielded new tools for producing functional vascular tissues in the near future.

Keywords: Nanotechnology, Microtechnology, Tissue Engineering, Vascularization.

CONTENTS

1. INTRODUCTION

Nanotechnology and microtechnology have made significant contributions to the field of medicine in recent years. In particular, microtechnology and nanotechnology have significantly been involved in a new scientific discipline know as tissue engineering (TE).[1] The goal of TE is to fabricate, repair, and/or replace tissues and organs using cell technology, medicine, advanced materials, and engineering approaches (Fig. 1).[2,3] Although several advances have been achieved in this field, more work is required for their clinical application of engineered tissues.[4] Today, clinical application in human regenerative medicine has been achieved for only skin, cornea, and cartilage transplants.[5,6] Only these tissues can be supplied with nutrients and oxygen via diffusion from distant blood vessel systems.[7,8] The relative lack of functional engineered tissues can be attributed to our current inability to fabricate engineered blood vessels within engineered tissues.

Tissues and complex organs in the body depend on a branched blood vessel system with a maximum separation of $< 200\ \mu m$[9,10] within vessels. These tissues are

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irrigated with blood by a highly branched system of large blood vessels *in vivo*[11] (Fig. 2). Strategies proposed to introduce vascularization in fabricated tissues include a cell-based approach, where endothelial cells from various sources are induced to form new vessels within the cells forming the tissues, with the process being referred to as neonangiogenesis.[12] This strategy relies on cell–cell interactions, growth factors, and the pluripotent properties of used embryonic stem cells or inducible pluripotent stem (*i*PS) cells.[13, 14] Another strategy focuses on giving external support to endothelial cells to generate a three-dimensional (3D) capillary network. Following this, novel scaffolds and devices that potentially confer fine control over cellular positioning, organization, and interactions have been developed. Continuing development in microtechnology and nanotechnology has profoundly contributed to the improvement of both production methods and analytical tools in this strategy.[15–19] The two strategies

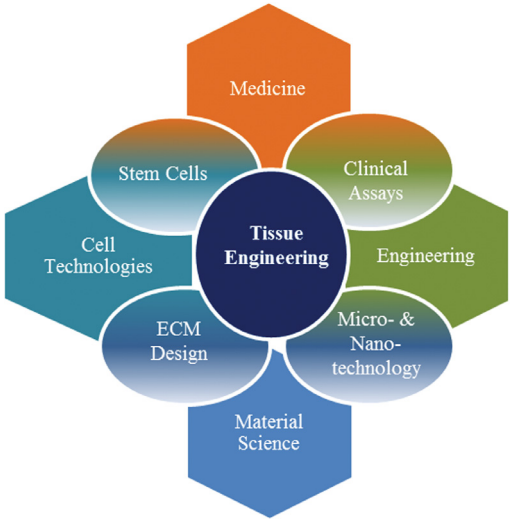

Figure 1. Schematic representation of the different areas of expertise involved in a tissue engineering (TE) field of research. TE is a multidisciplinary science that aims for the integration of nanotechnology and microtechnology with medicine and cell knowledge.

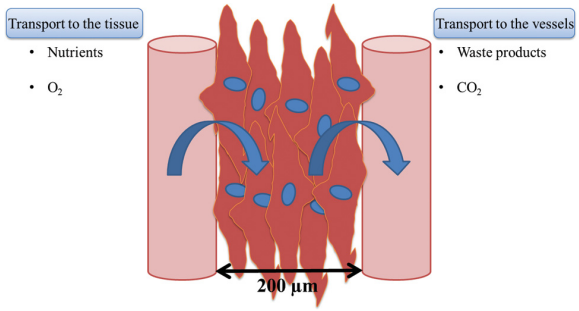

Figure 2. Schematic description of diffusion and transport processes in vascularized tissues *in vivo*. The surrounding tissue is supplied with oxygen and nutrients via the vasculature. Waste products and CO_2 are carted away from the tissue into the blood vessels. The maximum distance between these capillaries is 200 μm, which correlates with the diffusion limit of oxygen.

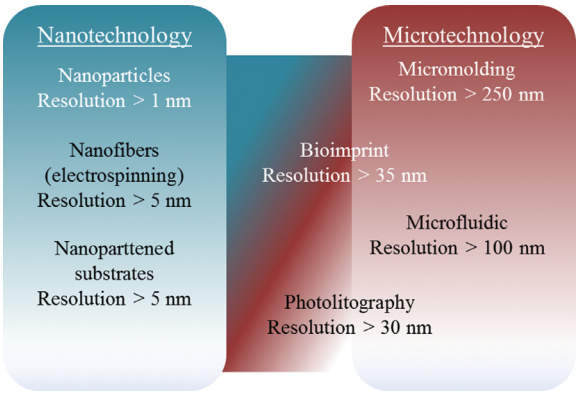

Figure 3. Summary of the nano-microtechnologies most used in vascularized engineered tissues.

cannot be clearly differentiated because endothelial cells play a key role in both approaches.[10,20] To create vascular structures, different behaviors of endothelial cell, such as migration and growth are regulated in a complex manner by the surrounding biomaterial surface, supporting cells, growth factors, and hemodynamic forces.[21–23] Microtechnology and nanotechnology provide novel TE vascularization techniques. Positioning of cells, delivery of molecules, and design of the extracellular matrix (ECM) from the nanoscopic scale to the macroscopic scale can be achieved using these novel technologies.[24] Figure 3 shows the main technologies currently used in TE applications and their distribution between the microscopic and nanoscopic scales. By definition, nanotechnology deals with objects in the range of 1–100 nm, but as can be seen in the figure, some techniques could overlap both scales. This review focuses on the principles of microtechnology and nanotechnology, their use to fabricate vascularized tissues or promote tissue vascularization, and it highlights the most promising works in this research area.

