From Microscale Devices to 3D Printing
Advances in Fabrication of 3D Cardiovascular Tissues

Anton V. Borovjagin, Brenda M. Ogle, Joel L. Berry, Jianyi Zhang

Abstract: Current strategies for engineering cardiovascular cells and tissues have yielded a variety of sophisticated tools for studying disease mechanisms, for development of drug therapies, and for fabrication of tissue equivalents that may have application in future clinical use. These efforts are motivated by the need to extend traditional 2-dimensional (2D) cell culture systems into 3D to more accurately replicate in vivo cell and tissue function of cardiovascular structures. Developments in microscale devices and bioprinted 3D tissues are beginning to supplant traditional 2D cell cultures and preclinical animal studies that have historically been the standard for drug and tissue development. These new approaches lend themselves to patient-specific diagnostics, therapeutics, and tissue regeneration. The emergence of these technologies also carries technical challenges to be met before traditional cell culture and animal testing become obsolete. Successful development and validation of 3D human tissue constructs will provide powerful new paradigms for more cost effective and timely translation of cardiovascular tissue equivalents. (Circ Res. 2017;120:150-165. DOI: 10.1161/CIRCRESAHA.116.308538.)

Key Words: biocompatible materials ■ heart ■ printing, three-dimensional ■ stem cells ■ tissue engineering

At the intersection of stem cell biology and tissue engineering resides enormous potential for patient-specific drug screening, disease modeling, and tissue equivalents that offer hope to treat some of the most devastating cardiovascular diseases. Tissue engineering comprises the optimization of 3 primary components: (1) the type or types of cells being implanted such as somatic cells, induced pluripotent stem cells or embryonic stem cell–derived cells, adult stem cells, and cardiac progenitor cells, (2) type of scaffolds supporting the cells (ie, the mechanical cues provided to the cells), and (3) type of small molecules, extracellular matrix (ECM), and growth factors conditioning the cells, (ie, the chemical cues provided to the cells). In addition, the conditions (eg, fluid flow, oxygenation, and temperature) in which the construct is cultured can have a significant impact on its maturation, making the development of novel bioreactors a major part of tissue engineering. Bioreactors can be used to aid in the in vitro development of new tissue by providing biochemical and physical regulatory signals to cells and encourage differentiation and production of extracellular matrix (ECM) before in vivo implantation.1 Bioreactor technology is integral to the emergence of microfluidic lab-on-a-chip, organs-on-chips, and bioprinted 3-dimensional (3D) tissues. These techniques are emerging as a supplement to traditional 2D cell cultures and preclinical animal testing as the standard for drug and tissue development. This review summarizes the bioengineering basis for these technologies and how they are shaping the future of cardiovascular tissue engineering. We begin our review by defining microfluidic chip technology and its application to studying some basic mechanisms governing behavior of cardiovascular cells. We briefly discuss 3D organs-on-chips. We then focus mainly on 3D tissue printing methods and the relationship to cardiovascular bioprinting with an emphasis on the fabrication of vascular networks. We further discuss bioprinting using cell spheroids and methods to manipulate spheroids to produce their own ECM without the use of natural or synthetic polymer scaffolding. Finally, we summarize what is already possible with these technologies and their limits as compared with more traditional cardiovascular tissue engineering methods.

3D On-Chip Technologies
During embryonic development, the fate specification of stem cells differentiation is regulated by the 3D microenvironment, in which not only a variety of biochemical factors but also the biophysical signals are presented within the 3D extracellular matrices. These seamless signaling pathways, and spatial, temporal factors together dictate stem cell differentiation and maturation.2

Why 3D Is Outpacing 2D in Cell Culture Technologies
Cell cultures were developed in the first half of the 20th century by Harrison.3 Despite the significant contributions and their demonstrated value in biomedical research, they are unable to recapitulate the tissue-specific functions of many...
differentiated cell types or accurately predict the in vivo effects of drugs. These limitations prompted development of more complex 2D tissue culture models, such as those that incorporate multiple cell types or involve cell patterning. In the case of cardiomyocytes, paracrine signals from endoderm-like cells, endothelial, cardiac fibroblasts, and other stromal cell types have been shown to support normal physiology and maturation of cardiomyocytes. Similarly, patterning of cell adhesion molecules or fabricating channels of appropriate microgeometry can promote cardiomyocyte function and alignment. However 3D models are rapidly gaining favor as they have the capacity to better represent the structural and functional complexity of living tissues (Figure 1). The cost–benefit analysis of 3D versus 2D approaches for cardiovascular tissue engineering includes consideration of cell–cell and cell–matrix interactions, the ability to modulate culture stiffness to mimic that of the native heart with development or disease, the capacity to impose mechanical and electric stimulation akin to that experienced in the heart, and the inclusion of perfusable vasculature to carry not only nutrients but also relevant cytokines and other signaling molecules (Table 1). As one pertinent example, a recent study showed that cardiomyocytes maintained in 3D hydrogels composed of fibrin exhibit higher conduction velocities, longer sarcomeres, and enhanced expression of genes involved in contractile function than 2D monolayers matched in age and purity of myocytes. For this reason, many 3D model systems for cardiomyocyte culture have emerged with the goal of optimizing scaffold formulation, supporting cell content, and electromechanical stimuli to promote cardiomyocyte maturation. The 3D models in use today, often termed engineered heart tissue, are more suitable than conventional or 2D cultures for studying the molecular basis of cardiac function and represent better disease models for studying signaling pathways and drug responsiveness (Figure 2). In 3D cultures, cells can be exposed to normal physical factors, such as mechanical tension/stress, compression or fluid shear stress, which affect tissue architecture, organ development, and function. The absence of fluid flow in 2D tissue models also precludes the study of the interaction of cultured cells with circulating perfusion or the cytokines released.

**Microscale Devices: Transition to 3D Platforms**

More recently, microscale devices have emerged to recapitulate some functional properties and features of minimal functional units of tissues of organs. These devices, sometimes called organs-on-chips, can harbor channels or reservoirs in which hydrogels support multiple cell types and are typically perfused by microfluidic vascular conduits designed to model human physiology in vitro. An organ-on-a-chip is a microfluidic cell culture device created with microchip manufacturing methods that contain continuously perfused chambers inhabited by living cells arranged to simulate tissue- and organ-level physiology. These platforms provide a useful alternative to macroscale 3D model systems as they allow scaling down of reagents and cells and are also easily amenable to continuous perfusion, drug dosing, and imposition of mechanical and electric stimulation.

Manufacturing of microscale devices often involves a replica molding technique wherein a liquid polydimethylsiloxane prepolymer is cast into the mold and peeled off after polymerization. Critical to the success of microscale devices in this context is consideration of the channel or reservoir material. For decades, polydimethylsioxane was used as support material, but this material suffers 2 primary limitations: (1) evaporation because of permeability to water vapor and (2) bulk absorption of hydrophobic components including protein. To avoid these drawbacks, polystyrene, cyclo-olefin copolymer, and Teflon have been implemented recently with success.

