4

TISSUE FABRICATION TECHNOLOGY

Learning Objectives

After completing this chapter, students should be able to:

1. Describe self-organization technology for the fabrication of 3D artificial tissue.
2. Explain the process of cell sheet engineering for the fabrication of 3D artificial tissue.
3. Describe cell and organ printing as it applies to the tissue fabrication process.
4. Explain scaffold-based tissue engineering as a process of bioengineering 3D artificial tissue.
5. Discuss solid freeform fabrication of bioengineering 3D scaffolds.
6. Describe the concept of soft lithography as it relates to microfluidics and explain how this technology has been used to develop "organ-on-a-chip" models.
7. Describe technologies for cell patterning.
8. Design an idealized system to support fabrication of 3D artificial heart muscle.

Introduction to Tissue Engineering: Applications and Challenges, First Edition. Ravi Birla.

CHAPTER OVERVIEW

In this chapter, we will present techniques for the fabrication of 3D artificial tissue. We begin with a discussion of scaffold-free methods, which make use of the extracellular matrix that has been generated by the cells for artificial tissue fabrication. Cell-sheeting engineering is another scaffold-free tissue fabrication strategy; in this case, temperature- sensitive surfaces are used to fabricate 2D cells sheets that can be stacked together to form 3D artificial tissue. We will also discuss scaffold-based tissue engineering, including acellular grafts, polymeric scaffolds, and biodegradable hydrogels for tissue engineering. Scaffold-free technologies and scaffold-based strategies are the most commonly used methods of fabricating 3D artificial tissue. However, over the years, many new methods have been developed to support tissue fabrication, some of which are covered in this chapter. We discuss cell and organ printing as it relates to tissue engineering. Cell/organ printing makes use of 3D printing technology to fabricate 3D artificial tissue one layer at a time. This method has far-reaching applications in the tissue engineering space. A similar technique has been applied when fabricating 3D scaffolds and is known as solid freeform fabrication; in this case, complex 3D scaffolds are fabricated by printing one layer at a time. Cell patterning is another technique that has received a lot of attention and is based on techniques that regulate the spatial positioning of isolated cells. Cell patterning techniques are designed to replicate the complex organization of multiple cell types found in mammalian tissue. Microfluidic techniques are designed to fabricate microchannels, which are often used to simulate flow conditions within capillary networks and can also be used to regulate the spatial positioning of cells. Collectively, the strategies we present in this chapter provide researchers with a tool kit of fabrication techniques that can be applied toward the fabrication of 3D artificial tissue.

4.1 INTRODUCTION TO TISSUE FABRICATION TECHNOLOGIES

Tissue fabrication technologies have been developed to fabricate 3D tissue artificial tissue. As we have seen throughout the course of this book, 3D artificial tissue is fabricated by coupling isolated cells with biomaterials to support functional integration; tissue fabrication technologies have been developed to support this process. The objective of these technologies is to fabricate 3D artificial tissue that is similar in form and function to mammalian tissue. In nature, mammalian tissue is highly organized with multiple cell types and extracellular matrix components interacting in an orchestrated way to support formation and function of tissue. During early days of tissue engineering, 3D artificial tissue was fabricated by direct injection of isolated cells within 3D scaffolds. However, over the years significant advancements have been made in the development of novel tissue fabrication technologies to support fabrication of 3D artificial tissue. In this chapter, we will study many of these technologies.