2. NANOTECHNOLOGY

In the last decade, the number of nanotechnology-related papers in TE journals has dramatically increased. Nanotechnology has had a great impact in the fields of regenerative medicine and TE. New biomaterials fabricated using nanotechnological approaches can be designed at the molecular level, improving the interactions of cells with the materials. Even though the size of cells is on the micrometer scale, they are strongly influenced by the topographical and structural properties of ECM at the nanoscale level. Nanostructuralized and nanopatterned scaffolds can control the differentiation of pluripotent stem cells toward the vascular phenotype. Several nanotechnological strategies for using nanoparticles in vascularized TE have been emerged, with most of them being based on nanoparticles with magnetic properties for magnetic cell positioning and the delivery of biomolecules. To date, interesting reviews of nanotechnological approaches to solving the TE vascularization problem are available in the literature.[19,25–30]

2.1. Nanoparticles

The main feature of nanoparticles is their facile attachment or incorporation into cells by endocytosis. This property leads to two main applications for nanoparticles in vascularization, i.e., (1) delivery of biomolecules and (2) cell positioning using magnetic forces. Nanoparticles play an important role in the delivery of biomolecules.[31] Biomolecules (drugs, deoxyribonucleic acid, and growth factors) can be encapsulated within or on nanoparticles and released in a controllable manner in a localized area. Vascular endothelial growth factor (VEGF) is one of the most important and widely used growth factors for vascularization. VEGF has been attached via heparin to a nanoparticle/fibrin complex.[32] Endothelial cells treated with these complexes have shown efficient vascularization. The local and sustained release of growth factor avoids the administration of high doses of VEGF, which can lead to severe side effects including undesired vascularization of non-target areas, tumor release, hypotension, and edema.[32,33] In addition, since VEFG rapidly degrades, its attachment to heparin/chitosan nanoparticles preserves its biological activity.[34] It is also feasible to covalently introduce functionalized nanoparticles into decellularized scaffold. Therefore, the release and activity of attached growth factors to nanoparticles were sustained for several weeks.

Cells coated with iron oxide nanoparticles can be driven by magnetic forces to the desired locations. Therefore, it is possible to control the cell seeding, adhesion, and attachment.[35,36] Several approaches have employed magnetic particles for vascular TE.[37–42] In one approach, cells coated with magnetic microparticles were introduced into a decellularized tubular scaffold and magnetically moved to the internal surface of the scaffold.[41,42] In a similar approach, iron oxide nanoparticles were used in combination with endothelial cells[38] (see Fig. 4(a)). In another interesting approach, a cylindrical magnet was rolled into a cell sheet, forming a tube around the magnet. The magnet was then removed, leaving behind a tubular structure.[39] Magnetic force-driven techniques have also been combined with organ-printing techniques for vascularization.[43,44] Recently, 3D white adipose tissue with engineered vascular vessels has been also synthesized using magnetic nanoparticles and cell seeding.[45] It is important to assess the cytotoxic effects of nanoparticles on endothelial cells. Although nanoparticles can induce endothelial activation,[46] they can be internalized by cells. Because of their large surface area, these particles can react in different ways inside the cell.[47] More studies will be necessary before these techniques can be applied in the clinical setting.

Carbon nanotubes (CNTs) are nanoparticles that could be used as scaffolds to promote vascularization. CNTs