Microscale devices enable modeling and analyses of a whole variety of physiological processes. Such analyses are often not feasible for static 3D cultures or bioreactors. Another important advantage of the organ-on-chip devices is the potential to control cell patterning. Different cell types can be plated in the microchannel in distinct patterns or in direct juxtaposition on the same planar substrate. Tissue–tissue interfaces can be engineered by culturing 2 different cell types on opposite sides of a permeable (porous) membrane to model universal interactions between a vascular endothelium and parenchymal tissues. Furthermore, electromagnetic
fields, applied to chip-based tissue models, are capable of stimulating wound healing or contractile movements (pacing) of muscle tissues. Finally, integration of electronic microsensors, constructed by using microchip fabrication technologies, enables monitoring cell migration, fluid pressure, or other factors of

<table>
<thead>
<tr>
<th>Feature</th>
<th>3D Cardiovascular Tissues vs 2D Systems</th>
<th>Advantage</th>
</tr>
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<tbody>
<tr>
<td>Cell–cell and cell–matrix</td>
<td>Incorporation of multiple supportive cell types and formulations of extracellular matrix proteins</td>
<td>Feature can be tuned to reflect attributes of the native CV system with receptor engagement on all cell surfaces</td>
</tr>
<tr>
<td>Tissue stiffness</td>
<td>Various biomaterials and associated processing steps can be used to control tissue stiffness</td>
<td>Feature can be tuned to reflect that of developing, healthy or diseased tissue</td>
</tr>
<tr>
<td>Mechanical strain</td>
<td>Strains can be imposed that allow auxotonic contraction</td>
<td>Feature supports alignment and maturation of cardiomyocytes</td>
</tr>
<tr>
<td>Electric stimulation</td>
<td>3D engineered tissues can be paced at constant or increasing frequency</td>
<td>Feature supports alignment and maturation of cardiomyocytes</td>
</tr>
<tr>
<td>Vascularization</td>
<td>Only possible in 3D and being implemented in a range of forms</td>
<td>Feature provides nutrient perfusion and paracrine functions vital for cardiac homeostasis</td>
</tr>
<tr>
<td>Intercellular electric coupling</td>
<td>Can be assessed by calcium handling or reporters for gap junctions in combination with confocal or multiphoton laser scanning microscopy</td>
<td>Electric and structural coupling are more accurately assessed in 3D where the coordinated effect of multiple Z planes can be included</td>
</tr>
<tr>
<td>Mature cardiac cell function</td>
<td>Can be assessed by quality of physiological and pharmacological responses</td>
<td>Action potential, abundance of sarcomeric and sarcoplasmic proteins, quality of Frank–Starling behavior, force–frequency relationship, reaction to calcium, isoprenaline, carbachol found to be more akin to tissue response</td>
</tr>
</tbody>
</table>

3D indicates 3-dimensional.

Figure 2. In vitro testing of cells and tissues may occur in several ways. Microfluidic systems (A) have emerged as a tool for basic science studies of the effect of highly controlled fluid mechanical and solid mechanical forces on single cell types or cocultures. Microfluidic systems are also gaining favor as a diagnostic tool and a platform for drug development. Organoid cultures (B) are described as organ buds grown in culture that feature realistic microanatomy and are useful as cellular models of human disease. These cultures have found utility in the study of basic mechanisms of organ-specific diseases. Spheroid cultures (C) feature sphere-shaped clusters of a single cell type or coculture sustained in a gel or a bioreactor in order to interact with their 3D surroundings and are useful in testing drug efficacy and toxicity. (D) Engineered heart tissues are constructed by polymerizing an extracellular matrix–based gel containing cardiac cell types between 2 elastomeric posts or similar structures allowing auxotonic contraction of cardiomyocytes. This allows approximation of the normal conditions of the heart contracting against the hydrostatic pressure imposed by the circulation. This type of tissue construct has been used for testing toxicity of drugs and basic studies of muscle function and interplay between multiple cardiac cell types.
the artificial tissue/organ microenvironment. Modulation of flow rates or microfluidic channel sizes enables monitoring fluid shear stresses independently of physical and chemical gradients.

**Organs-on-Chips**

Utilization of induced pluripotent stem cells for organs-on-chips and 3D tissue engineering holds potential for organ modeling in disease-specific and in a patient-specific manner. Studies using these cells may lead to the development of personalized humans-on-chips systems with all cellular components derived from a patient. Over the past decade, researchers have constructed organ-on-chip model systems for studying functions of different organs including kidney,11–14 intestine,15,16 lung,17–19 liver,20–24 heart,25,26 smooth and striated muscle tissue,27 fat,28–30 bone,31 marrow,30,32 cornea,33 skin,34 blood vessels,35–37 nerves,38,39 and even blood–brain barrier.40,41 However, because of the single cell type composition, many of the above systems cannot be considered organ models. Nonetheless, fluid flow and shear stress alone have been demonstrated by some of those microchips to have a profound structural and functional impact on cells. In addition, oxygen pressure variations applied to organs-on-chips allowed better recapitulation of hypoxia-caused diseases such as myocardial ischemia25 or vaso-occlusion in sickle-cell disease42 and facilitated drug screening.

The feasibility of integration of muscular cell layers into microfluidic chips43 or confined to microscale pillars opened perspectives for examining the contribution of fluid flow, tissue–tissue interactions, as well as mechanical and electric signals to the development of cardiovascular diseases. A simple microfluidic model of cardiac ischemia/perfusion injury has been created by culturing primary porcine cardiomyocytes under variable oxygenation to mimic hypoxic conditions or hypoxia/normoxia transition.25,43

Microscale devices with gas control and oxygen monitoring functionalities have also been used for culturing cardiac tissue and physical stimulation of cardiomyocyte contractile function.44 Those cardiac model systems have been used for assaying loss of membrane potential and cytochrome C release as an early manifestation of apoptosis typically observed after ischemia reperfusion. This chip and related approaches have proven to be useful tools for inducing ischemia/reperfusion injury in primary cardiomyocytes and for determining the kinetics of apoptosis with cardiomyocyte loss.5,25

Multiple on-chip models of angiogenesis and microvascular function have been reported. Those were primarily composed of microfluidically ported microchannels permeating an ECM stroma to create functional capillary networks with free sprouting potential in response to soluble gradients of angiogenic factors. The use of ECM to embed internal networks of microchannels filled with sacrificial materials enabled independent cell seeding in either the channels (endothelial cells) or surrounding ECM (tumor cells, fibroblasts, etc). Once functionally integrated into the existing vascular network, the newly formed microvessels were perfused by connection to external flow via a gasket that also served to house the 3D ECM. Such models of angiogenesis allowed studying 3D morphogenetic processes, including the functional mechanism of angiogenesis inhibitors, and helped our understanding of how spatial diffusive gradients influence angiogenic sprouting.45–47

A more complex model of vascular networks, reported recently, was based on an electric circuit design and involved an array of nearly identical human microtissues with interconnected vascular networks. The authors applied resistive circuit concepts to design pressure dividers in serially connected microtissue chambers, thereby creating a controlled microphysiological environment within fibrin scaffold-containing microchambers. This methodology enabled culturing a large array of microtissues with interconnected vascular networks for biological studies and applications such as drug development.48