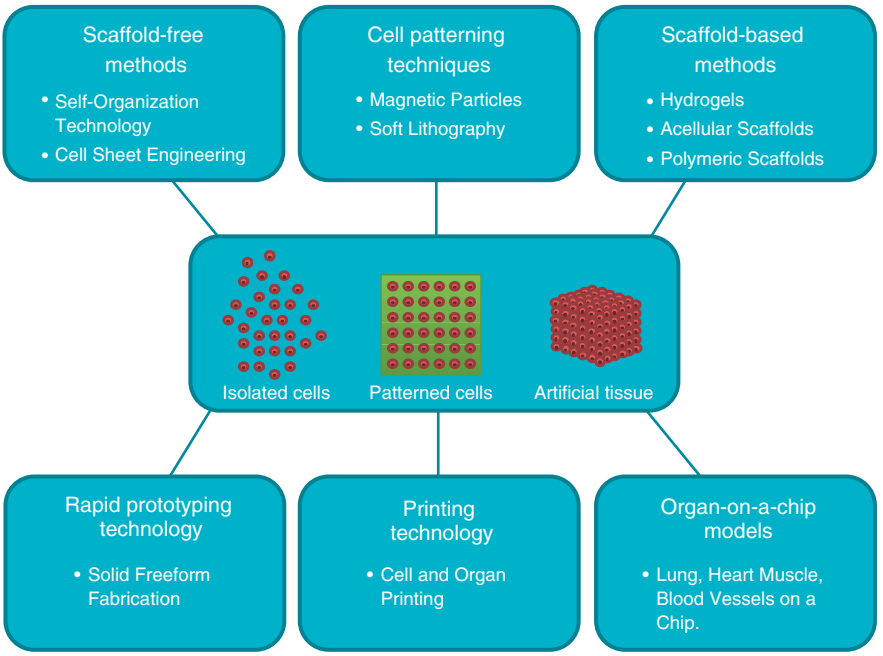

Figure 4.1 Overview of Tissue Fabrication Technologies—There are six categories of tissue fabrication technologies: scaffold-free methods, cell patterning techniques, scaffold-based methods, rapid prototyping technology, printing technology, and "organ-on-a-chip" model.

Tissue fabrication technologies can be classified into six categories, as shown in Figure 4.1, which include scaffold-free methods, cell patterning techniques, scaffold-based methods, rapid prototyping technologies, printing technology, and "organ-on-a-chip" models.

Scaffold-free methods have been developed to support tissue fabrication in the absence of any external scaffolding; rather, the extracellular matrix is generated by cells (1,2). These methods are based on the hypothesis that ECM fabricated by cells will prove to be superior to any external synthetic scaffolding material. Scaffold-free methods are based on the theory that extracellular matrix fabricated by cells will be superior in form and function to synthetic materials and therefore will provide a perfect scaffold to support cell-matrix interaction and formation of functional artificial tissue. Some examples of scaffold-free technologies that have been developed to support artificial tissue fabrication include self-organization strategies and cell sheet engineering.

Scaffold-based tissue fabrication models extensively used in the tissue engineering field (3). As we saw in Chapter 1, seminal work in the field of tissue engineering was based on this technology. The primary advantage of using scaffold-based methods is the ability to regulate material properties like 3D architecture, fiber geometry, and pore size, orientation, and alignment. This ability to control scaffold properties

leads to process customization and the ability to bioengineer specific scaffolds for specific applications. Acellular scaffolds, polymer scaffolds, and biodegradable scaffolds are examples of scaffolds used in tissue engineering.

Rapid prototyping systems have recently come into play and represent a very interesting area of research. These systems are designed to fabricate scaffolds or entire artificial tissue by creating one layer of the tissue/scaffold at a time in the x-y-direction and then building additional layers in the z-direction (4,5). Using this strategy, the process for scaffold/tissue fabrication can be spatially controlled. Solid freeform fabrication has been used in the production of scaffolds, while cell and organ printing has been used for cell patterning and tissue fabrication.

Soft lithography (6,7) and microfluidics (8–10) have been used for the development of the "organ-on-a- chip" model. Soft lithography is used to create a specific pattern of microchannels on a culture substrate and can be been used to fabricate microvascular networks. This technology has been used for cell patterning, which allows spatial regulation in the placement of cells on a 2D culture surface. Recently, this technology has also been adapted for "organ-on-a-chip" models. "Organ-on-a-chip" models are used to understand organ functionality in an isolated *in vitro* system; the models are not developed for the fabrication of transplantable tissue.

Cell patterning technologies are designed to create 2D patterns of cells on a culture substrate, including specific positioning of cells to control alignment and orientation of cells (11–14). Several strategies have been developed to pattern cells on 2D culture surfaces, including cell printing, soft lithography, and use of magnetic nanoparticles. Cell patterning has been extended to fabricate 3D artificial tissue using printing technologies to control 3D positioning of cells; this is referred to as organ printing.