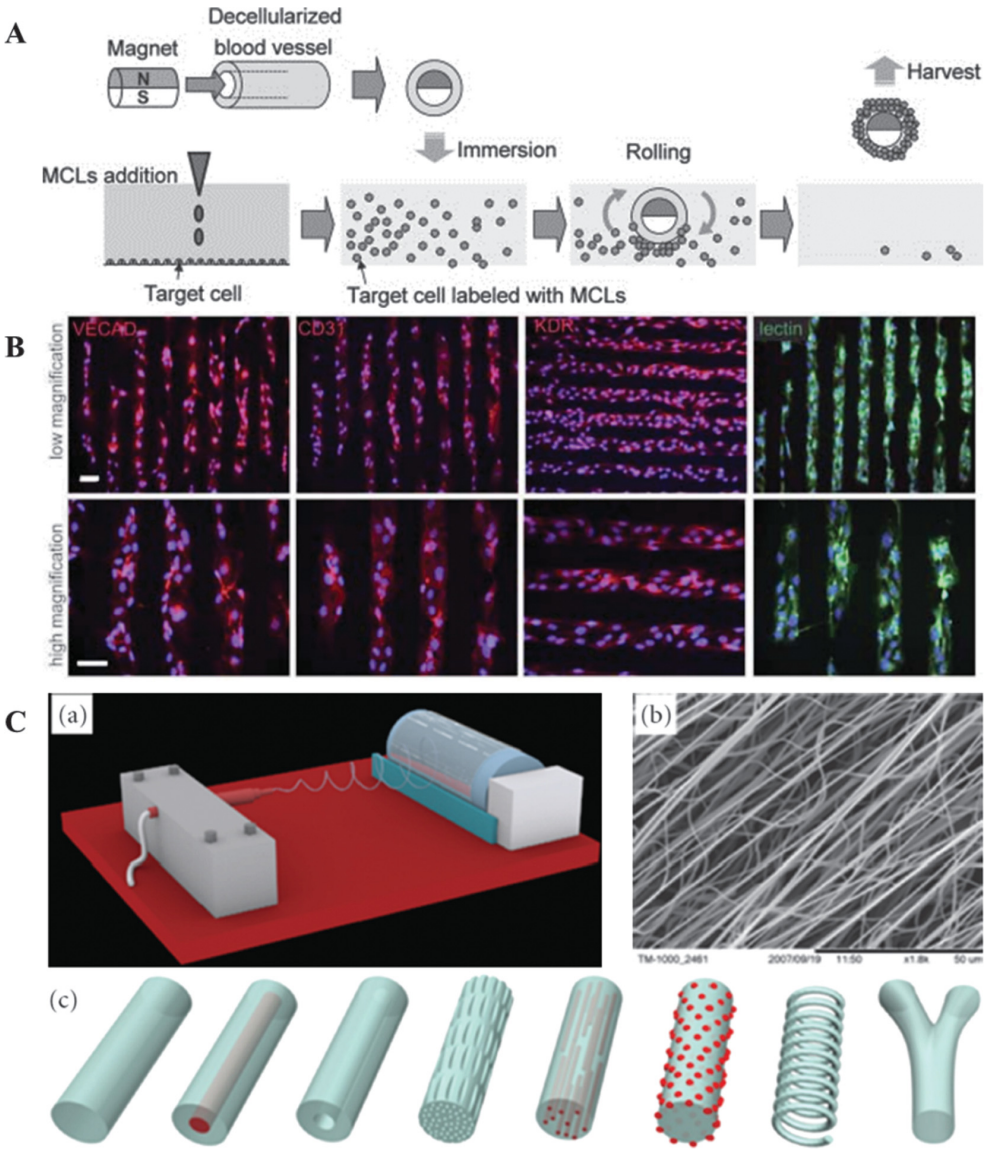

Figure 4. Nanotechnological approaches to fabricate blood vessels. (A) Magnetic nanoparticles were added to the medium to label target cells magnetically. Decellularized blood vessel into which a magnet was inserted was immersed in the cell suspension and rolled in the suspension to seed cells evenly. Reprinted with permission from [38], A. Ito, et al., *Tissue Eng.* 11, 1553 **(2005)**. © 2005, Elsevier. (B) Human endothelial progenitor cells cultured on a nanopatterned fibronectin structure. Reprinted with permission from [54], K. Seunarine, et al., *J. Vac. Sci. Technol. B* 24, 3258 **(2006)**. © 2006, The Royal Society of Chemistry. (C) (a) Scheme demonstrating principle of electrospinning technology and electrospinning apparatus, (b) scanning electrode image of an electrospun, (c) scheme showing different classes of nanofibers that can be obtained by electrospinning for blood vessels fabrication. Reprinted with permission from [30], D. A. LaVan, et al., *Nat. Rev. Drug Discov.* 1, 77 **(2002)**. © 2002, Elsevier.

have advantageous properties for TE applications, including high conductivity, high surface area, and strong mechanical properties. Especially interesting is the use of CNTs in combination with established scaffolds to produce a new type of hybrid materials, combining the properties of both materials.[48] CNTs were recently used in combination with poly(vinylidene fluoride) polymer to promote cell response, and their biological response was evaluated.[49] The hybrid CNT-polymer material resulted in a significant increase of vascularization after implantation in animal models.

2.2. Nanopatterned Surfaces

Surface-patterning techniques integrate several fabrication methods including electron-beam lithography; nanoimprint lithography; photolithography, including micro-electrical-mechanical systems (MEMS); nanocontact printing; micromachining; and 3D printing.[50] Patterned surfaces

with proteins and growth factors on 2D surfaces have been found to promote alignment and organization of endothelial cells along the patterned regions.[51,52] Nanopatterned surfaces can precisely pattern endothelial cells into parallel structures, mimicking their cellular alignment in blood vessels. Spatial patterning of endothelial cells can also affect their phenotype and modulate their morphology.[53] The main disadvantage of this method is the lack of a 3D structure that closely mimics vascularized structures *in vivo*. To circumvent this limitation, Gerecht et al. added a hydrogel component to the nanopatterned surfaces to provide a 3D structure for the cells[54] (see Fig. 4(b)). Seunarine et al. in another approach, used X-ray exposure to print an array of dots onto a curved surface, adding a new dimension to the nanopatterned surfaces.[55] The experiment demonstrated that X-ray printing is a suitable technique for printing 3D surfaces that mimic vessel structure. Scaffolds with nanopatterned surfaces have also been studied. Zorlutuna et al. fabricated a collagen scaffold with a nanopatterned structure to orient smooth muscle cells by topographical cues.[56] The resulting constructed vascular tissue showed superior mechanical properties due to the orientation of the smooth muscle cells. Finally, nanotechnologies may pattern biocompatible polymers to simulate capillary-like channels. Hoganson et al. created a patterned template that could be used to mold silicon wafers with a branching pattern similar to vascular branching. Molded scaffolds on these silicon wafers had an arrangement of channels simulating a lung capillary network.[57]

2.3. Nanofibers

We can found different approaches for fabricating fibers from natural or synthetic materials for TE applications, such as (1) electrospinning, (2) wet spinning, (3) biospinning, (4) interfacial complexation, (5) microfluidic spinning, and (6) melt spinning (extrusion). Electrospinning is fabrication technology for the generation of nanofibers. Nanofibers have been used in recent years to fabricate TE scaffolds with tunable properties. Electrospinning technology allows the fabrication of a large variety of nanostructured scaffolds with special features and functionalities, whose shape, size, composition, and porosity can be controlled. The literature contains several reviews of this technology,[58–60] but the present chapter focuses on the application of nanofiber scaffolds for vascular TE.