Lining a fibronectin-coated polycarbonate membrane with human brain microvascular endothelium on one side and human astrocytes on the other allowed development of a human blood–brain-barrier-on-a-chip.49 Another complex microfluidic system was developed as a neurovascular model of neuroinflammation by lining a porous membrane with rat brain microvascular endothelial cells on the one side and a mixture of astrocytes, neurons, and microglia, on the other. The cultured neural cells showed capability of building inhibitory and excitatory potentials, whereas engineered endothelium retained good barrier function and activated the adjacent microglia and astrocytes via tumor necrosis factor-α, analogous to neuroinfectious disease.49

**Three-Dimensional Printing Approach in Tissue and Organ Engineering**

To date, 3D printing techniques have been primarily used for fabrication of acellular 3D scaffolds and molds50,51 that in some cases could be subsequently filled up with live cells. A more advanced strategy of 3D printing, known as bioprinting, that allows printing tissue constructs by direct deposition of cells or cell aggregates has also recently been explored.52–54

To reproduce structural architecture and functional characteristics of a natural tissue and ultimately whole organs with high degree of accuracy, engineered constructs have to embody several key components such as cells, ECM, and vascular networks, which have to be assembled together with precise 3D-patterning using contemporary bioink (building blocks containing cells/biomaterials mixture or spheroids) deposition technologies. Perhaps the most important of the above components is the vasculature that provides nutrients, signaling molecules/factors, and efficient clearance/excretion of metabolites (waste transport) to matrix-seeded cells. Three-dimensional tissue constructs without adequate vascularization quickly develop necrotic regions within a few hundred microns of the boundaries/edges of the construct.55

Bioprinting technology allows fabrication of biomimetic and even anatomic 3D structures by using patients’ images obtained using medical imaging technologies, eg, computer tomography and magnetic resonance imaging. As one of the most advanced tissue/organ fabrication technologies, 3D printing uses automated processes and standardized materials as building blocks and enables creation of 3D objects from personalized computer-aided designs (CADs). Three-dimensional
printing, also referred to as additive manufacturing or solid free form fabrication, has already been utilized by cardiovascular surgeons to fabricate personalized organ models for visualization of anatomic structures.60 Personalized bioprinted models can better reflect structural abnormalities than traditional models or cadavers, and as a result can improve the choice of surgical approach and offer a platform to practice the procedures.

To precisely recapitulate complex objects, 3D bioprinting technology utilizes a computer-generated 3D design file created by virtually decomposing the shape of the object (obtained through medical imaging) into a series of 2D layers. The 3D bioprinter deposits bioinks in a layer-by-layer manner based on the design file. Each layer is then bonded to the previous layer to fabricate the 3D constructs. The layer-by-layer bioprinting can be accomplished by different methods depending on the type of printed material.57,58

There are 4 methods of 3D printing compatible with biopolymers typically used for generating scaffolds and ECM in tissue engineering applications: (1) selective laser sintering,59 (2) stereolithography,60 (3) fused deposition modeling,61 and (4) pressure-based extrusion.52,62 In brief, selective laser sintering uses CO2 laser to locally melt a thin layer of powder to fuse particles into a solid object. Stereolithography utilizes UV or visible light to trigger polymerization of a thin layer or a small focal volume of a photocrosslinkable resin-containing solution. Fused deposition modeling melts and extrudes a polymer through a nozzle onto a flat substrate to build up a 3D structure. Pressure-based extrusion is based on a differential pressure, generated by a syringe pump or an upstream pressure reservoir, to drive the material through a nozzle. Although selective laser sintering and stereolithography are typically faster printing methods than fused deposition modeling and pressure-based extrusion, they both require expensive lasers and optics. Selective laser sintering printing might require higher temperatures and thus is ideal for ceramics or metals, but could be prohibitive for bioprinting applications. Stereolithography, although fast and straightforward, requires materials (typically plastics) compatible with photocurable chemistries. Although both fused deposition modeling and pressure-based extrusion represent potentially simpler systems, they can be slower than other bioprinting methods used for cardiac tissue engineering.

Three-dimensional printing for tissue engineering applications has the flexibility to use inks with or without cells. In the former case, scaffolds of complex geometries should be printed using only biocompatible materials. The technology of engineering artificial tissues by directly encapsulating cells as part of the ink (called bioink) during the printing process is known as bioprinting.51,54 Although 3D printing, using routine methods for industrial applications, allows a direct use of commercial printers without modifications, bioprinting technologies may not be compatible with commercial printer use and would require custom-built printing devices and biocompatible ink materials. Bioinks comprising cells suspended in hydrogels that serve as ECM are currently being developed and used in cardiovascular tissue engineering to directly print implants in the form of myocardial tissue, heart valves, and coronary arteries (see below). Furthermore, 3D printing has the ability to integrate electronics into tissue-engineered constructs to provide additional functionality, such as sensing and actuation.65-68

The main technologies used for deposition and patterning of biological materials include laser-assisted printing, multiphoton excitation (MPE)–based fabrication, inkjet printing, and microextrusion (Table 2; Figure 3). The features of these technologies should be discussed in conjunction with the most important factors in 3D bioprinting, such as feature resolution, cell viability, and the biological materials used for printing77 (summarized in Table 2).

Laser-Assisted Bioprinting
Laser-assisted bioprinting (LAB) is based on laser-induced forward transfer (LIFT)69,70 of the printed material/ink. Initially developed to transfer metals, LIFT technology has been successfully applied to biological material, such as peptides, DNA, and cells. Although being less common than inkjet or microextrusion bioprinting, LAB is often used for tissue engineering and organ engineering applications (Figure 3A). LAB functions using focused laser pulses on the absorbing layer of a ribbon that has a donor transport support usually made from glass that is covered with a laser-energy–absorbing layer (eg, gold or titanium), to generate a high-pressure bubble propelling a cell-containing material toward the collector substrate. The important advantage of LAB devices is that they do not use nozzle, which avoids clogging with cells or materials, the problem limiting performance of other bioprinting technologies. In addition, LAB is compatible with a range of viscosities (1–300 mPa/s) and can print mammalian cells with negligible effect on cell viability and function. LAB can deposit cells at high density (up to 10⁶ cells/mL) with microscale resolution (1 cell per drop, minimum drop size is ≈20 μm) and 5-kHz laser pulse repetition rate with overall printing speeds up to 16 cm/s. However, high LAB resolution requires fast ink gelation kinetics, which is hard to achieve, and practically leads to a relatively low overall flow rate. Besides, preparation of each individual ribbon, often required for each printed cell or hydrogel type, is time-consuming, costly, and may be technically challenging for depositing multiple cell types. Accurate targeting and positioning cells can be difficult owing to the nature of the ribbon cell coating and may require cell-recognition scanning technology to enable the laser beam to select a single cell per pulse or using a ribbon with very high cell concentrations. The high cost of LAB systems is also a concern for basic tissue engineering research, although as is the case with most 3D printing technologies, these costs are rapidly decreasing (Table 2).

