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Vascularization of Three-Dimensional Engineered Tissues for Regenerative Medicine Applications

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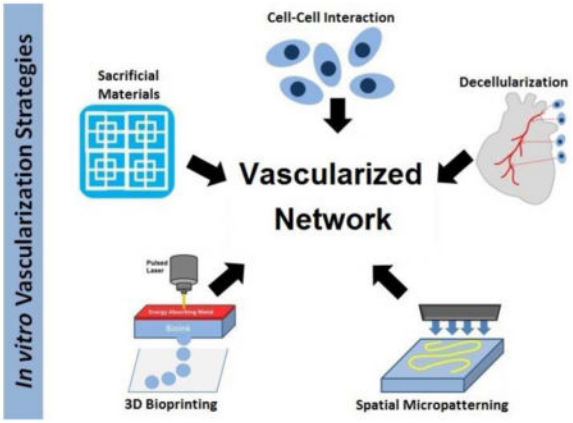
Abstract

Engineering of three-dimensional (3D) tissues is a promising approach for restoring diseased or dysfunctional myocardium with a functional replacement. However, a major bottleneck in this field is the lack of efficient vascularization strategies, because tissue constructs produced *in vitro* require a constant flow of oxygen and nutrients to maintain viability and functionality. Compared to angiogenic cell therapy and growth factor treatment, bioengineering approaches such as spatial micropatterning, sacrificial materials, tissue decellularization, and 3D bioprinting enable the generation of more precisely controllable neovessel formation. In this review, we summarize the state-of-the-art approaches to develop 3D tissue engineered constructs with vasculature, and demonstrate how some of these techniques have been applied towards regenerative medicine for treatment of heart failure.

Graphical Abstract



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Keywords

vascularization; endothelial cell; cardiac engineering; tissue engineering; biomaterials; extracellular matrix; 3D bioprinting

1. Introduction

In the United States, congestive heart failure accounts for over 300,000 deaths in the US annually and $39 billion in total hospital burden [1]. With the increasing demand for heart transplantation [2], meeting this demand is becoming increasingly difficult with current heart transplantation approaches. To address this limitation, the field of tissue engineering has made notable progress towards regenerative medical approaches to repair damaged or diseased myocardium using a combination of biocompatible materials and patient-specific cells grown in the laboratory [3, 4]. However, the biggest hurdle for the clinical translation of three-dimensional (3D) tissue engineered constructs is their functional vascularization. 3D engineered myocardial tissues require a constant supply of oxygen and nutrients, as well as a route to remove wastes that are generated in the tissue, in order to maintain long-term survival and functionality. *In vivo,* living cells must be within 100–200 μm from a blood capillary in order to survive and function [5], due to the diffusion limit of oxygen through biological tissues [6]. Thus, when designing 3D myocardial tissue *in vitro*, it is critical to consider this requirement in order to prevent significant cell death and promote optimal cell function. Furthermore, the strategy to induce vascularization should be compatible with integration into the host's circulatory system.

The realization of efficient, robust and reproducible methods to provide relatively large tissue-engineered constructs with functional vasculature will facilitate the mass production and widespread adoption of such constructs for clinical applications. As such, much financial and intellectual investment on this front continues to be made [7]. This review will highlight the latest progress and most promising vascularization strategies in tissue engineering to date, highlighting the application of these strategies towards engineering vascularized cardiac tissue.

2. Physiological Properties of Vasculature

2.1 Development: Vasculogenesis to Angiogenesis

Physiologically, human vasculature is composed of several cell types that function concurrently to provide specific mechanical and chemical microenvironments for the optimal transport of blood and nutrients throughout the body [8]. Successful *in vitro* vascularization strategies are dependent on their ability to most closely mimic *in vivo* physiology. Thus, it is crucial to comprehensively understand the biological properties of native blood vessels when designing vascularization approaches *in vitro* [9]. Important aspects of blood vessels to consider include their development, function, and physical/chemical properties. Since detailed reviews about the angiogenesis process can be found elsewhere [10, 11], here, we will briefly describe its salient characteristics.

Blood vessels are first formed *de novo* through a process known as vasculogenesis that occurs in parallel with hematopoiesis [12, 13]. In the embryonic yolk sac of mammalian embryos, early endothelial progenitor cells and hematopoietic stem cells aggregate to form distinct blood islands, which give rise to endothelial cells that gradually fuse and form lumen structures within the extracellular matrix (ECM), and subsequently develop the primitive capillary plexus [12]. This plexus is the foundation for continued vessel construction, in which the ECM is actively remodeled and developed through a process known as angiogenesis to form the circulatory system [14]. Angiogenesis can occur via sprouting or splitting (intussusception) routes, where sprouting expands the vascular network via extension from existing capillaries guided by tip cells [15], and splitting involves the internal division of an existing capillary into multiple capillaries. The sprouts can also migrate towards pre-existing vessels and integrate with them through inosculation. The process of angiogenesis is highly influenced and regulated by cell-cell and cell-ECM interactions via crosstalk between vascular endothelial growth factors (VEGF) and Notch signaling pathways [16]. New vascular networks are stabilized by the recruitment of cells from the surrounding stroma by the coordinated secretion of platelet-derived growth factor (PDGF) and VEGF secreted by endothelial cells and vascular smooth muscle cells [17]. Perivascular cells form around and stabilize the microvasculature, and have been known to recruit proangiogenic factors, whereas smooth muscle cells are recruited to larger vessels [18]. Interactions with these supporting cell types are highly important in the angiogenesis process, and is reviewed elsewhere [19–21].