Synthetic polymers, natural proteins, or a combination of both materials have been successfully employed in electrospinning. A broad spectrum of synthetic polymers has been used to create vascularized tissues, including polylactide–caprolactone,[61,62] polyethylene terephthalate,[63] poly-*N*-acetyl glucosamine,[64] chitosan fibers,[65] poly(lactic-co-glycolic acid),[66] polyurethane in combination with carbon nanofibers,[67] poly(propylene carbonate),[68] synthetic elastin,[69] pullulan/dextran polysaccharides,[70] polyhydroxybutyrate,[71] and a mixture of polyurethane and poly(ethylene glycol).[72] On the other hand, reported stability of vascular scaffolds fabricated by electrospinning of only natural proteins is still far from desirable. To tackle this problem, an alternative approach is electrospinning of natural proteins with synthetic polymers, such as electrospinning of collagen and elastin.[34,73–76] Indeed, the best results have been obtained from combinations of synthetic materials with natural proteins,[77–84] where the stability and mechanical properties of synthetic materials are combined with the improved cell–cell adhesion and cell-ECM interactions within natural proteins.

One of the main advantages of nanofibers produced by electrospinning technique is production of structured scaffolds, each with different functionalities and special characteristics. The obtained nanofibers can be constructed with different shape, size and composition, and they can be solid, hollow, porous, helical, or branched[85,86] (see Fig. 4(c)). This large variety of electrospun nanofibers presents a broad range of opportunities for the improvement of vascular functionalities. For example, nanofibers can be treated with growth factors and act as a drug-delivery system at the same time as scaffolds.[66,87–90] Finally, one of major advances in electrospinning is the one-step fabrication of a vascular scaffold with cells integrated in it.[91] The main limitation of scaffolds constructed with nanofibers is the difficulty in achieving high cell density and good cell infiltration throughout the scaffold. Encapsulation of living cells in electrospun nanofibers solves this problem and eliminates the need of bioreactors for the cell seeding.

3. MICROTECHNOLOGY

Microtechnologies including photolithography, soft lithography, organ printing, and cell sheet engineering have been widely used to engineer tissue constructs containing functional vascular systems. In the following sections, principles and applications of these technologies to fabricate vascularized tissues are discussed.

3.1. Photolithography

Photolithography is a useful technique for transferring patterns onto a substrate and has been commonly used to pattern cells on a substrate[92–94] or to immobilize cells within microengineered scaffolds.[95–98] This technique can also be used to conjugate chemical entities to hydrogels with controlled spatial resolution. Several studies have demonstrated that encapsulated cells and/or bioactive molecules within photocrosslinkable microgels can be used to generate hydrogels for vascularization applications.[54,99–103] For example, Moon et al.[104] prepared protease-sensitive poly(ethylene glycol) (PEG) hydrogels.[105] Human umbilical vein endothelial (HUVE) cells and 10T1/2 cells (mesenchymal stem cells) were encapsulated in the PEG hydrogels and then were exposed to ultraviolet light to photopolymerize the precursors into hydrogels, and used as provisional matrices for angiogenesis both *in vitro*

and *in vivo*. They observed that 10% wt PEG hydrogels produced a robust angiogenic responses after 6 days of culture *in vitro*. Using this formulation, they implanted prototypically degradable PEG hydrogels into mouse corneas using a micropocket angiogenesis assay. Seven days after implantation, blood vessels in the hydrogels were created and become functional and perfused with the host's circulatory system. Du et al.[97] prepared by photolithography arrays of microgels in oil using photolithography and a sequential assembly approach in oil and obtained microchannels similar to vascular channels. In this preliminary study, they obtained, using a two-step photolithographic method, concentric microgels containing an internal HUVE cell layer and outer layer of smooth muscle cells. Although they could construct complex microarchitectures with a high level of control, more work would be needed to obtain a perfusable and mature vascular system. More recently, Masuda et al.[106] using maskless grayscale photolithography, created a 3D microwell with a horseshoe-shaped bottom on polydimethylsiloxane (PDMS), using maskless grayscale photolithography. Using this platform, they obtained cellular aggregates with a toroidal-like geometry and a microlumen. Using directed assembly of multicellular aggregates, they could fabricate channels for vascularization. Lei et al.[107] used photolithography for creating peptide micropatterns on polyethylene terephthalate (PET) films. They observed that tube formation and vascular network formation of HUVE cells could be regulated and guided by micropatterning peptides on PET. This technique could be transferred to a biodegradable scaffold to promote vascularization *in vivo*. Finally, in another approach Ramon et al. used a photopolymerizable semi-natural hydrogel in combination with dielectrophoresis to fabricate 3D cell patterns. Myoblast (C2C12) and endothelial (HUVE) cells were electrically patterned, encapsulated in the hydrogel and co-cultivated for several days.[108] In posterior works, cells have been differentiated and electrically stimulated to obtain highly complex microscale tissues.[109–113] These tissues have been used to study the effect of the electrical stimulation on glucose consumption.[114]

3.2. Soft Lithography

Although a photolithography technique has been used most extensively for patterning proteins and cells is photolithography, it cannot be used to pattern on nonplanar substrates. The high costs associated with the equipment also make this technique inconvenient for biological applications. Soft lithography is an alternative method for photolithography to tackle these aforementioned problems. Soft lithography is a valuable tool for the fabrication of micro/nanostructures on a surface or within a channel. First introduced by Whitesides et al.[115,116] it is a versatile, effective, and low-cost technique that can create 3D features and fabricate patterns on planar and nonplanar surfaces.[117] A prototype is usually fabricated via photolithography or silicon etching. The prototype is used to emboss structures onto an elastomeric material, most commonly PDMS. Polyurethanes, polyamines, or Teflon® can also be used either as molds or as stamps. Microcontact printing and micromolding are other alternatives.