MPE-Based Fabrication
MPE photochemistry can also be used to 3D print synthetic materials and native proteins.71,78,79 This method is analogous to multiphoton laser scanning microscopy in that the excitation, and thus the photochemistry, is restricted to the focal volume80–85 (Figure 3B). Previously, MPE fabrication technology was shown to crosslink layer-by-layer soluble and structural proteins into 3D matrices and fiber patterns
with spatial fidelity of >85%. The crucial component of this approach was implementation of a new photochemistry allowing efficient cross-linking of all types of collagen (eg, type I, II, and IV), laminin, fibronectin, and other proteins with limited solubility into 3D structures without the use of synthetic polymers. Extensive studies have characterized several scaffold materials and investigated stem cell–ECM interactions within fabricated structures. The major advantage of this rapid prototyping approach to CAD-guided MPE fabrication is that, in contrast to the best commercially available 3D printing technologies with 5- to 10-μm resolution limit, it allows fabrication of complex models with submicron-level resolution enabled by the MPE point spread function. A primary drawback of this 3D printing modality is limited macroscale size. To date, tissue fabrication is limited to microscopic fields of view. Bioprinting of larger structures thus would require merging fields of view and would result in structural seams significantly limiting printing speed, throughput, and fidelity to the digital template.

Table 2. Features of the Current 3D Bioprinting Approaches

<table>
<thead>
<tr>
<th>Feature</th>
<th>Laser-Assisted</th>
<th>Multiphoton Excitation</th>
<th>Inkjet</th>
<th>Microextrusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>High</td>
<td>Very high</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Droplet size</td>
<td>&gt;20 μm</td>
<td>300 nm to 3 μm</td>
<td>50 to 300 μm</td>
<td>100 μm to 1 mm</td>
</tr>
<tr>
<td>Printer speed</td>
<td>Medium (200–1600 μm/s)</td>
<td>Slow (1 mm²/h)</td>
<td>Fast (1–10 000 droplets/s)</td>
<td>Medium (10–1000 μm/s)</td>
</tr>
<tr>
<td>Cell viability</td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Cost</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>Primary advantage(s)</td>
<td>Single cell manipulation, no clogging associated with nozzles, wide viscosity range</td>
<td>Can print ECM exclusively, not dependent on high viscosity of bioink</td>
<td>Gradients can be generated by altering droplet size, low cost</td>
<td>High cell density can be used</td>
</tr>
<tr>
<td>Primary disadvantage(s)</td>
<td>High cost, time-consuming, technically challenging</td>
<td>Cells cannot be deposited with printing, end product mm small scale</td>
<td>Nozzle clogging, low droplet directionality</td>
<td>Limited number of biomaterials used to date</td>
</tr>
<tr>
<td>References</td>
<td>69, 70</td>
<td>71</td>
<td>72–75</td>
<td>76</td>
</tr>
</tbody>
</table>

ECM indicates extracellular matrix.

Figure 3. Bioprinting is usually accomplished using a combination of gel and cells. Laser-assisted bioprinting (A) using Laser-induced forward transfer relies on the focused energy of a laser onto an energy absorbing ribbon to induce bioink droplet formation. This technique is advantageous because it avoids the problem of clogging of the bioink nozzle that plagues other bioprinting techniques. Multiphoton excitation-based printing (B) is accomplished via photocrosslinking of proteins or polymers in the focal volume of the laser and excels in its high resolution and ability to polymerize many native proteins that do not form hydrogels spontaneously outside the body. Inkjet printing (C), one of the most common printing techniques, relies on a vapor bubble or a piezoelectric actuator to displace material to extrude the bioink from a nozzle. Robotic dispensing (D) uses other mechanical means of displacing bioink under robotic control. ECM indicates extracellular matrix.
Inkjet Printing

Inkjet printers (also known as drop-on-demand printers) are the most commonly used printers for both nonbiological and biological 3D printing applications. The key property of the inkjet technology is automated (computer-assisted) delivery of controlled volumes of liquid (ink) to predefined locations. Modified versions of commercially available 2D inkjet printers, where the cartridge ink was replaced with a biological material, and an electronically controlled z-axis elevator stage replaced paper, were the first 3D bioprinters used for tissue fabrication applications. Contemporary, custom-designed inkjet bioprinters utilize thermal or acoustic force-based liquid ejection mechanisms to print biological materials at increasing resolution, precision, and speed. The functional principle of thermal inkjet printing is based on electric heating of the print head to produce pulses of pressure that force droplets from the nozzle (Figure 3C). Localized heating, ranging from 200 to 300°C has been demonstrated by several studies to have no detrimental effect on either the viability or function of mammalian cells or stability of biological molecules, such as DNA. This is because the short duration of the heating pulse (≈2 µs) raises the temperature in the printer head by only 4 to 10°C. Another advantage of inkjet printing is the potential to create concentration gradients of cells, materials, or growth factors throughout the 3D structure by altering drop density or drop size during the printing process. Despite the fact that thermal inkjet printers are cost effective and offer high speed of bioink deposition along with the cell gradient capability, they pose numerous disadvantages for use in 3D bioprinting. Those include exposure of cells and materials to thermal and mechanical stresses, nonuniform droplet size, low droplet directionality, frequent nozzle clogging, unreliable cell encapsulation, and low cell densities. Another common drawback of inkjet bioprinting is the requirement for the biological material to be in a liquid form to enable droplet formation. As a result, the printed bioink must subsequently form a solid 3D structure with structural organization and functionality.

Microextrusion Printing

Microextrusion ink deposition technology is commonly used for nonbiological 3D printing applications and is becoming more wide-spread for bioprinting applications. The most typical microextrusion bioprinters consist of the following components: (1) a dispensing system controlled by temperature, (2) a computer-controlled 3D moving stage; (3) a video camera controlling 3D stage movements for precision control; (4) a photoinitiator-activating light source that illuminates the area of ink deposition; and (5) a piezoelectric humidifier (Figure 3D). The mechanism of microextrusion printer function is based on material deposition onto a substrate by robotically controlled extrusion through nozzles or needles connected to ink-loaded microextrusion head (cartridge) by continuous flow (line) of the printed (bio) ink material rather than deposition of liquid droplets. The material is deposited in 2D fashion, directed by the CAD-computer-aided manufacturing software, and could occur also in the form of small beads as in the case of bioprinting spheroids illustrated in Figure 4A. Inkjet and extrusion printing are thus the 2 major printing technologies, fully compatible with 3D printing of cell-laden constructs under physiological conditions. Although bioprinting of viscous bioinks using inkjet printing is relatively challenging, it has been widely used for 3D printing of cell-laden constructs owing to relatively higher cell viability provided by this method as compared with microextrusion printing. The z-axis material deposition can be achieved by either stage or the microextrusion head movements, whereby each deposited layer creates a foundation for the next layer. Materials compatible with microextrusion-based printers include biocompatible copolymers, hydrogels, and cell spheroids. The most common forces used in extrusion printers for dispensing of biological materials are pneumatic and mechanical (Figure 3D). Because of the delay associated with the gas volume compression in pneumatic systems, they might provide less direct control over the material flow than printers with mechanical force-driven dispensing. Mechanical force extrusion printers with screw-based dispensing mechanism are thought to be optimal for printing of highly viscous hydrogels although pneumatic systems could also be used for printing such materials.