Besides soluble factors, the ECM also plays an important role by providing a structural framework and dynamic signaling cues throughout the angiogenesis process. Angiogenesis initiates with disruption of the basement membrane, followed by the influx of plasma-derived fibrinogen, vitronectin, and fibronectin to form a provisional matrix [22–24]. The provisional matrix guides their invasion into the tissue stroma, which is composed of collagen isoforms I and III. As new vessels form, the basement membrane acts to stabilize the vessels. The importance of cell-ECM interactions in modulating angiogenesis is reviewed in greater depth elsewhere [22, 25, 26].

2.2 Heterogeneity of Endothelial Cells

Throughout the process of angiogenesis, blood vessels specialize into arteries, veins or their connecting capillaries through distinct biochemical and biophysical cues [27, 28]. Thus it is important to consider the differences in vessel types when fabricating vasculature *in vitro.* After sufficient maturation, different blood vessel types allow for the perfusion of blood throughout the circulatory system in different flow directions and pressures [29]. Arteries exhibit thick elastic muscular walls composed of concentric layers of vascular smooth muscle cells, which allow for high pressure blood flow and shear stress. Veins, on the other hand, contain a thin layer of vascular smooth muscle cells surrounding their inner endothelial cells, which are subject to relatively low pressure blood flow and shear stress. Veins are also equipped with valves that open unidirectionally to prevent backflow.

The connection between arteries and veins is achieved via capillaries, which are the major exchange vessels in blood circulation, comprising the bulk of the surface area [10].

Capillaries exhibit the thinnest vessel walls, which are mainly comprised of capillary endothelial cells stabilized by pericytes and the surrounding extracellular matrix. In addition, blood flow is relatively slow through capillaries, which allows for adequate diffusion [10]. It is therefore important to consider these differences in blood vessel types that are associated with specific biochemical and physical *in vivo* properties when designing vasculature *in vitro.* Taking into account the physiological process of angiogenesis, we will next highlight some of the state-of-the-art approaches to engineer vasculature *in vitro*.

3. In Vitro Approaches for Vascularizing Biomaterials

3.1 Biomaterials used in Vascularization Approaches

It is well-recognized that interactions between endothelial cells and biomaterials play an important role in modulating neovessel formation in engineered tissues [30]. Early vascularization engineering efforts utilized non-biodegradable materials (i.e. silicon) mainly by employing soft lithography and photolithography approaches [31, 32]. Although these early efforts led to important discoveries [33–35], clinical translation was limited by the inability of those engineered constructs to be safely implanted and integrated in the body due to the lack of biodegradability. Thus, subsequent investigations placed a greater emphasis on utilizing materials that were biodegradable and feasible for implantation [36]. Some of these materials include aliphatic polyester elastomers [37, 38], polyurethanes [39], polylactones [40], polyphosphazenes [41], and tyrosine derived polycarbonates [42, 43] (Table 1). Based on the physical properties of the polymer type, different fabrication approaches have been used to synthesize 3D constructs that promote cell attachment, survival, growth, differentiation and function. The chemistry of these polymers makes them uniquely suitable for specific engineering strategies. The strategies that are relevant to engineering vasculature will be reviewed below.

3.2 Vascularization by Cell-Cell Interaction

Many early efforts to form 3D networks that mimic *in vivo* vasculature rely on interacting with endothelial cells in co-culture. Among these early efforts, Levenberg *et al.* created vascularized engineered construct models of skeletal muscle tissue by co-culturing myoblasts, embryonic fibroblasts and endothelial cells on porous poly-L-lactic acid/ polylactic-co-glycolic acid (PLLA/PLGA) scaffolds fabricated by salt-leaching [44]. The optimal ratio among cell types to maximize vascularization as assessed by endothelial cell area, number of lumens and total lumen area was 40:13:47 ratio of myoblasts: fibroblasts: endothelial cells. They demonstrated stable vessel formation and maintenance in mouse models by pre-vascularizing the scaffolds prior to *in vivo* implantation [44]. Using a porous collagen scaffold with an average pore width of 80 μm and pore length of 110 μm to enable cellular penetration through the scaffold, another group endothelialized reconstructed skin composed of keratinocytes, fibroblasts and endothelial cells [45]. This construct formed a capillary-like network *in vitro,* which when transplanted *in vivo* could inosculate with the host vasculature [46]. In another approach, the fabrication of hollow channels within silk fibroin scaffolds enabled the formation of extensive vascular networks that anastamosed with the host vasculature when implanted *in vivo* [47]. Collectively, vascularization by co-

culturing with endothelial cells has demonstrated improvement in vessel network growth and stability when implanted *in vivo.*

Another technique that has been increasingly investigated in tissue engineering is the fabrication of scaffold-free cell sheets, which allows for a layer-by-layer construction of 3D constructs. Sasagawa *et al.* used such a strategy to layer sheets of human aortic endothelial cells interspersed between layers of fibroblast sheets [48]. These pre-vascularized cell sheets were subcutaneously transplanted and ultimately integrated into immunodeficient rats [48]. Another group used a similar approach to engineer pre-vascularized liver tissue composed of a layer of hepatocytes and a layer of fibroblasts, which integrated with the vasculature in the subcutaneous murine tissue [49]. Owing to the reproducibility of this approach and the ability to control cellular composition, this is a promising approach for the fabrication of thin vascularized tissue constructs for patch-based applications.