Control of cell positioning in an organized pattern on a substrate has become increasingly important for the development of cellular biosensor technology, especially in TE applications. Microcontact printing uses stamps, mainly of PDMS, to pattern a substrate via surface adsorption or a self-assembled monolayer.[118] This technique is useful for spatially positioning cells and biological molecules, controlling their form and function.[119–123] Gao et al.[124] prepared capillary blood vessels *in vitro* on gelatin *in vitro*. For this purpose, they cultured human microvascular endothelial (HMVE) cells on microgrooves by microcontact printing of the surface with cell-resistant PEG/poly (L-lactide). Hannachi et al.[125] used microcontact printing to pattern surfaces onto commercially temperature-responsive culture dishes to obtain micropatterned cell sheets for TE.

Organized vasculature is crucial for TE applications. In most *in vitro* methods, tubule formation is randomly distributed throughout the matrix, given that there is little control over initial cell position. However, new approaches allowing spatial control during the generation of complex vascularized tissues have emerged. Among others is micromolding that is based on the same concept of microcontact printing, but can generate micropatterns in 3D structures. Using this technique, the engineering of microvessel structures with spatial control was demonstrated with endothelial cells on solid materials, such as glass substrates,[54,126,127] PDMS,[128,129] and films.[130] Micromolding techniques are applicable to a wide range of materials, including, recently, hydrogels to create microwell structures,[131,132] exploiting a physiological environment with high water content, high porosity, and mechanical support.[102] For example, Raghavan et al.[128] spatially patterned endothelial cells within micromolded collagen gels. They encapsulated bovine adrenal microvascular endothelial cells and HUVE cells inside the collagen gel and stimulated tubule formation with VEGF and basic fibroblast growth factor. With this method, they could modulate the tube diameter and generate tubes with a desired branched architecture. More recently, Jiang et al.[16] cocultured HUVE cells and human neonatal skin fibroblasts on microgrooved hyaluronic acid-dextran (HA-dextran) and agarose hydrogels. They observed that cells presented optimal attachment, alignment, and organization while the width of the microgroove was < 50 μm. Endothelial and fibroblasts cells formed a double-layered organotypic assembly in HA-dextran hydrogels compared with agarose gels owing to the greater stability of collagen entrapped in microgroove regions on HA-dextran gels than that in agarose hydrogels. Zheng et al.[133] prepared structures within a matrix of collagen to direct

the growth and the vascularization of tissues *in vivo*. They showed that the guidance of cells invasion and vascularization in a murine wound model was possible using their microstructured tissue templates (MTTs). The invasion of tissue and functional blood vessels was higher in collagen templates than in Integra®, a commercial tissue template. These MTTs could be a useful tool in surgery and regenerative medicine, given that they could be designed to direct cellular migration and vascularization into physiologically appropriate, heterogeneous architectures.

3.3. Microfluidics

Conventional *in vitro* studies of angiogenesis are typically performed in static cultures. The integration of microfluidic systems would present several advantages for studying angiogenesis, anastomosis, and other basic and applied topics related to vascular biology, and could provide a good platform for drug screening.[134–140] Microfluidic networks have been embedded directly within cell-laden hydrogels[141, 142] or biocompatible polymers[143–146] (see Fig. 5(A)) to enable efficient transport of nutrients and other soluble cues throughout the 3D scaffolds. Cuchiara et al.[147] developed a perfusable prevascularized tissue *in vitro* using a synthetic pro-vasculogenic hydrogel fabricated in a previous work,[104] combining it with hydrogel microchannels (50–1000 μm) and self-assembled microvascular networks (15–50 μm). This integration allowed controlled convective transport across a wide range of vessel length and diameters and has the potential to result in improved anastomotic interfaces. In an earlier work, Yeon et al. prepared blood vessels with different dimensions with HUVE cells; however, they connected the blood vessels with one another through a gap in the ladder structure, thus obtaining a perfusable capillary network.[148] Kim et al.[149] fabricated a microfluidic platform to form perfusable and functional 3D microvessels *in vitro*. The chip consisted of a mold of PDMS with five embedded parallel channels: a central channel for culturing HUVE cells and two outside channels to coculture normal human lung fibroblast cells, which secrete soluble factors supporting microvessel formation. Between culture cells channels there was a fluidic channel. After 4 days of culture, they obtained perfusable microvasculature networks with intact barrier functions and long-term stability. This platform may be a useful tool for performing drug screening and vascular physiology studies on human disease models. More recently, Mu et al.[150] fabricated a 3D vascular network to mimic a nephron in hydrogel. They prepared the network based on two principles: fibrillogenesis of collagen and liquid molding. In fibrillogenesis, the collagen molecules in the bottom of a hydrogel solution layer connect with residues of collagen fibrils in the top hydrogel layer, forming new collagen fibrils across the hydrogel interface. They used water as a liquid mold until the gelation of the hydrogel solution. At the end of the process, they obtained a vascular network in two layers of the hydrogel. By repeating the process, they constructed a three-layer hydrogel vascular network with microchannels in different layers. After 5 days of culture, primary HUVE cells formed a monolayer around the inner walls of the hydrogel microchannels. The constructed vascular network had a controlled structure, and it was mechanically stable and cytocompatible. To mimic passive diffusion in a nephron, they seeded stained Madin–Darby canine kidney cells and stained primary HUVE cells into the hydrogel vasculature to mimic renal tubules and vessels, respectively. After 3 days, they observed passive diffusion between microchannels.