4.2 SELF-ORGANIZATION TECHNOLOGY

Introduction—Self-organization technology was pioneered in 2001 for the fabrication of artificial skeletal muscle and later adapted for the fabrication of 3D artificial heart muscle (1,2,15). Self-organization technology is based on the fabrication of extracellular matrix by cells that then use the newly formed ECM to support artificial tissue fabrication. This technology is an example of a scaffold-free tissue fabrication process and does not require external or synthetic scaffolding; rather, scaffolding is produced by cells. The hypothesis is that extracellular matrix produced by cells is superior in form and function to synthetic scaffolding and can support formation of 3D artificial tissue superior to that of synthetic scaffolding. The formation of 3D artificial tissue is by organization and alignment of cells relative to newly formed extracellular matrix. Artificial tissue formation is governed by self-organization of cells and ECM in the absence of any external signals or cues. There are two important concepts related to self-organization technology: 1) there are no synthetic scaffolds, and extracellular matrix is produced by cells, and 2) fabrication of 3D artificial tissue is by spontaneous remodeling of cells and not by external cues or based on the geometry of synthetic scaffolding.

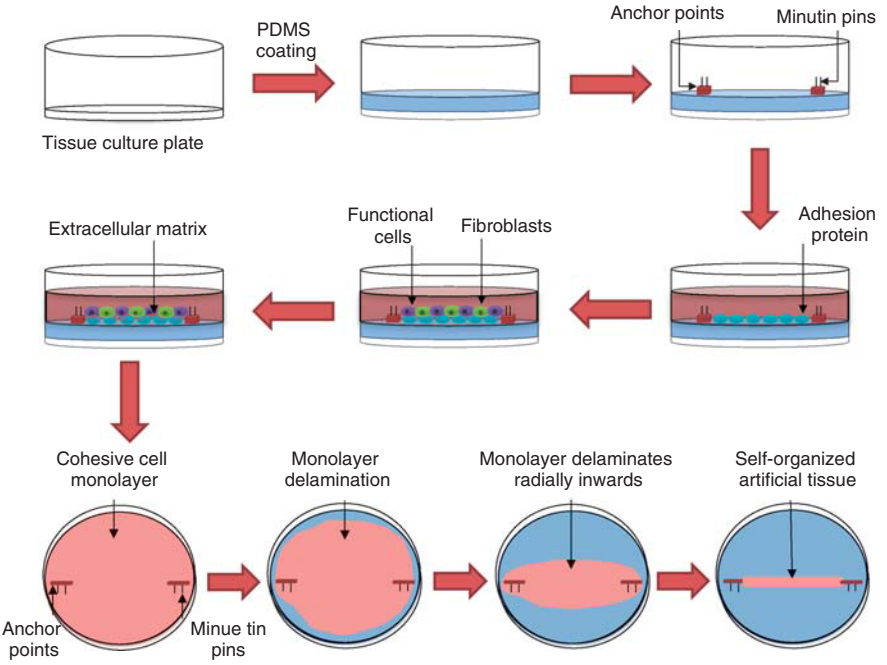

Figure 4.2 Self-Organization Technology for Fabrication of 3D Artificial Tissue—A tissue culture surface coated with PDMS and anchor points are positioned at the center of the culture surface. The culture surface is coated with an adhesion protein. Isolated cells are plated on the culture surface and attached to the adhesion protein. During culture, cells proliferate and form a cohesive cell monolayer; cells also generate extracellular matrix that serves to provide scaffolding during tissue remodeling and tissue fabrication. During time in culture, the extracellular matrix degrades with regular media changes. The cohesive cell monolayer delaminates, either due to the spontaneous contractions or due to the basal tension of cells. Monolayer delamination starts at the periphery of the culture surface and progresses radially inwards toward the center of the plate. At the center of the tissue culture plate, the delaminating monolayer attaches to anchor points that were pre-engineered onto the culture surface. Subsequent remodeling results in the fabrication of 3D artificial tissue.

Methodology—The methodology to support the fabrication of artificial 3D tissue using self-organization technology is presented in Figure 4.2.

The first step in the process is coating a culture surface with polydimethylsiloxane (PDMS), a culture surface that does not support cell adhesion very well (the function of the PDMS surface will become clear at the end of this discussion). Anchor points are positioned at the center of the tissue culture and consist of silk sutures that are secured in position using minutin pins (one of the functions of the PDMS is to allow placement of pins, as PDMS is a rubber like material). The culture surface is then coated with an adhesion protein; laminin has been used extensively to support artificial tissue fabrication, although other adhesion proteins like fibronectin and collagen have worked well in concert with self-organization

technology. PDMS does not support cell adhesion, and therefore the function of the adhesion protein is to support attachment of cells to the culture surface. The specific adhesion protein used and the concentration at which it is used are experimentally determined variables and vary between tissue applications.