However, despite the promise of co-culture techniques for the *de novo* vascularization of engineered tissue constructs, this technique is limited by the lack of precision in network formation and architectural specification, which inhibits the ability to generate spatially controllable vascular networks. For this reason, the use of bioengineering approaches that employ biochemical and biophysical cues may enable greater control of the angiogenesis process, leading to efficient construction of vascular networks *in vitro*. Some of these techniques are described below.

3.3 Release of Angiogenesis Growth Factors

The formation of vascular networks can be expedited by functionalizing scaffolds with angiogenic growth factors or small molecules. The controlled localization and immobilization of growth factors to scaffolds has been a widely used strategy to promote angiogenesis. Angiogenic growth factors such as VEGF and angiopoietin-1 can be covalently immobilized to collagen via 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) crosslinking to promote endothelial tube-like formation by endothelial cells [50]. Besides chemical crosslinkers, photopolymerization using laser scanning lithography is another approach to covalently bind and immobilize RGDS cell binding domain and VEGF onto polyethylene glycol (PEG) hydrogels. Endothelial cells cultured on this hydrogel formed capillary tube-like structures within two days [51]. Immobilization of growth factors can also be achieved by employing non-covalent interactions. For example, growth factors such as basic fibroblast growth factor and epidermal growth factor were immobilized to synthetic ECMs through non-covalent coiled-coil interactions, which resulted in enhanced cell proliferation and tube-like formation [52].

The controlled release of growth factors in different biodegradable material constructs for the promotion of angiogenesis has also been widely investigated. Hydrogels are often employed for controlled release of soluble factors because of their biocompatibility and tunable release profiles using chemical- or photo-crosslinking. For example, PEG-based hydrogels promoted the controlled and sustained release of the chemokine stromal cell-derived factor-1 (SDF1), and the release properties were easily controlled by the degree of crosslinking density of hydrogel [53]. These functional hydrogels were fabricated by simply blending Poly(ethylene glycol) diacrylate (PEGda) with stromal cell-derived factor 1 –

glycoprotein VI (SDF1-GPVI) and crosslinking at a wavelength of 365 nm with a UV light intensity of 5 mW/cm^2 using a UV crosslinker [53]. Using a similar system, sustained delivery of SDF1 preserved ventricular function in a rat model of myocardial infarction (MI) [54]. The tunability of VEGF release was also demonstrated in a protein-PEG hybrid hydrogel by varying the molecular weight and proline-rich domains on the peptide-PEG component, which was mixed with PEG [55]. When these custom hydrogels were used to control the release kinetics of VEGF and deliver induced pluripotent stem cell-derived endothelial cells into a mouse model of hindlimb ischemia, the animals demonstrated greater cell survival and muscle regeneration compared to those treated with saline alone [55]. Another approach to deliver angiogenic growth factors employed composite biomaterials consisting of a PEG and PLLA hydrogel containing poly(lactic-co-glycolic acid) (PLGA) microspheres that released fibroblast growth factor 1 (FGF-1) and platelet derived growth factor-BB (PDGF-BB), which were loaded into the microspheres via gravity and capillary force [56]. Although these studies have been effective for regulating spatiotemporal growth factor release, they are collectively less precise for generating desired vascular networks and supporting active flow dynamics. Thus, increasingly proactive presentation of fluid dynamics has also been investigated.

Perhaps one of the most extensively investigated methods for generating vascular networks is by using microfluidic devices to produce concentration gradients of angiogenic growth factors. One group developed a microfluidic device that mimics the physiologic gradient distributions of angiopoietin-1 and VEGF to examine the chemotactic response of endothelial cells during angiogenic sprouting [57]. They reported that precise concentration gradients of both angiopoietin-1 and VEGF are required for the proper induction of tip cells and migration and connection of tip and stalk cells [58–60].

Despite the promise of growth factor guided vascular network formation, this approach has limited ability to control the architectural formation of vascular networks. With immobilization of growth factors, abnormal cellular behavior can occur due to continuous stimulation to the cells despite their negative feedback, leading to potentially deleterious effects [61]. Accordingly, bioengineering efforts to more precisely fabricate vascular network architecture are described below.

3.4 Sacrificial Materials for Vascular Channel/Network Formation

Sacrificial materials have been widely investigated as a way to form desired geometrical features by molding a non-sacrificial material around a sacrificial component, followed by removal of the sacrificial component (Figure 2A). In the context of vascular networks, this can result in a complex network mimicking *in vivo* vasculature. For example, Chrobak *et al.* surrounded a stainless steel needle (120 μm diameter) located within a microchannel containing collagen I, followed by removal of the needle. Seeding of primary endothelial and perivascular cells into the hollow collagen tube resulted in the formation of vascular tubes that were perfusable and mimicked native vessels by displaying endothelial barrier function [62] (Figure 1A). Another group used a similar fabrication approach by curing sucrose/agarose solutions in a custom polydimethylsiloxane (PDMS) mold outfitted with a cylindrical metal tube, where removal of the tube produced a single channel for flow [63].