3.4. Bioprinting

Although still in the early steps in the development, bioprinting technology seems to hold great promise for advancing TE, given that it offers great precision in the spatial placement of cells and biomolecules. Bioprinting or direct cell printing uses bioadditive manufacturing technologies including (a) laser-based writing, (b) inkjet-based printing, and (c) extrusion-based deposition that are discussed as follows.

3.4.1. Laser-Based Writing

This technology allows continuous and accurate cell printing with high cell viability. It can be used with many materials, given that it does not require an orifice as does inkjet-based printing, thus eliminating the nozzle-clogging risk. Laser direct writing was introduced in 1999 by Odde et al.[151] to process 2D cell patterning and since then has been employed several times to produce vascularized tissues. For example, Nahmias et al.[152] used laser-guided direct writing to pattern HUVE cells in 2D and 3D on Matrigel™. They showed that this technology allows direct preparation of *in vitro* vascular networks with micrometer precision on biological gels. More recently, Wu et al.[153] using biological laser printing (BioLP)[154] fabricated defined branch/stem structures of HUVE cells and human umbilical vein smooth muscle cells to mimic vascular networks (see Fig. 5(B)).

3.4.2. Inkjet-Based Printing

Inkjet printing technology prepares and releases droplets of fluid on demand, precisely depositing these droplets on a surface. It is a rapid, versatile, and inexpensive technique and offers the advantage of noncontact printing. It also eliminates the use of a photomask, commonly used in cell patterning techniques such as microcontact printing and microfluidic channel flow patterning. There are two forms of inkjet printing: continuous inkjet printing and drop-on-demand (DOD) inkjet printing. Owing to its good controllability and less contamination, DOD inkjetting, introduced in the early 2000s, is currently the most commonly used. Nakamura et al.[155] studied the