The structure of pneumatic force-driven 3D printers is relatively simple. Force limitations are determined by air pressure capabilities. Printers with mechanically driven mechanisms contain more complex, but compact components, and provide better spatial control. However, the latter often comes at the price of reduced force capabilities. Microextrusion bioprinting technology offers an important advantage over the other types of bioprinting approaches in that it enables depositing cells with very high densities (Table 2). However, the resulting high viscosity of the bioink might be detrimental for cell viability and requires suitable bioink formulation. Therefore, bioinks that allow physiological density of the cells/tissues of interest in engineered tissues and organs are critical for the success of the bioprinting field.

Commercial Bioprinters

Currently, there are several major commercial bioprinter devices. Some of the better known companies, at this writing, are BioFactory by RegenHu (hard and soft tissue bioprinting), BioAssemblyBot by Advanced Solutions Life Science (6-axis robotic arm with up to 8 syringe barrels for dispensing bioink), and Bio3D SYN from Bio3D Technologies (designed to be modifiable for research and scientific purposes). In general, they have each developed their own unique approaches to the theme of building 3D tissue structures. Other examples are the 3D-Bioplotter System developed at the Freiburg Materials Research Centre in Germany. This bioprinter is compatible with the use of a large variety of biomaterials ranging from soft hydrogels and polymer melts to hard ceramics and metals. Three-dimensional CAD models derived from patient-specific computer tomographic data are used in Bioplotter for fabrication of 3D scaffolds with well-defined outer shape and an open inner structure, critical for tissue engineering and controlled drug release. The 3D-Bioplotter is specifically designed to work in the sterile environment of biosafety cabinets, which is crucial for biofabrication of scaffolds from alginate cell suspensions. In contrast to other rapid prototyping techniques, the technology used in 3D-Bioplotter is simple and
straightforward. The world’s first commercial 3D bioprinter, NovoGen MMX bioprinter, contains 2 separate robotically controlled precision print heads: one for depositing cells and the other for hydrogel scaffold or support matrix. Although the bioprinter was initially designed to fabricate tissues, like blood vessels and nerve conduits, it could potentially be utilized for printing more complex anatomic structures such as heart and its integral tissue components.

Bioprinting Technology Applications

Fabrication of engineered tissue constructs typically involves manual procedures, which impose limitations on the complexity, by which materials of varying properties and dimensions can be interfaced. Three-dimensional printing offers a means to automate the process of fabricating tissue mimics from a variety of compatible materials. The ability to print 3D microenvironments opens new perspectives for the development of new drug screening methods and facilitates fundamental studies in the fields of wound healing, angiogenesis, and stem cell biology. On further optimization, the existing tissue engineering techniques may lead to the rapid manufacturing of functional 3D tissues and, possibly, even artificial organs. Studies are currently underway to determine the optimal ways to produce fully vascularized, engineered tissue constructs by combining biological self-assembly with 3D printing approaches.95

Strategies for Cardiac Tissue Engineering

The effectiveness of cell therapy applications for cardiac diseases depends on the ability of the implanted cells to survive and properly integrate into the recipient’s heart tissue with the resulting improvement in cardiac function.96 Currently, strategies to build thick multilayer tissues involve integration of vascular structures into the implant before transplantation. This has to date resulted in fabrication of artificial tissues with thickness on the order of 100 µm, which still suffer from cell death at the center.97 Because viability of printed tissues heavily depends on oxygen supply, thin tissue constructs that can receive oxygen simply by diffusion demonstrate better survival than thick patches with limited accessibility of deep cell layers to freely diffusing oxygen.

In this regard, to generate viable bioprinted tissues, the following minimal requirements have to be met: (1) a functional vascular system that can easily be integrated with the recipient/host tissue should be incorporated into the transplant tissue, (2) cells have to be precisely patterned and oriented in the context of a hierarchical structure on microns to millimeters scale, and (3) the engineered tissue structure should incorporate materials that induce and maintain proper phenotype of the cells and do not elicit any adverse reaction from the host, such as inflammation or immune response. However, the advances in technologies to improve the transplanted cell survival for the first 1 to 3 days are going to be the most important and useful. This is because if the cells in fabricated tissue can survive for the first 3 days after the cardiac transplantation, sprouting of pre-existing vessels of the recipient tissue could occur into the grafted tissue to supply it with oxygen and nutrients as heart is an organ with a very robust angiogenesis potential.

Typical materials utilized in cardiovascular bioprinting include synthetic and natural bioactive hydrogels such as gelatin, collagen, fibrin, and peptides with cell adhesion-supporting capacity.98–100 Microcarriers are another option and offer a highly specific surface area and bioactive environment for quick cell attachment and proliferation.101 Cells can be encapsulated within microcarriers and further incorporated within bioinks for bioprinting. Scaffold-free cell spheroids (Figure 1), generated by biofabrication approaches like hanging drop, micromolded, microfluidics, and spinner flasks, represent another biological substrate for bioprinting. Spheroids can fuse together and quickly generate mature constructs.

Figure 4. Scaffold-free bioprinting uses cell spheroids and does not utilize a gel as a carrier. Robotic dispensing of spheroids (A) typically occurs through a nozzle onto a carrier substrate. Cell spheroids can be delivered to form various shapes including blood vessels (B). Cell spheroids will self-organize, fuse, and begin forming their own extracellular matrix (C). Under appropriate mechanical stimulation, fused spheroids can develop enough mechanical integrity to become suitable for implantation as a load-bearing tissue replacement.
with heterogeneous cell populations and better biomimicry (Figure 3C). This enables coculturing different cardiovascular cell types such as cardiomyocytes, endothelial cells, smooth muscle cells (SMCs), and cardiac fibroblasts. However, cell spheroid–based scaffold-free constructs are not stable enough and require a support structure to stabilize the structure initially and several weeks to undergo remodeling and full maturation.

Generation of bioinks from natural decellularized ECM (dECM) represents a new approach that can be broadly used in the context of extrusion-based 3D printing. ECM derived from various native tissues represents a new source of bioink with broad utility for bioprinting applications. ECM is considered as an essential structural element of tissue with important role in biochemical signaling, particularly, pertaining to stem cell differentiation and survival.102,103 Bioinks composed exclusively of ECM, simply containing exogenous ECM or producing endogenous ECM as a result of biological activity of spheroids hold enormous potential for 3D printing technology. To produce ECM-based bioink, ECM is first decellularized and then dissolved/concentrated into paste-like material.104 To make consistent and component controllable bioinks, it is important to standardize the process of ECM decellularization based on tissue sources. To improve the mechanical properties and bioprintability of a tissue scaffold, synthetic hydrogel-based bioinks are often combined with decellularized ECM, which can be performed in the context of a supportive frame to be printed first. Bioinks made of decellularized ECM, obtained by decellularization of whole organs, may be capable of better maintaining proper cellular phenotype owing to the ability to provide instructional signals to seeded cells. In support of this concept, decellularized ECM bioink induced higher expression of cardiac-specific genes (Myh6 and Actn1) and higher expression of cardiac β-myosin heavy chain after 4 days in culture as compared with collagen-based construct.104

As an alternative, and more easily standardized method, bottom-up approaches have been used to create ECM formulations from combinations of different types and amounts of individual ECM proteins. By pairing this concept with design of experiments statistical approaches, ECM formulations supportive of cardiac cell types have been developed105 and could be further optimized in future.