Besides being physically removed, sacrificial materials can also be chemically dissolved away to leave behind a network within a non-sacrificial biomaterial. A negative mold of a network can be formed using dissolvable hydrogels or polymers. The pre-formed mesh or network can be encapsulated in a 3D scaffold structure before being dissolved away from the scaffold, leaving behind interconnected channels within the scaffold. One group used micromolded meshes of gelatin as the sacrificial material, followed by encapsulating gelatin meshes in a collagen hydrogel. Upon removal of gelatin via heating to 37°C and flushing out with phosphate-buffered saline (PBS), interconnected channels were formed in the hydrogel. Human endothelial cells seeded into these channels could be perfused by microfluidic approaches [64]. Another group used sacrificial sugar structures to form microchannel networks in PLLA scaffolds. They proceeded to seed endothelial cells into these scaffolds and demonstrated that the cells were able to permeate the entire scaffold and remain viable after 7 days of culture [65].

Sacrificial material networks can also be formed by an additive process, resulting in high precision of the geometrical features of the network. In the advent of additive manufacturing, 3D printers have been increasingly used in biological applications (as described in section 3.6). Using a 3D printer, one group created 3D filament networks of carbohydrate glass as a sacrificial vascular network within various gels, including agarose, alginate, fibrin and matrigel gels, and PEG hydrogels. The cylindrical network was shown to be able to be lined with endothelial cells and was perfusable with blood under high-pressure pulsatile flow [66]. The investigators also demonstrated the sustainment of metabolic function of hepatocytes in this vascularized construct as evidence of tissue-like function.

In summary, strategies employing sacrificial approaches to construct vascular networks are diverse in regards to chemical composition of the materials as well as fabrications techniques used. Owing to the consistent demonstration of functionality, this approach remains promising as a strategy to ultimately create complex vasculature within 3D tissue engineered constructs. However, the complete removal of the sacrificial material is not always trivial, and when not removed completely, residual materials can be cytotoxic and/or harmful to the host.

3.5 Spatial Micropatterning

In stark contrast to vascularization methods using molding or sacrificial materials, micropatterning of geometric features or chemical cues to regulate cellular assembly has also been extensively investigated for vascularization of biomaterials. For example, Chaturvedi *et al.* created endothelial cell cords of varying diameter (i.e. 50, 150 and 500 μm) by culturing endothelial cells suspended in collagen into microfabricated PDMS channels [67]. These constructs acted as a template after implantation for the guided formation of patterned capillaries that were ultimately integrated into host mouse tissue. Another group fabricated scaffolds containing microvessels that were 100 μm in diameter out of polyglycerol sebacate (PGS) elastomer molded from etched silicon wafers. By perfusing medium at 10 μl/min for five days to cultured human endothelial cells within the device, the resultant scaffolds were implanted in the small intestines of nude rats [68]. Another approach utilized soft lithography to engineer an organized capillary network between an

artery and a vein by inducing directed capillary sprouting from vascular explants on micropatterned substrates containing thymosin B4-encapsulated collagen-chitosan hydrogel [69]. These substrates consisted of channels ranging from 25–100 μm in width and 65 μm in height. The capillary outgrowth process was expedited by the application of soluble VEGF and hepatocyte growth factor, and the endothelial cells formed tubules with lumens expressing characteristic biomarkers for functional vascular endothelial cells [69].

Aside from soft lithography, lasers have also been utilized to create desired scaffold features. A photolithography approach uses laser beams to selectively photopolymerize biomolecules to form 3D hydrogel structures (Figure 1B). One group used a two-photon laser to micropattern the ligand RGDS in hydrogels to guide cell migration along predefined 3D pathways. Using *in vivo* tissue sample data as blueprints, micropatterned growth factors were created in hydrogels at 3 μm spacing for a total pattern depth of 150 μm, which guided the spatial organization of human endothelial cells and murine fibroblasts into complex tubule networks [70].

Cell patterning has also been used to generate vascularized cardiac patches [71, 72]. Liau *el al.* generated a 3D fibrin-based hydrogel matrix seeded with mouse embryonic stem cell-derived cardiomyocytes and cardiovascular progenitor cells (CVPs). In another example, the authors applied a versatile soft lithography technique to fabricate PDMS molds containing arrays of mesoscopic posts (1.2 mm × 0.2mm × 1.5mm: LxWxH), which were used to generate parallel-aligned engineered cardiac tissues in 3D [73]. After 21 days, the cardiac patches became uniformly densely populated and aligned. Functionally, the cardiac patches had high electrophysiological interaction with rapid conduction velocity (22–25 cm/s), and had significant contractile forces (up to 2 mN) [71]. Moreover, the CVPs were shown to differentiate into endothelial and vascular lineages, which may have contributed to cardiac function and/or maturation. More recently, the same group applied the same approach to human embryonic stem cell-derived cardiomyocytes [72]. In comparison with 2D monolayer culture, 3D fibrin-based cardiac patch showed higher conduction velocity (>25 cm/s), longer sarcomeres, and enhanced gene expressions related to contractile functions, including cardiac troponin-T and α-myosin heavy chain. In addition, the 3D cardiac patch had significant positive inotropy to β-adrenergic stimulation in a dose dependent manner [72].

The use of patterning ligands and ECM components to navigate cellular behavior is an area that has gained much attention lately. Most of these studies involve short peptide sequences that are patterned and immobilized to control cellular adhesion, cytoskeletal shape and function. Lee *et al.* developed peptides that contain a protective group on its integrin receptor-binding site that can be seamlessly removed using light exposure to activate the peptide. This approach allows for both spatial and temporal control of ligand presentation, which was applied to regulate the vascularization of biomaterials [74]. Due to the high spatial precision of micropatterning strategies, this technique could be a powerful tool to be used in conjunction with other engineering strategies to incorporate micro- and nano-scale details into tissue engineered constructs. However, despite its many advantages, many micropatterning techniques require background blocking in non-patterned regions, which can potentially substantially change important substrate properties. In addition, the size-

scale of spatial micropatterned substrates is currently limited, thus strategies that enable larger scale vascular networks are advantageous in this regard.