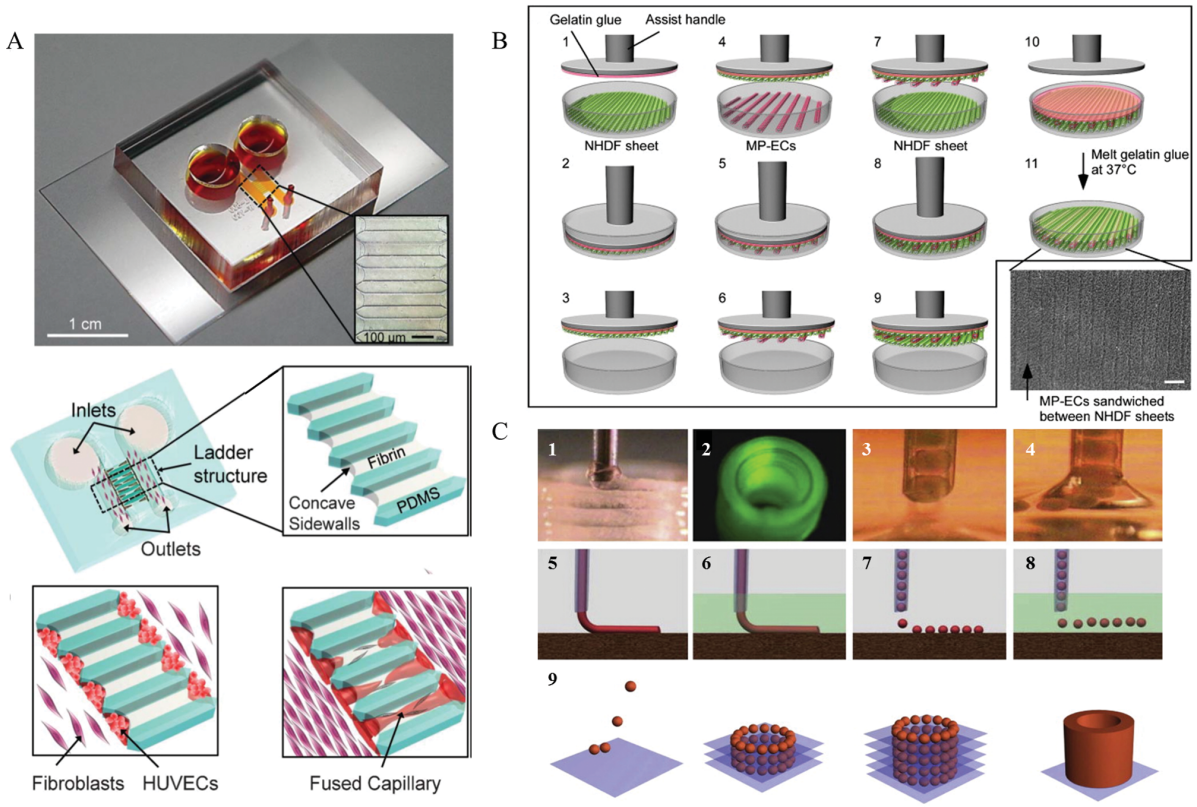

Figure 5. Microtechnology: fabrication of vascularized tissues. (A) A photograph of a microfluidic device for capillary formation. The device consisting of two main channels and ladder microstructures filled with fibrin gel with the concave sidewalls. HUVE cells were attached on the concave sidewalls and fibroblasts were loaded into the main channels after HUVE cells attachment on the fibrin gel. Reprinted with permission from [144], C. Fidkowski, et al., *Tissue Eng.* 11, 302 **(2005)**. © 2005, The Royal Society of Chemistry. (B) Principles of bioprinting technology: (1) general view of bioprinter; (2) multiple bioprinter nozzles; (3) and (4) tissue spheroids before and during dispensing; (5) and (6) continuous dispensing in air and in fluid; (7) and (8) digital dispensing in air and in fluid; (9) sequential steps of layer-by-layer tissue spheroid deposition and tissue fusion process. Reprinted with permission from [157], X. F. Cui and T. Boland, *Biomaterials* 30, 6221 **(2009)**. © 2009, Elsevier. (C) Schematic illustration of 3-D manipulation of cell sheets with the gelatin-coated stacking manipulator to create three-layer cell constructs *in vitro*. (1)–(9) The layering manipulator affixed with gelatin is placed onto a fibroblast sheet after 20 min the fibroblast sheet attached with gelatin glue is lifted and transferred onto a micro-patterned endothelial cells sheet. After 20 min the bilayer sheet is placed onto another fibroblast sheet. To create five-layer cell constructs *in vitro*, the process (1)–(9) is repeated. Reprinted with permission from [166], T. Sasagawa, et al., *Biomaterials* 31, 1646 **(2010)**. © 2010, Elsevier.

biocompatibility of a inkjet head with microseeding living cells, preparing a bovine vascular endothelial cell suspension used as "ink" and ejected onto a culture dish. They used an electrostatically driven inkjet system to prevent damage to cells by the heat of thermal inkjet printers. Recently, Xu et al.[156] fabricated a 3D complex heterogeneous tissue using inkjet printing technology. They used human amniotic fluid derived stem cells (hAFSCs), canine smooth muscle cells (dSMCs), and bovine aortic endothelial cells (bECs) mixed with calcium chloride as a crosslinker, and printed into sodium alginate-collagen composites using a modified thermal inkjet printer based on a DOD mechanism. Vascularization of the bEC constructs was evaluated *in vivo* on constructs implanted subcutaneously in athymic mice, and after 8 weeks, bECs implants presented vascular networks integrated into the host vasculature.

3.4.3. Extrusion-Based Deposition

This technology is based on the extrusion of continuous filaments made of cell-laden biomaterials with a precise deposition of encapsulated cells. It is more efficient than inkjet printing for generating large tissues but has limitations including low resolution and accuracy. It also requires a high viscosity for the biomaterial suspension, restricting the range of suitable biomaterials. Mironov et al.[157] introduced the concept of bioprinting and assembly in hydrogel media, and it has since been applied to vascularized tissues. Cui et al.[158] using a thermal inkjet printer, simultaneously deposited HMVE cells and fibrin gel to form microvasculature. They showed that printed endothelial cells proliferated to form tubular structures sealed inside fibrin channels. After 21 days of culture, the microvasculature showed high integrity. Norotte et al.[159] using tissue spheroids made from fibroblasts and smooth

muscle cells, produced scaffold-free small-diameter vascular constructs. Linear and branched tubular structures were engineered using various cell types. Tissue spheroids were printed layer by layer with agarose rods as the molding template. Upon fusion of tissue spheroids followed by a maturation process after printing (3 days), the support material was removed to generate the lumen. Using this technology, they prepared double-layered vascular tubes whose inner and outer layers were composed of smooth muscle cells and fibroblasts, respectively. Although the fusion process was long and labor- and spheroid-intensive and led to a somewhat inhomogeneous construct, they obtained vascular tubes with ODs ranging from 0.9 to 2.5 mm. Recently, Khatiwala et al.[160] using the NovoGen MMX Bioprinter™, fabricated small-diameter (260 or 500 μm) blood vessels with primary human adult aortic smooth muscle cells, endothelial cells, and dermal fibroblasts. After bioprinting, the vascular grafts required maturation in a perfusion bioreactor for further cellular and matrix reorganization. After 7 days, the blood vessels showed good mechanical integrity, but endothelialization took place only on vessel walls and did not migrate to the lumen.

3D vascularized tissues or organs require the use of heterogeneous aggregates to mimic natural counterparts.[157] This requirement may be fulfilled with the Novogen MMX Bioprinter, which allows printing heterocellular tissue aggregates and hydrogels as support material, or via an alternative strategy that combines printing cellular assembly with microfluidic systems. The idea is to print semipermeable microfluidic channels in tandem with a cellular assembly using a Multi-Arm Bioprinter. Zhang et al.[161] prepared microfluidic channels within multilayer bulk hydrogel that enabled the transport of media to the cellular assembly and also supported the mechanical integrity of the 3D cellular environment. They proposed that the bioprinter platform could be used to create tubular tissue scaffolds for vascular TE by printing living cells such as endothelial and smooth muscle cells loaded within hollow filaments.