Once the culture surface has been coated with adhesion protein, cells are plated on the surface. It is important to plate a mixed cell population that consists of at least two cell types: the functional cell type, which would be cardiac myocytes for heart muscle fabrication, and fibroblasts, which produce extracellular matrix. The ratio of functional cell type to fibroblasts is an experimentally determined variable. Once attached, functional cells like cardiac myocytes begin the process of remodeling and formation of intercellular connectivity between neighboring cells. In addition, fibroblasts begin the process of extracellular matrix production. The cells functionally couple with the extracellular matrix to form a cohesive cell monolayer that covers the entire culture surface.

The formation of a cohesive cell monolayer is a critical prerequisite for the fabrication of artificial tissue and is supported by the adhesion protein, as cells remain anchored to the underlying protein. A few days after formation of the cohesive cell monolayer, the cell monolayer detaches from the underlying culture surface in a process known as monolayer delamination. This occurs due to the spontaneous contractions of the cells (if they are cardiac myocytes) or due to the basal tension created by other cell types. During the course of the culture period, adhesion protein dissolves, exposing cells to underlying PDMS surface; this process aids delamination of the cohesive cell monolayer. Over time, monolayer delamination continues, and the cohesive cell monolayer progresses toward the center of the culture plate. This process is spontaneous and does not require any external intervention like chemical or electrical stimulation. At the center of the plate, the delaminating cell monolayer attaches to anchor points, resulting in the formation of 3D artificial.

Discussion of Self-Organization Technology—The novelty of this technology is that 3D tissue formation is spontaneous and regulated by cells without any external stimuli. Chemical or electrical stimuli are not required for tissue formation, and even the delamination process is self-regulated. Delamination of the cohesive cell monolayer is regulated by spontaneous contractions of cardiac myocytes or basal tension of other cell types, while 3D tissue formation is a result of self-assembly of the cohesive cell monolayer. Extracellular matrix is produced by fibroblasts and has clear advantages over synthetic scaffolding; the composition is physiological, has attachment sites for cells and mechanical properties required for fabrication of artificial tissue, and is closer in form and function to mammalian tissue.

4.3 CELL SHEET ENGINEERING

Cell sheet engineering is another example of a scaffold-free technology and does not rely on external scaffolding to support fabrication of artificial tissue

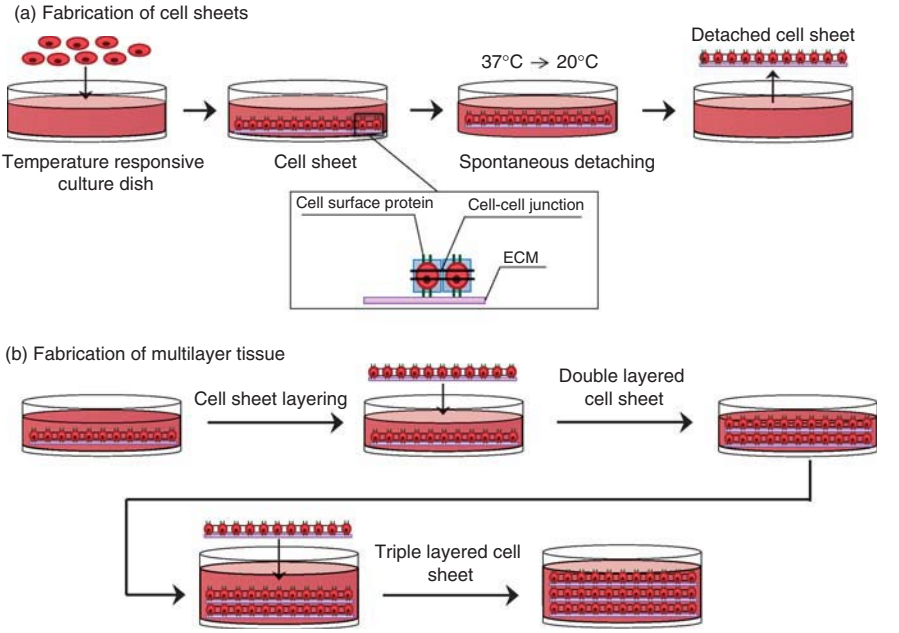

Figure 4.3 Cell Sheet Engineering for the Fabrication of Artificial Tissue—(a) Fabrication of Cell Sheets—Isolated cells are plated on the surface of a culture plate coated with a temperature-sensitive molecule. At 37°C, the temperature-responsive surface supports cell attachment. During the initial phases of cell culture, cells attach to the temperature-responsive surface, proliferate, and form a cohesive cell monolayer. Once a cohesive cell monolayer has been formed, the culture temperature is reduced to 20°C. At the lower temperature, the temperature-responsive surface does not support cell attachment. This in turn results in detachment of the cohesive cell monolayer, which at this point is known as a cell sheet. (b) Fabrication of Multilayer tissue—Individual cell sheets can be stacked together to form 3D artificial tissue, which consists of multiple layers of cell sheets stacked on top of each other.