3.6 Decellularized Tissues as Templates for Vascularization

Another approach to neovascularization is by decellularization of a vascularized organ or tissue, followed by repopulation of the vascular channels with vascular lineages. The use of native tissues or organs eliminates the need to design vascular networks *de novo* and often preserves the 3D native vasculature within the organ of interest. Since the decellularization process often involves harsh conditions that may result in ECM damage, one group developed a process to preserve global tissue architecture and ECM components in decellularized porcine lung by using Triton X-100 and sodium deoxycholate (SDC) as chemical detergents [75]. Another group decellularized a human heart with fully preserved 3D geometrical features and vascular network. When the decellularized heart was re-cellularized with human cardiac progenitor cells, bone-marrow derived mesenchymal cells, human umbilical vein endothelial cells and cardiomyocytes in a bioreactor, the authors report that the endothelial cells were able to form a lining of endocardium and vasculature, whereas the differentiated cardiomyocytes organized into nascent muscle bundles and demonstrated mature calcium dynamics as well as electrical coupling [76]. Another group decellularized cadaveric hearts via coronary perfusion with detergents, which preserved their underlying extracellular matrix and retained 3D architecture of the heart tissue and vascular networks. When the decellularized scaffolds were re-cellularized with cardiac cells and rat aortic endothelial cells, followed by maturation of the tissue in a bioreactor for 28 days, the re-cellularized heart tissue remained functional based on electrical conductivity and the ability to perfuse blood *in vitro* [77]. Besides decellularized hearts, decellularized porcine jejunal segments could also be generated with preserved vascular structure. When re-cellularized with porcine endothelial cells and hepatocytes, the re-cellularized tissue could be maintained viable for three weeks *in vitro*. Furthermore, the hepatocytes maintained their morphology and had stable metabolic activity [78]. Recently, a group further improved on the re-cellularization of a decellularized porcine whole liver by conjugating anti-endothelial cell antibodies to promote the targeted attachment of endothelial cells to vessel walls (Figure 1C). When transplanted heterotopically with inflow from the renal artery and the outflow to the renal vein into healthy Yorkshire pigs, the re-endothelialized whole livers were able to conduct blood flow for a period of 24 hours [79]. Since decellularized tissue constructs currently most closely mimic *in vivo* tissue architecture and vascular network organization, this is the most clinically translatable approach to reconstituting vascular networks, although antigenicity from xenogenic tissues remains a concern [79].

3.7 3D Bioprinting

In recent years, 3D printing has been increasingly applied to biological applications. A key advantage of 3D printing is the ability to have precise control within 3D space. Different approaches for 3D printing in tissue engineering have evolved to accommodate the printing of cells in a process known as 3D bioprinting. The printing of complex geometrical features found *in vivo* requires a comprehensive map of the tissue/organ of interest. For this reason, medical imaging technologies such as computed tomography and magnetic resonance imaging are vital in providing spatial information to guide the computer-aided design (CAD)

blueprints of 3D printed tissues/organs. The three main approaches used in 3D bioprinting today are inkjet, laser-assisted and extrusion-based techniques (Figure 2) [80].

The most commonly used bioprinter is based on inkjets that use thermal or piezoelectric mechanisms to deposit drops of material (i.e. bioink) onto a substrate. Thermal inkjet bioprinters electrically heat a print head to produce pressure pulses that eject droplets from a nozzle. On the other hand, piezoelectric inkjet bioprinters create an acoustic wave inside the print head to create droplets of bioink at regular intervals. Inkjet bioprinting has been used to print human microvasculature using bioink composed of human microvascular endothelial cells and fibrin, which resulted in functional 3D tubular structure [81]. These printed endothelial cells proliferated to form a confluent lining for up to 21 days of culture, and demonstrated mechanical integrity with an elastic modulus of 2.9 MPa and estimated burst pressure of 2955 mm Hg [81]. Recently, one of the most sophisticated 3D bioprinters, named an integrated tissue-organ printer (ITOP), was described [82]. The authors demonstrated ITOP's ability to print defined cellular patterns, fabricate mandible and calvarial bone, cartilage and skeletal muscle. This study represents perhaps one of the most advanced studies of 3D bioprinting for clinical applications to date. In another recent study, vascularized human bone tissue blocks, which were able to conduct flow through active perfusion with growth factors [83], was generated using a 3D bioprinting approach.

Laser-assisted bioprinting is based on laser-induced forward transfer, and employs a "ribbon" consisting of a laser energy absorbing layer of metal (i.e. gold or titanium) and an underlying layer of bioink. A laser vaporizes the metal layer to induce droplet formation of the bioink, which is then deposited onto a collecting substrate. This approach was used to print spots of human adipose-derived stem cells and endothelial colony forming cells, which resulted in the development of stable vascular-like networks [84].