3.5. Cell Sheet Engineering

Cell sheet technology has been developed as an alternative to previous scaffold-based methods. This technology yields monolayers of cells (homogenous or heterogenous) that can be attached directly to host tissues without the need for biodegradable scaffolds, mediators, or sutures. Okanos' group have developed a new approach using temperature-responsive culture surfaces[162] (see Fig. 5(c)). At 37 °C or higher, the surface becomes hydrophobic and is suitable for cell culture, but when the temperature is decreased to 32 °C or lower, the surface becomes hydrophilic and the cell sheets can be spontaneously released from the dish. With noninvasive cell harvesting, cell–cell junctions and ECM proteins can thus be maintained. Using these temperature-responsive dishes, Shimizu et al.[163, 164] prepared neonatal rat cardiomyocyte sheets and transplanted them *in vivo*. After 1 or 2 days, they observed a rapid vasculature by the host within the transplanted triple-layer grafts. Exploiting the ability of existing vessels to induce vascular network perfusion in implanted tissues, they obtained myocardial tissue with vascularization having approximately 1-mm thickness with a well-organized microvascular network after 10 transplantations. However, this method is not applicable in patients, in view of the medical risk of multiple surgical procedures. A practical solution of this problem is to create endothelial cell networks in engineered tissues prior to transplantation. Sekine et al.[165] cocultured neonatal rat cardiomyocytes with green fluorescent protein-positive rat-derived endothelial cells. After the formation of endothelial cell networks, the cardiac cell sheets were harvested and transplanted to an infarcted heart surface. After 4 weeks of transplantation, they observed a greater number of new microvessels able to connect to host vessels, thus allowing direct blood flow between them and the possible improvement of the cardiac function of infarcted hearts.

Tsuda et al.[166] using a new cell sheet-stacking technology, fabricated prevascularized tissue combining micropatterning technology and 3D cell sheet engineering. They fabricated a multilayered tissue combining monolayers of neonatal normal human dermal fibroblasts cells with patterned HUVE cell sheets. After 5 days of tissue culture they observed a network organization similar to microvessels. With this technique, they could construct 3D tissues with a precise multicellular architecture without using synthetic polymers or collagen as supports. Using the same stacking technology, Sasagawa et al.[167] cultured HUVE cells sandwiched between two human skeletal muscle myoblast sheets. They prepared five-layer myoblast sheets that presented capillary-like networks after 4 days of culture and were transplanted to the dorsal subcutaneous tissue of nude rats. After 1 week following transplantation, they observed that the partially formed microvessels contained red blood cells and were connected to the host vessels. In both cases using this technique, they could control the cellular density and orientation of cultured endothelial cells. Most recently, Okanos' group showed the importance of the 3D design when engineering 3D tissue requiring a well-organized microstructure.[168–173]

Sekine et al.[174] prepared endothelial cells cocultured with cardiac cell sheets to fabricate *in vitro* functional 3D cardiac tissues. Using rat femoral muscle, they fabricated a vascular bed perfused within a novel bioreactor system to promote angiogenesis. They overlaid a triple-layer endothelial cell-cardiac cell sheet on the system. After 3 days of culture, they perfused black ink through the constructs and observed that endothelial cells contributed to blood vessel formation and that fibroblast growth factor 2 in the perfusion media promoted blood vessel communication between the graft and the vascular bed. They

then overlaid a triple-layer endothelial cell-cardiac cell sheet. After 6 days of cultivation, they obtained a functional and perfusable 3D tissue. Sakaguchi et al. conducted a similar experiment, but fabricated an artificial culture with microchannels able to mimic a subcutaneous vascular structure.[175] Triple-layer endothelial cell-cardiac cell sheets were repeatedly layered, as in the previous report, until a 12-layer construct was obtained. With this new multi-step bioreactor, they fabricated a vascularized thick 3D cardiac tissue, but more biological and mechanical optimization was needed, given that live/dead cell viability assays revealed focal cell death.

In parallel, other researchers have developed sheet-based bioengineering concepts for the recreation of tissues including human blood vessels. L'Heureux et al.[176] using human smooth muscle cells (SMC) and fibroblast cell sheets, constructed vascular grafts without any synthetic material in the presence of ascorbic acid. SMC and fibroblast sheets were wrapped around a tubular support to produce the media and the adventitia, respectively, of blood vessels. The tubular support was removed after a maturation period of 8 weeks, and endothelial cells were seeded in the lumen. At the end of the process approximately 3 months afterward, they obtained blood vessels with a well-defined structure and strong mechanical properties. Using this approach, L'Heureux et al.[177] reported short-term to mid-term *in vivo* results in animal models, using blood vessels built with human cells derived from a single xenografted patient. They also implanted small-diameter vascular grafts in humans for hemodialysis access.[178–180]

Guillemette et al.[181] demonstrated that the perfusion of engineered tissue *in vivo* could be improved by *in vitro* addition of a capillary system to the adventitial layer. They cultivated human skin fibroblast cell sheets with ascorbic acid. After a 21-day cultivation period, they seeded HUVE cells on top of the fibroblast sheet over 7 days, to allow the formation of capillary-like structures inside the fibroblast sheet. After 28 days, they rolled the fibroblast-HUVE sheets onto a mandrel and matured them *in vitro* for 14 days more. The resulting tissue-engineered vascular adventitia with vasa vasorum was implanted in athymic mice without surgical anastomoses. After 2 weeks, they observed functional vasa vasorum *in vivo*; after 90 days, hybrid vessels containing human and mouse endothelial cells with intact perfusion were observed.

4. CONCLUSIONS AND FUTURE TRENDS

In recent decades, microtechnology and nanotechnology have been gradually integrated with medical research and are now an essential part of TE research. One of major challenges for engineered tissues is the lack of vascularized network tissues, which largely limits their biological functions. Recently, there have been numerous studies using *i*PS cells to engineer tissue. For instance, Takebe et al.[14] fabricated vascularized liver buds by mixing *i*PS and endothelial cells. The size of the liver bud was limited, but with the incorporation of some microtechnology and nanotechnology explained in this review, the fabrication of vascularized organs/tissues might be achieved. It is becoming increasingly obvious that in the near future, the ongoing integration of nanotechnology, microtechnology, stem cells and TE approaches would result in artificially fabricated, functional vascularized tissues and organs.

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