Like many other tissue engineering approaches, 3D printing of myocardial tissue is limited primarily by low resolution of complex structures and availability of appropriate cells for the bioink, ie, implantable human cardiac cells. Cardiac progenitor cells or induced pluripotent stem cells hold promise for bioprinting tissues and organs as unlimited source of cardiac cells.

**Three-Dimensional Bioprinting of Arteries and Microvascular Structures**

Vascular networks are essential not only for oxygen transport, delivery of nutrients and immune cells, or removal of metabolic waste products from cells and tissues but also for the process of regeneration of cardiovascular and other tissues. Coronary artery disease resulting from deficient blood supply of cardiac tissue still accounts for >30% of all human deaths and is responsible for ≈ 1.2 million hospitalizations each year. Standard of care for this disease includes statins, antiplatelet agents, nitrates, coronary angioplasty, and coronary artery bypass grafting surgery.

Vascular tissue engineering holds promise for fabrication of artificial coronary bypass grafts. An ideal tissue-engineered graft should be nonthrombogenic, properly endothelialized and possess biomechanical properties comparable to the native blood vessel. Biomimetic (denotes synthetic methods, systems or elements of nature that mimic biochemical processes) blood vessels can be engineered by using 2 main approaches: (1) a scaffold-guided method, in which scaffolds using natural, synthetic biomaterials, or decellularized ECM are built to support cell attachment, infiltration, and proliferation during the in vitro tissue development; (2) a cell-sheet-based approach, in which a monolayer of 2D-cultured cells is rolled on a mandrel to produce an artery mimicking tubular conduit.105 Despite recent efforts, most small lumen artificial coronary bypass grafts failed to achieve the longevity and the performance of natural autologous grafts, and therefore none of them to date has been successfully commercialized for clinical use. Major problems remain to be the loss of the endothelial cell layer and early vessel closure after transplantation.

Three-dimensional printing can greatly contribute to the creation of microvascular networks and individual, replacement vessels by enabling generation of scaffolds with patient-specific geometries or direct printing of differentiated endothelial cells, fibroblasts and SMCs, or mesenchymal and hematopoietic stem cells in the context of biocompatible scaffold materials (eg, hydrogel). However, 3D printing has not yet been used for fabrication of coronary bypass grafts. Instead, the research efforts have been focused primarily on generation of in vitro vascular models through lining the inner surface of patterned microchannels with endothelial cells to fabricate microvascular networks for studying angiogenesis and thrombosis or for supplying nutrients and oxygen to engineered tissue.

Three-dimensional bioprinting technology enables fabrication of vascular networks with patient-specific patterns and clinically relevant size of perfusable channels. The following vascularization strategies are used in vascular tissue engineering: (1) generation of vascular constructs by self-assembly of cells; (2) generation of microvessels by inkjet-based bioprinting; (3) generation of bioprinted constructs with growth factor delivery capabilities; (4) fabrication of vasculature using coaxial nozzle-assisted 3D bioprinting; and (5) generation of constructs through channel-based vascularization. Self-assembly of cells utilizes similar adhesive properties of certain cell types to form spontaneous and stable aggregates or structures without external stimuli. The examples of such structures are spheroids and self-assembling tubular structures.65,106

Spheroids generated from cell suspensions were used in a recent study as building blocks to fuse into vasculature-like constructs62 (Figure 3). Tubular structures with controllable channel diameter, wall thickness, and branching pattern could be fabricated by fusion of multicellular spheroids on agarose rods as templates. Vascular construct with double-layered wall and specific branching pattern could recently be obtained by depositing multicellular cylinders composed of human SMCs and human skin fibroblasts.54
Kucukgul et al.\textsuperscript{107} 3D-bioprinted aggregates of mouse embryonic fibroblasts instead of spheroids to form an arterial (aortic) tissue construct. Simultaneous deposition of human microvascular endothelial cells and fibrin as a scaffold material allowed Cui and Boland\textsuperscript{108} to bioprint microvascular constructs by using a modified commercial inkjet printer. The scaffold of the fabricated construct retained proper shape after printing, whereas endothelial cells spontaneously formed tubular structures on proliferation.

Hollow calcium alginate filaments (channels) were fabricated by using a coaxial nozzle-assisted 3D bioprinting system, dispensing sodium alginate solution (with or without cells) that was cross-linked after coming into contact with calcium chloride solution in the inner chamber of the coaxial nozzle. The hollow filaments were further used as building blocks for bioprinting vascular constructs.\textsuperscript{109} A similar strategy was used in yet another study by Yu et al.\textsuperscript{110} This strategy allowed bioprinting of vasculature of defined geometry, length, and orientation. Coaxial nozzle-assisted 3D bioprinting technology is limited by the availability of bioink. Only alginate-based bioink is currently used with this technology owing to its fast cross-linking capacity.

Besides direct printing of vascular channels, many research teams generate vascular networks by using sacrificial materials within engineered tissue constructs, which involves 3D printing of water soluble material-based filament networks into a supportive matrix material (typically cell-laden hydrogel) with subsequent dissolving of the filaments with special solvents or altering temperature. A new 3D printing-based approach for creating vascularized, heterogeneous tissue constructs was reported by Kolesky et al.\textsuperscript{95} The authors initially fabricated a multilayer tissue construct by coprinting 2 inks at 20 to 22°C: the fugitive Pluronic F127 ink and a cell-laden gelatin methacryloyl ink with green fluorescent protein–expressing human neonatal dermal fibroblasts. Then they deposited pure gelatin methacryloyl ink at 37°C to fully encapsulate the printed features, followed by photopolymerization of the gelatin methacryloyl matrix by cross-linking. The fugitive ink was subsequently liquefied and removed from the 3D construct so that the evacuated channels could be endothelialized. The authors clearly observed both the green fluorescent protein–expressing human neonatal dermal fibroblasts in gelatin methacryloyl and the red-human umbilical vein endothelial cells (HUVECs) lining the embedded 3D vasculature by confocal microscopy.\textsuperscript{95} Thus, the 3D printing platform allows fabrication of artificial tissue constructs by programmed deposition of multiple cell types along with vascular structures within extracellular matrices.