Currently, bioprinting using mechanical extruder techniques is perhaps the most widely utilized approach, and it is compatible with a wide range of different biomaterials and cells. This method uses mechanical extruders to print cells and/or biomaterials onto a supporting structure, where layer-by-layer structural complexities can be realized. One group used this approach to print decellularized ECM bioinks encapsulated with cells to form 3D tissue-specific constructs in raster snake labyrinths that support long-term stability and cellular compatibility [85]. Another group used extrusion 3D printing to present growth factor cues (i.e. BMP-2 and VEGF) to prevascularize bone tissue. The authors found that bone regeneration was significantly faster in prevascularized bone tissue, with the additional angiogenesis from host tissue after transplantation into a mouse model [86]. Although much of this work was proof-of-concept, the results hold promise for the eventual development of functional tissues/organs via additive bioprinting.

Although 3D bioprinting is the least developed among the different strategies discussed thus far, innovations in hardware, printing strategies and bioink formulations are continually being developed to advance the field. 3D bioprinting is perhaps the most promising approach for the future of engineering organs due to its precision, reproducibility and relatively low cost of operations. However, many more technical hurdles must be overcome before 3D bioprinting is able to be used widely for clinical applications.

4. Towards *in Vivo* Application

The ultimate goal of all tissue engineering efforts is to translate *in vitro* engineered tissues or organs to human patients as a therapeutic treatment. Since it is critical for tissue patches to integrate with the host's vasculature to optimally restore function to the damaged area, vascularization has remained a critical area of interest. Owing to the experimental progress of engineering vascularized tissue constructs *in vitro*, many engineered tissues have progressed into pre-clinical testing phase for testing of safety and efficacy. One area of *in vivo* translation of vascularization strategies is for treatment of heart failure.. It is characterized by reduced heart function, leading to impaired ability to deliver blood and oxygen to other vital organs. Heart failure is often preceded by single or multiple episodes of MI, in which coronary arteries become occluded, leading to death of cardiomyocytes that are responsible for the heart's contractility. In order to maintain contractile function, the cardiomyocytes are highly metabolic and require an extensive vascular network to provide continuous nutrients and oxygen. For this reason, engineering of vascularized cardiac patches are in high in demand. Using the vascularization strategies described in Section 3, researchers now seek to create neovasculature within cardiac tissue constructs that could promote cardiomyocyte function and survival, or promote anastomosis of the engineered construct to the myocardium. Some of the approaches for vascularizing cardiac tissue constructs are described below.

4.1 Vascularization of Cardiac Patch by Co-Culture Interaction

A number of strategies have been employed to create vascularized cardiac patches. The simplest approach involves co-culture of cardiomyocytes with endothelial cells [87–89]. Sekine *et al.* co-cultured primary rat cardiomyocytes with endothelial cells within cell-sheets at varying cardiomyocyte:endothelial cell ratios of 12:1, 6:1, or 3:1 [87]. These cell sheets were fabricated by co-culturing cardiomyocytes and ECs on 35 mm dishes coated with thermoresponsive poly(N-isopropylacrylamide), which resulted into transferable, intact cell sheets after 4 days of culture. When implanted into an athymic rat model of MI for four weeks, the co-cultured cell sheets with 6:1 or 3:1 relative ratios demonstrated a significant increase in fractional shortening as a measure of myocardial function. Furthermore, the pre-formed vascular networks within the co-cultured sheets with 6:1 ratio were able to reduce the formation of fibrosis in the infarct scar. More importantly, immunostaining of isolectin B4 and green fluorescence protein has revealed that blood vessels from the cardiac patch successfully bridged to the capillaries of the host heart, and formed fused vessels containing endothelial cells from both the host and the co-cultured sheets. Another group generated a cardiac patch by combining human embryonic stem cell-derived cardiomyocytes, endothelial cells and fibroblasts at a ratio of 1:1:0.5 [90]. *In vitro*, it was reported that the human $CD31^{+}$ endothelial cell networks resembled a vascular plexus *in vitro,* and the tri-cell cardiac patch could be paced electrically up to 3 Hz. Moreover, when these patches were delivered in rodent hearts, they formed viable human myocardium, and the human coronary microvessels integrated with the host vasculature by forming vessel-like lumens that contain leukocytes and Ter-199-expressing red blood cells. More recently, another group fabricated a cardiac patch composed of a fibrin gel scaffold laden with human induced pluripotent stem cell-derived cardiomyocytes and human vascular pericytes, which was subsequently

implanted onto athymic rat hearts. These patches were viable and vascularized upon *in vivo* transplantation, and microvessels were more abundant in the host myocardium border zone. These patches ultimately improved cardiac function by reducing the infarct size while improving fractional shortening, in comparison to the no treatment control condition [91].