(16–22). As with self-organization technology, cell sheet engineering relies upon extracellular matrix produced by cells; the newly formed extracellular matrix provides scaffolding during artificial tissue formation and remodeling. As we have seen before, extracellular matrix produced by cells has the right composition and distribution of proteins and other components required for cell attachment and 3D tissue fabrication.

The process of fabricating artificial tissue using cell sheet engineering is shown in Figure 4.3.

At the heart of the process is a temperature-sensitive molecule known as poly (N-isopoplyacrylaminde) (PIPAAm) (21). Under normal cell culture conditions at 37°C, PIPAAm can support cell attachment and proliferation. However, if the temperature is reduced to below 32°C, the PIPAAm surface properties change and can no longer support cell attachment; at lower temperatures, attached cells

detach from the culture surface. In essence, the PIPAAm molecules change from a "*cell-friendly*" surface to a "*cell-unfriendly*" surface as the temperature is reduced from 37°C to 32°C; this process is at the heart of cell sheet engineering.

The first step in the tissue fabrication process using cell sheet engineering is to coat a standard polystyrene culture surface with PIPAAm. Isolated cells are then plated on the modified tissue culture surface. During initial stages of cell attachment and culture, the temperature is maintained at 37°C to support cell attachment on the PIPAAm surface; cells attach to the culture surface and proliferate to form a confluent monolayer (similar to the case with self-organization technology). The cells form a cohesive monolayer, supporting cell-cell interaction and cellular remodeling and begin the process of extracellular matrix production, which requires presence of fibroblasts or other cell types known to engage in generation of extracellular matrix.

After formation of a cohesive cell monolayer, the culture temperature is reduced from 37°C to a temperature below 32°C (usually 20°C), which changes the properties of PIPAAm from "*cell-friendly*" to "*cell-unfriendly*." This change results in detachment of the cell monolayer from the culture surface. The detached cell monolayer is referred to as a cell sheet since it is exactly that: a sheet of cells. The cell sheet remains intact after detachment from the culture surface, retaining intercellular connectivity. Once an individual sheet has detached from the culture surface, it can be physically transferred and placed on top of another cell sheet. This process results in the fabrication of 3D artificial tissue with a thickness of two cell monolayers. This process continues, and multiple cell sheets can be added on top of eachother, supporting fabrication of multilayer artificial tissue.

The process of cell sheeting engineering can be compared with self-organization technology. Let us look at the similarities first. Both processes are based on scaffold-free technology and depend on extracellular matrix produced by cells. In addition, formation of a cohesive cell monolayer is required for both processes, and the presence of extracellular matrix-producing cells is essential for both technologies. There are also significant differences between the two processes: in the case of self-organization technology, the process is regulated by adhesion proteins like laminin, while in the case of cell sheet engineering, the process is regulated by temperature responsive culture surfaces.

4.4 SCAFFOLD-BASED TISSUE FABRICATION

Scaffold-based tissue fabrication has been seminal in the development of the field of tissue engineering (23–25), as much of the early work was based on this technology. Indeed, one of the seminal papers in the field, which first demonstrated the feasibility of fabricating artificial liver tissue equivalents, was based on the use of this technology. Due to the prevalence of scaffold-based tissue engineering in the field, we described the process of tissue fabrication based on scaffolding technology during our discussion in Chapter 1. Scaffold-based models continue to receive