**3D Bioprinting of the Myocardium**

Myocardial infarction (MI) accounts for nearly half of the 7.3 million heart disease–related deaths each year.\textsuperscript{111} If the coronary blood supply is not recovered quickly within 60 minutes, a large number of cardiac cells, including cardiomyocytes, within the blood-deprived myocardium are lost. A prolonged vigorous inflammatory response and postinfarction left ventricular remodeling ultimately lead to heart failure. Because cardiomyocytes, being the main building blocks of the heart tissue,\textsuperscript{112} have limited capacity to proliferate and replace damaged cells, the ischemia damaged heart fails to recover after MI.\textsuperscript{113} The native population of cardiac progenitor cells is very limited and decreases significantly on aging, thereby compromising the myocardial repair potential.\textsuperscript{114}

Although preclinical and clinical studies of cell therapy demonstrated promising results,\textsuperscript{115–117} this approach is limited by low long-term grafts.\textsuperscript{118} Heart transplantation, as the last therapeutic option for severe heart failure, is limited by shortage of organ donors on the one hand, and allogeneic transplant rejection, on the other.\textsuperscript{119} A myocardial tissue regeneration strategy, as a cutting edge treatment option for MI, relies on tissue engineering technologies including 3D printing. A successful fabrication of myocardial tissue from chick embryonic cardiomyocytes mixed with collagen solution was performed back in 1997 by Eschenhagen et al.\textsuperscript{120} leading to establishment of the first coherently contracting 3D model of heart tissue that allowed direct measurement of isometric contractile force. Fabrication of human myocardial tissue equivalent using human induced pluripotent stem cells holds potential for replacing some of the conventional therapies in the future.\textsuperscript{121,122}

Using high fidelity cardiac magnetic resonance imaging information to guide the engineering of human myocardial tissue equivalent with regards to the shape and size of the artificial tissue implants that would mimic the essential characteristics of the natural myocardium is the major goal of 3D printing of human myocardial tissue equivalent.

Fabrication of cardiac tissue implants, in addition to proper vascularization and efficient oxygen exchange, requires proper density of cardiomyocytes and various supporting cells.\textsuperscript{123} These conditions can be achieved by various tissue bioprinting techniques providing unique capabilities for patterning and assembling cells with defined density and spatial distribution. Gaebel et al.\textsuperscript{123} used LIFT cell printing approach to fabricate a cardiac patch seeded with HUVECs and human mesenchymal stem cells in a defined pattern. The authors’ incentive for coprinting human mesenchymal stem cell with HUVEC was based on the recent finding that multicellular spheroids could inhibit apoptosis of endothelial cells under hypoxic condition, thereby increasing their survival, and stimulate angiogenesis. Specific vascular patterns were successfully generated by LIFT printing of fluorescently labeled HUVEC (green) and human mesenchymal stem cell (red) arranged in a capillary-like pattern on a polyester urethane urea cardiac patch, whereas control patches were generated without LIFT by random seeding of equal amounts of each cell type. Patches with LIFT-patterned cells were cultivated further and transplanted onto infarcted zones of rat hearts after left anterior descending-ligation. Cardiac performance was assessed 8 weeks post infarction and showed that the LIFT-generated cell patterning stimulated growth of cocultured HUVECs and human mesenchymal stem cells, leading to significant improvement of functional characteristics of the infarcted hearts. This study also demonstrated an increased capillary density and integration of transplanted cells into the recipient’s vascular system by functional connection to its blood vessels, suggesting functional benefit of LIFT-generated cardiac patch for wound healing and functional preservation during MI treatment.
Recently, Gaetani et al.\textsuperscript{124} demonstrated that microstructure tissue printing using a combination of alginate scaffold with human fetal cardiomyocyte progenitor cells can be used to fabricate a cardiogenic patch with defined pore size and improved viability. To further improve this technology of myocardial tissue engineering, a new cardiac scaffold consisting of human fetal cardiomyocyte progenitor cells and a hyaluronic acid/gelatin (HA/gel)-based biomaterial was created. This advanced bioink enhanced attachment and survival of human fetal cardiomyocyte progenitor cells without affecting their growth and differentiation potential. Besides this, the cells’ commitment for the cardiac lineage was maintained as evidenced by upregulation of early cardiac transcription factors and expression of sarcomeric protein troponin T. The bioprinted tissue patch demonstrated excellent cell survival and engraftment when tested in a murine model of MI. Hearts receiving the human fetal cardiomyocyte progenitor cells scaffold transplantation showed improved cardiac function after MI.\textsuperscript{125}

**Fabrication of Heart Valves by 3D Printing**

Three-dimensional printing also holds strong potential for fabrication of engineered heart valves. Currently, heart valve replacement surgery involves implantation of either mechanical or chemically cross-linked tissue heart valves.\textsuperscript{126} The advantage of synthetic valves over biological valves is that they are mechanically robust and typically have a longer lifetime.\textsuperscript{127} However, patients with prosthetic valves are required to take anticoagulants. Biological valves, made from either an allogeneic or xenogeneic source, do not require the patient to take anticoagulants.\textsuperscript{128} In addition, neither of the current valve replacement types are able to grow and remodel with the patient. Three-dimensional bioprinting could in principle address all of the current limitations of valve replacements.

To date, the engineered heart valves have been fabricated by using an extrusion-based 3D printer from 2 types of photocrosslinkable hydrogels: one rigid (\textapprox 75 kPa) hydrogel for the root and the other soft (\textapprox 5 kPa) hydrogel for the leaflets.\textsuperscript{129} The ability to simultaneously print 2 materials with distinct mechanical properties provided by the 3D printer, allowed better mimicry of the differential stiffness of the native heart valve tissues such as the leaflets and the root. Another study from the same group showed that interstitial cells from porcine aortic valve can survive for up to 3 weeks in the context of 3D printed artificial heart valves.

In yet another study, aortic root sinus SMCs and aortic valve leaflet interstitial cells encapsulated into the root and leaflet portions of the valve, respectively, remained viable for 7 days in culture. The valve leaflet interstitial cells could remodel the printed hydrogels by depositing their own collagen and glycosaminoglycan-based ECM. Encapsulated SMC and valve leaflet interstitial cell showed elevated expression of \(\alpha\)-smooth muscle actin and vimentin, respectively, demonstrating that anatomically complex and heterogeneously encapsulated aortic valve hydrogel conduits can be successfully fabricated using the 3D bioprinting approach.\textsuperscript{130} Despite the progress in heart valve tissue engineering, no functional testing of any of the printed valves has been performed to date.

**Whole-Heart Bioprinting**

Heart is mainly composed of 3 different types of cardiac tissues: myocardium, endocardium, and pericardium. The main cell types that make up the cardiac tissues are cardiomyocytes, cardiac fibroblasts, and endothelial cells. It has been reported that myocytes constitute up to 30% to 40% of the entire cell population of normal adult heart, and the rest of it is represented by nonmyocytes with the majority being fibroblasts.\textsuperscript{112} However, a recent study by Pinto et al.\textsuperscript{131} demonstrated that endothelial cells make up the largest portion of nonmyocytes in the heart.

A group of specialized pacemaker cells located in the right atrium and called the sinoatrial node can autonomously generate electric impulses to set off contractions of the myocardium. Anisotropic self-alignment and contractile synchronization of cardiomyocytes in the myocardium promotes the electric activation of the cardiac muscles. In addition to the intrinsic automaticity of sinoatrial node, its pacemaker activity is normally controlled by opposing input from the parasympathetic and sympathetic nerves of the peripheral nervous system.