4.2 Vascularization of Cardiac Patch by Growth Factors

Besides co-culture with different vascular cell types, a number of growth factors have also been examined for inducing angiogenesis as a form of myocardial repair. Miyagi *et al.* tested the hypothesis that covalent immobilization of VEGF onto a collagen scaffold could improve vascularization *in vivo* [92]. They reported that either low (~15 ng) or high concentration (~100 ng) VEGF-releasing scaffolds were able to enhance the growth of endothelial and bone marrow cells *in vitro*, in comparison to untreated scaffolds. When these cell-seeded scaffolds were transplanted *in vivo* to replace the right ventricular free wall in rat hearts, high concentration VEGF scaffolds resulted in increased blood vessel density after 28 days. In addition, both high and low concentration VEGF scaffolds resulted in thicker ventricular wall size, in comparison to control animals with non-treated scaffolds [92]. Another angiogenic growth factor, SDF1, was heavily studied and has been proven to improve neovascularization in different MI models [93, 94]. One group cultured endothelial progenitor cells (EPCs) on a vitronectin/collagen scaffold primed with SDF1, which they named EPC Matrix (EPCM) [93]. EPCM was tested *in vivo* by being sutured to the anterolateral left ventricular wall of a rat MI heart and then monitored for up to 4 weeks. Not only did the levels of VEGF increase in the EPCM group, but a more densely packed and organized capillary network has been observed in the border zone myocardial sections with significantly enhanced perfusion, in comparison to the treatment group receiving ECM seeded with EPCs without SDF1. More importantly, the EPCM animals showed preservation of function in echocardiography, pressure-volume conductance, and Doppler flow, along with reduced infarct scar size [93]. The same group further advanced the application of SDF1 therapeutically by testing an engineered SDF1 analog (ESA) in a preclinical ovine model of MI [94]. Injection of ESA to the border zone of infarcted hearts resulted in increased capillary and arteriolar density, reduced infarct size and a steeper slope of the end-systolic pressure-volume relationship, which indicating preserved contractility, when comparing to animals injected with saline solution. Furthermore, coronary angiograms demonstrated an increase in myocardial perfusion, along with the immunohistochemical analysis showing increased capillary and arteriolar density in the border zone. This data supported the idea of applying ESA in human patients with acute MI in order to prevent ischemic cardiomyopathy [94].

4.3 Vascularization of Cardiac Patch by Decellularization

Perhaps one of the more immediately promising approaches to generate autologous hearts for implantation is by repopulating a decellularized heart with a patient's own cells. In the seminal study by Ott *et al.*, the authors demonstrated that the decellularized rat whole heart was capable of vascular perfusion, showing patent vessels after heterotopic transplantation into a recipient rat [77]. A recent study by Robertson *et al.* highlighted the importance of re-endothelialization in creating a minimally thrombogenic heart scaffold [95]. In this study, three strategies were used to re-cellularize vasculature in perfusion-decellularized rat heart

using rat aortic endothelial cells: retrograde aortic infusion, brachiocephalic artery (BA) infusion, or inferior vena cava (IVC) plus BA infusion. The authors reported that IVC plus BA infusion resulted in whole heart distribution of the endothelial cells, increased scaffold cellularity, and increased number of vessels *in vitro*. In addition, the re-endothelialized scaffolds seeded with neonatal cardiac cells were able to enhance construct contractility *in vivo,* compared to constructs seeded with cardiac cells without re-endothelialization [95].

Together, these studies demonstrate the application of vascularization strategies to enhance cardiac function after MI. As technological and scientific advances in this field ensue, clinical translation of vascularization strategies is promising.

5. Conclusions and Future Remarks

Tissues and organs produced *in vitro* need a constant supply of oxygen and nutrients to maintain viability and function. Tissue engineering has continued to progress forward with new chemical/biochemical discoveries combining compatible mixtures of natural and synthetic components. However, the lack of efficiently designed vasculature *in vitro* has stunted its exponential growth. Currently, some of the major roadblocks of vascularization approaches include (1) inefficient network architecture; (2) inability to closely mimic physiological blood flow rates; (3) precise biological control of the growth and stability of specific phenotypes derived from stem cells; and (4) maintenance of nutrient perfusion after vascularization. Thus, there is a clear need to investigate a large range of biomaterials and design approaches, using pro-angiogenic cells primed by chemical and physical cues. In the advent of new engineering technologies and the continued improvement of existing strategies, progress in vascularization of engineered tissues continues to advance. Successfully overcoming the vascularization hurdle will pave the way for scalable production of implantable tissues and organs, saving countless lives of patients, and ultimately extending the average life-span of human beings.

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Statement of Significance

Tissue engineering is a promising approach to replace or restore dysfunctional tissues/organs, but a major bottleneck in realizing its potential is the challenge of creating scalable three-dimensional (3D) tissues. Since most 3D engineered tissues require a constant supply of nutrients, it is necessary to integrate a functional vasculature within the tissues in order to facilitate transport of nutrients. To address these needs, researchers are employing biomaterial engineering and design strategies to foster vessel formation within 3D tissues. This review highlights the state-of-the-art bioengineering tools and technologies to create vascularized 3D tissues for clinical applications in regenerative medicine, highlighting the application of these technologies to engineer vascularized cardiac patches for treatment of heart failure.