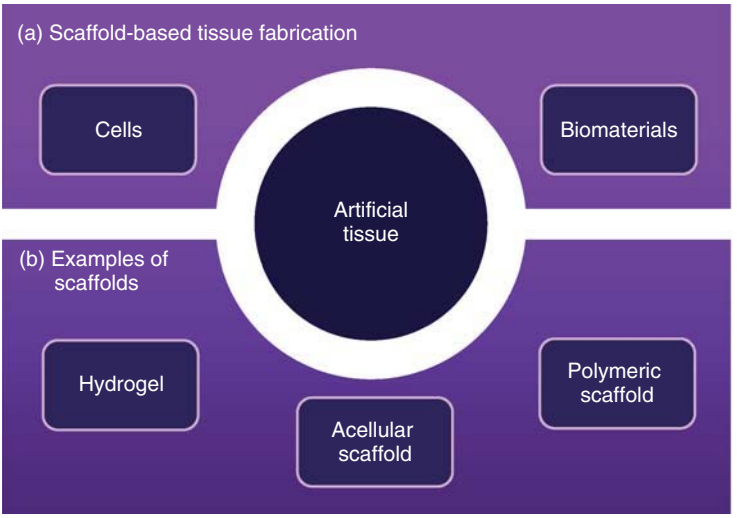

Figure 4.4 **(a) Scaffold-Based Tissue Engineering**—Technology is based on cellularization of 3D scaffolds with isolated cells. **(b) Examples of Scaffolds**—Acellular grafts, polymeric scaffolds, and hydrogels have been used to support the fabrication of 3D artificial tissue.

attention in recent tissue engineering studies, and this technology has significance in the field.

Scaffold-based tissue fabrication makes use of an external or synthetic 3D structure to simulate properties of the ECM (Figure 4.4).

Let us compare scaffold-based methods to self-organization strategies and cell sheet engineering. In the case of scaffold-free models, extracellular matrix is generated by cells during culture and utilized for artificial tissue fabrication. Compared to this approach, scaffold-based technologies rely on fabrication of a suitable scaffold to support formation of artificial tissue.

There are several advantages to the use of scaffold-based methods to support artificial tissue fabrication (23–25). The size, shape, and geometry of the scaffold can be controlled by changing processing variables, thereby allowing a greater degree of freedom in terms of determining scaffold geometry. Similarly, properties of the scaffold, including tensile properties, biomimetic properties, and biocompatibility, can also be modulated based on the specific tissue engineering application. Examples of variables that can be modulated include type and composition of individual monomer units and reaction conditions including temperature, pressure, and pH.

There are three strategies that have been used to fabricate artificial tissue using scaffold-based technology: acellular scaffolds, hydrogels, and polymer scaffolds (discussed in Chapter 3). Acellular scaffolds are obtained from tissue biopsies after complete removal of all cellular components, a process that leaves behind an intact extracellular matrix. As tissue specimens are obtained from mammalian

tissue biopsies, the composition and distribution of the extracellular matrix is perfect for tissue fabrication. Hydrogels contain a high percentage of water and often contain naturally occurring molecules like collagen, fibrin, and alginate, which support cell-matrix interactions leading to the fabrication of functional tissue. Polymeric scaffolds are rigid structures fabricated from monomer units using tightly controlled reaction conditions.

The elements of scaffold-based tissue engineering have been described before in several chapters and will be summarized here. The strategy of fabricating artificial tissue using scaffold-based technology has been described in Chapter 1. Depending on the choice of scaffolding used (acellular scaffolds, hydrogels, polymeric scaffolds), material fabrication and characterization efforts will be implemented and optimized, which has been discussed in Chapter 3. Prior to fabrication of artificial tissue, cell sourcing is important and requires knowledge about cell biology, stem cell engineering, and cell culture, all of which have been covered in Chapter 2. The implementation of bioreactors for scaffold cellularization is also important and is described later in Chapter 6.

There are several variables that need to be optimized for scaffold-based tissue fabrication technology. The number of cells, purity of the cells, and proportion of different cell types are important determinants of tissue function and need to be optimized experimentally. This is particularly important with the use of primary cells, as tissue digestion results in a mixed cell population consisting of functional cells, supporting structural cells, vascular, and nerve cells. As one example, digestion of hearts will result in a mixed cell population that consists of cardiac myocytes, cardiac fibroblasts, and vascular cells including endothelial cells and smooth muscle cells. During tissue fabrication, the number of different cell types will need to be calculated and the relative proportion of each cell type will need to be optimized for scaffold cellularization.