Endocardium, the innermost layer of the heart chambers and heart valves, is primarily made of endothelial cells that form overlapping regions to seal the heart chambers and connect the surrounding blood vessels. Apart from preventing blood leakage, it also plays important role of a blood–heart barrier controlling entry and exit of certain types of molecules. Pericardium is represented by a double-wall fibrous sac, enclosing the whole heart and the root of the blood vessels. The space between the 2 membranes of the pericardium, known as the pericardial cavity, contains pericardial fluid that serves as a lubricant facilitating membranes sliding over each other. In addition to the 3 major cardiac tissues, an important role in the heart structure and function also belongs to ECM, which controls cell fate and differentiation, and regulates protein expression. In normal myocardium, viscoelasticity of the collagen-enriched ECM and cardiomyocytes must be matched to generate actomyosin forces and pump of the heart.\textsuperscript{132}

Recent advances in cardiovascular tissue engineering led to the ability to fabricate various cardiac tissues and heart components by using state-of-the-art 3D bioprinting technologies. Despite demonstrated functionality and structural similarity of the engineered heart tissues to the native counterparts, their full structural and functional integration in the diseased organ may still be problematic, making successful implantation of myocardial patch, vascular (coronary or aortic) grafts or heart valves quite challenging. The importance of the structural integrity of the heart is predicated primarily by the electrophysiological coupling of cardiomyocytes, determining their highly synchronized ability to respond to the pacemaker activity of the sinoatrial node. Furthermore, repairing/curing the primary cause of cardiac malfunction may not always be sufficient to fully restore normal heart function because global anatomic and physiological changes to the whole organ may occur as a result the original, single cause. In light of this consideration, fabrication of the whole organ as opposed to its individual components (tissue implants) may become a more advantageous approach.
In fact, recent attempts in whole organ bioprinting demonstrated that the general structure of the whole heart can be fabricated. Hinton et al. developed a 3D bioprinting technology, suitable for fabrication of complex biological structures that was termed freeform reversible embedding of suspended hydrogels. This technology enabled 3D printing of hydrated materials such as alginate, collagen, and fibrin with an elastic modulus of <500 kPa. This method relied on direct bioprinting of the bioinks into a support bath of gelatin microparticles, which allowed depositing the supporting hydrogel under room temperature to construct large-scale volumetric objects that was impossible to achieve before. The support bath could then be liquefied at elevated temperature to release the bioprinted structures. In this study, the authors utilized CAD models of 3D optical, computed tomographic, and magnetic resonance imaging data. This technology of whole-heart bioprinting enabled fabrication of embryonic hearts with complex internal and external anatomic architecture at ≈200-μm resolution.

Despite the remarkable advances in bioprinting of whole organ models for educational and surgical guide purposes, to date bioprinting technology has not successfully printed any therapeutically relevant tissue constructs. One of the reasons is that each individual bioprinting technique has its own intrinsic disadvantages. In this regard, combining 2 or multiple bioprinting techniques or using bioprinting in conjunction with other tissue engineering technologies seems to be a more reasonable strategy for overcoming the above constraints.

**Spheroid Bioprinting Without Exogenous Structural Biomaterial**

To date, we have discussed cell and tissue engineering where the structural integrity of the cell-seeded system depends on the integrity of the substrate material to which the cells are attached. The underlying assumption is that engineered tissues require a scaffold (natural or synthetic) at the outset to support mechanical loading, particularly of vascular structures, as they become integrated into the host. However, formation of a tissue can occur in vitro without providing cells a supporting matrix in a process akin to embryonic development where morphogenesis of tissues is linked to cell-cell contact of originally isolated groups of cells followed by the formation of ECM. This approach is developing favor in tissue engineering and capitalizes on the idea that the ideal ECM is formed by the cells themselves as an adaptation to the in vitro or in vivo environment. Cell spheroids, collections of thousands of a single cell type, are ideal because no ECM is required, and they will self-assemble by forming cell-cell junctions. Spheroids are easily generated by centrifugation to form pellets, hanging drops, or micromolds.

Spheroids consisting of human dermal microvascular endothelial cells have also been shown in vitro to form dense tubular vessel-like networks within 72 hours and exhibit a significantly decreased rate of apoptotic cell death when compared with mono-culture HDMEC spheroids. After transplantation, these networks interconnected to the host microvasculature by external inosculation. In fact, spheroids have been shown to be more resistant against hypoxia and apoptotic cell death. Moreover, they secrete higher levels of proangiogenic growth factors such as vascular endothelial growth factor and fibroblast growth factor.

Cell spheroids can be loaded into biokin cartridges and dispensed using a bioprinter into a predetermined 3D configuration typically in a layer-by-layer deposition. This technique frequently relies on a gel material or biopaper to support the spheroids. Tissue or organ printing using self-assembled cell spheroids as a possible alternative to classic, solid, biodegradable, scaffold-based approaches could dramatically enhance and transform the field of tissue engineering by enabling large-scale industrial robotic biofabrication of living human organ constructs with built-in perfusable intraorgan branched vascular trees. It was recently demonstrated that a combination of human umbilical vein endothelial cells (40%), human aortic SMCs (10%), and normal human dermal fibroblasts (50%) could form multicellular spheroids of ~25,000 cells. These spheroids were robotically delivered to an array of needles and skewed in a tube-like configuration (1.5-mm ID×7-mm length) where they fused together after 4 days. After removal from the array, the tube was perfused for 4 additional days in preparation for implantation as a rat aorta vascular graft. After 5 days in vivo, the graft showed collagen production and some cellular rearrangement where endothelial cells were found in the lumen. This study was a clear demonstration of the capacity of cell spheroids to self-assemble and produce their own ECM.

Scaffold-free fabrication of cardiac patches was performed with contractile cardiac spheroids by plating a mixture of rat neonatal ventricular cardiomyocytes, human dermal fibroblasts, and human coronary microartery endothelial cells in ultralow attachment plates. Approximately 14,000 spheroids containing 1000 cells each were fused into a patch-like construct grafted into rat hearts. The patches were adherent to the surface of the heart and contractile after 7 days. Histological results showed that after 2 days, a microvascular network began forming in the spheroid. Furthermore, spheroids can be manipulated with robotic control to form a variety of physiologically relevant cardiovascular geometries composed of multiple cell types without the use of any ECM material.

**Future Perspective**

The technologies reported in this article comprise fundamental advances in the fabrication of engineered cardiovascular structures using variations of 3D printing. Bioprinting of soft tissues of the cardiovascular system relative to hard tissues (ie, bone and cartilage) necessitates special design criteria, and the technology is quickly advancing to address these criteria. Most critical is the need to match cell density and mechanical properties with the native structure while supporting cell viability and cell organization throughout the tissue. In addition, the speed of printing and fidelity with which printing can reproducibly replicate a native structure must be improved. When these criteria are realized, the concept of printing needed components for cardiovascular repair, including intact organs, in the operating suite may be within reach.

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