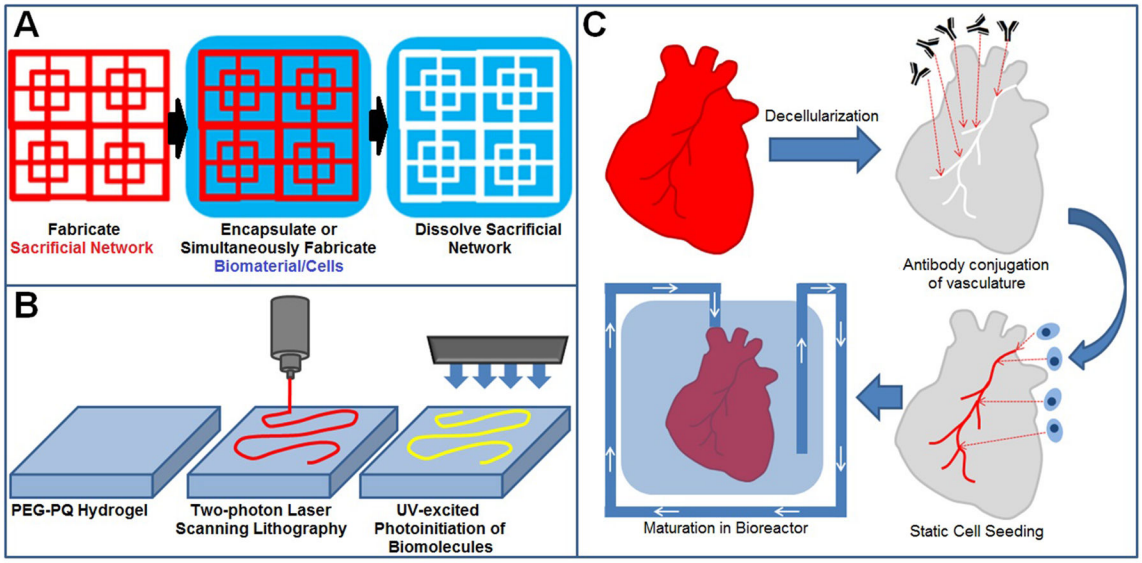


Figure 1. Engineering strategies for vascularization

(A) Channel formation using sacrificial materials. (B) Spatial micropatterning of biomolecules using two-photon lasers. (C) Decellularization and antibody-guided re-vascularization.

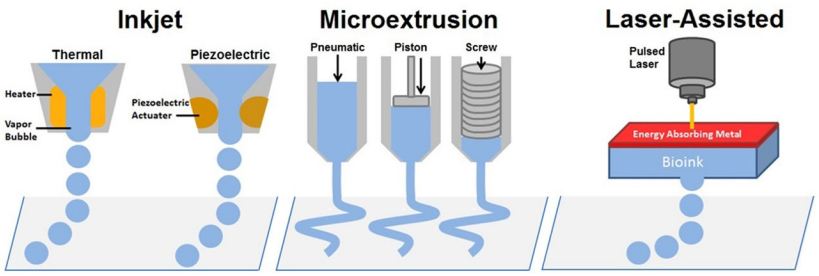


Figure 2. 3D Bioprinting approaches

Inkjet bioprinters use thermal or piezoelectric mechanisms to deposit drops of bioink onto a substrate. Microextrusion uses mechanical extruders to deposit bioink onto a supporting structure. Laser-assisted bioprinting is based on laser-induced forward transfer, and employs a “ribbon” consisting of a laser energy absorbing layer composed of a metal (i.e. gold or titanium) and a layer of bioink.

Table 1

Properties of common polymers used in vascular tissue engineering

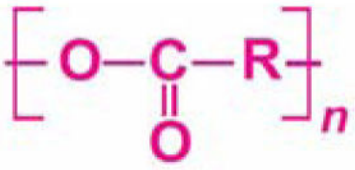
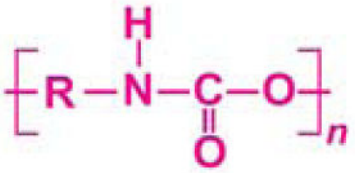
Type	General Chemistry	Description	Examples
Polyesters		Largest group of biodegradable polymers that are most widely used to date. Degradation rates and degradation products can be tuned according to composition, structure and molecular weight.	Polyglycolic acid (PGA), Polylactic acid (PLA), Poly-lactide-co-glycolide (PLGA), polycaprolactone (PCL)
Polyurethanes		Exhibit a segmented-block structure allowing for broad range of tunable mechanical, biodegradability and biocompatibility properties.	Polyurethane Acrylate
Poly-phosphazenes		Linear polymers with inorganic backbone of alternating phosphorous and nitrogen atoms with two side groups attached to each phosphorous atom, where manipulation of these side groups yield different properties.	Polydichloro-phosphazene
Tyrosine Derived Polycarbonates		Contain three hydrolysable bonds: amide, carbonate and ester. Via variation of the R group structure, different mechanical properties, degradation rates and biocompatibility can be achieved.	Polydesaminotyrosyl tyrosine ethyl ester carbonate

Table 2

Comparison of vascularization approaches

Strategy	Materials or Instruments Required	Advantages	Disadvantages	Example studies
Cell-Cell Interaction	Proangiogenic cells	Effective vasculogenesis, controlled composition	Lack of precision/resolution in network architecture	[44–48]
Functionalized Scaffolds	Angiogenic growth factors, biodegradable polymers	Controlled release of growth factors	Lack of precision/resolution in network architecture	[49–55]
Microfluidics	Active flow source (i.e. pump), soft-lithographized polymer	Ability to administer and study defined flow rates, ability to generate growth factor gradients	Lack of precision/resolution in network architecture	[56–60]
Sacrificial Materials	Dissolvable polymers or removable molds	Increased precision for defined network architecture compared to other approaches	Possible cytotoxicity with undissolved material	[62–66]
Spatial Micropatterning	Soft-lithographized or photo-lithographized polymers	High geometrical precision, controlled cellular and growth factor patterning/ presentation	Size-scale is limited Background blocking can change substrate properties	[67–75]
Decellularized Scaffolds	Donor tissue/organ, chemical detergents	Native vascular network is largely preserved	Chemical detergents can destroy integrity of tissue; requires donor tissue/organ; antigenicity from xenogenic tissues	[76–80]
3D Bioprinting	3D Printer, Bio-ink	High precision in 3D structure	3D printers for biological applications are still in infancy	[82–87]