Cell retention is an important variable when working with tissue fabrication technologies. A specific number of cells are added to the scaffold; however, only a certain percentage of cells are retained within the 3D scaffold. The remaining cells are washed out with regular media changes, particularly within the first 24 hours. Cell retention is defined as the number of cells within the scaffold at any given time, expressed as a percentage of the total number of cells used to populate the scaffold; cell retention needs to be experimentally determined and optimized. Cell retention is less of a challenge with scaffold-free technologies like self-organization strategies or cell sheet engineering, as a high percentage of cells are retained once integrated within the cohesive cell monolayer or cell sheet; cells are not lost with consecutive media changes.

Scaffold-based tissue engineering continues to be a preferred model for tissue fabrication and has found widespread applications in tissue engineering. Together with scaffold-free technologies, these methods continue to be explored for a variety of tissue applications and for the development of artificial tissue. Scaffold-based and scaffold-free technologies have relative advantages and disadvantages and are suited for different tissue fabrication applications.

4.5 CELL AND ORGAN PRINTING

We have looked at several strategies of fabricating 3D artificial tissue including scaffold-based and scaffold-free technologies like self-organization and cell sheet engineering. These approaches focus on building macroscopic artificial tissue starting with predefined structures: 3D scaffolds for scaffold-based methods, cohesive cell monolayers for self-organization, or cell sheets for cell sheet engineering. Cell and organ printing uses a different concept altogether and is based on a ground-up fabrication approach, starting with a single building block that consists of a few cells trapped within a hydrogel to fabricate or build artificial tissue by controlling spatial positioning of these building blocks (26–32). This approach may be compared to any fabrication methodology, including building a house, which requires placement of one brick at a time, or a building project with LEGOs, which again requires placement of individual blocks to fabricate complex 3D structures. The advantage of using cell and organ printing for tissue fabrication is the ability to control spatial placement of cells relative to other cells or ECM proteins; this in turn provides the ability to "build" 3D artificial tissue without any predefined geometrical constraints imposed by the scaffold, cell monolayer, or cell sheet.

Cell and organ printing may be compared to inkjet printing, in which individual droplets of ink are transferred through a nozzle and deposited on the surface of paper. In comparison, cell and organ printing technologies are based on transferring cells or groups of cells through a fluid nozzle onto a biologically adapted printing surface. While cell/organ printing is innovative, it is not without challenges, some of which include: what carrier will be used to deliver cells through a fluid nozzle? What will be done to ensure cells are not damaged in the transfer process? What will be used as the paper equivalent to inkjet printing—the surface on which the cells will be delivered? How will the spatial resolution be controlled? How will cells adhere to the printing surface on which they are delivered? How will individual cells or groups of cells bond with each other to form 3D structures?

Let us begin with some definitions related to this cell/organ printing (33,34):

- *Bioprinting*—use of computer-aided transfer processes for patterning and assembling living and non-living materials with prescribed 2D or 3D organization.
- *Bioink*—cell or biomaterial containing solution used in bioprinting technologies. For cell printing, cells are suspended in culture media and supplemented with a thickening agent that must be biocompatible (e.g. an alginate solution). Maintaining homogeneity of the cell suspension with time is crucial for reproducible bioprinting.
- *Biopaper*—surface onto which the bio-ink is deposited during bioprinting. This can be compared with traditional paper during inkjet printing.
- *Cell printing*—bioprinting process used for 2D cell patterning by depositing bio-ink on the surface of biopaper.
- *Organ printing*—bioprinting process used for fabrication of 3D tissue by depositing bio-ink on the surface of biopaper.

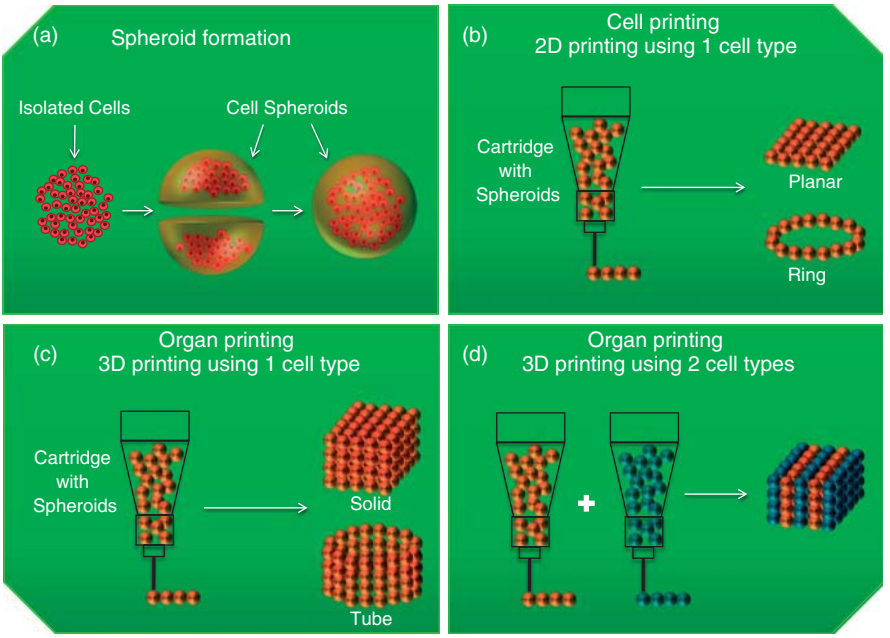

Figure 4.5 Cell and Organ Printing—(a) Spheroid Formation—Isolated cells are secured within a hydrogel to support formation of cell spheroids, which are building blocks for cell and organ printing technology. (b) Cell Printing–2D Printing using 1 cell type—Spheroids can be transferred from a nozzle to the surface to form several patterns, including planar, ring, and linear patterns. (c) Organ Printing— 3D Cell Printing using 1 Cell Type—A second layer of spheroids can be printed on the surface of the first spheroid to support formation of 3D artificial tissue. (d) Organ Printing—3D Cell Printing using 2 Cell Types—Complex organs can be fabricated using organ printing technologies with two or more different cell types.

We next provide a general description of the cell/organ printing process (Figure 4.5).

Cells are first suspended in a hydrogel solution (e.g. alginate or collagen) to form spheroids. Spheroids are the fundamental unit for cell and organ printing; isolated cells will be damaged during travel through the fluid nozzle in the bioprinter. The choice of hydrogel, concentration at which it is used, size of an individual spheroid, and cell density are experimental variables that need to be optimized based on the specific application. Once spheroids have been prepared, they are loaded into the bioprinter. A custom culture surface needs to be prepared for bioprinting of spheroids; hydrogels can be used as biopaper to receive and bind the cell-loaded spheroids. Other examples of culture surfaces include binding agents that secure the hydrogel upon contact or gelling agents, which polymerize upon contact and stabilize the hydrogel.

The bioprinter transfers spheroids from a hold reservoir through a fluid nozzle to the biopaper using robotic controls and software algorithms. Computer-aided

design (CAD) is used to design 2D cell patterns or 3D artificial tissue, while computer aided machining (CAM) is used to control the bioprinting process. Spatial resolution of up to 1 μm can be achieved. Spheroids are positioned on the biopaper using CAD drawings, and adhesives are used to secure individual spheroids to one another. A single layer of spheroids is patterned and is followed by a second layer and then a third layer and so on. This process results in fabrication of multilayer 3D artificial tissue. Depending on the complexity of the bioprinter, the number of fluid nozzles can be increased to accommodate different cell types. A two-nozzle system is required for dispensing two cell types, and a three-nozzle system is required for three cell types. In addition to dispensing cells, hydrogels can also be transferred from the bioprinter to the biopaper, and complex patterns of cells and hydrogels can be formed. Planar, solid, and tubular structures can be generated using bioprinting technology and can be combined to fabricate vascularized tissue; tubular structures can be generated using endothelial cells, and complex patterns of capillary networks can be generated.

Cell and organ printing has tremendous potential to have a significant impact on tissue engineering. From a conceptual standpoint, the bottom-up strategy for tissue fabrication and spatial resolution offered by bioprinters make this technology very appealing. However, a word of caution is warranted, and it should be appreciated that the current state of the technology is at the feasibility stage. While proof of concept studies have been successful, fabrication of complex multicellular functional tissue has not been demonstrated. In addition, the capital cost of bioprinters is currently high and can limit widespread acceptability of this technology.

4.6 SOLID FREEFORM FABRICATION

Solid freeform fabrication (SFF) refers to a group of technologies that build 3D scaffolds using a layer-by-layer approach (35,36). Collectively, these technologies are known as rapid prototyping (RP) methods. Cell and organ printing also fall within the general classification of RP technologies. SFF methods are bottom-up strategies and are designed to fabricate scaffolds with precise control over 3D architecture and morphology. The detailed architecture of the scaffold is designed using computer-aided design (CAD), and the CAD drawings are used to drive prototyping systems to fabricate the 3D scaffold. Compared to traditional scaffold fabrication technologies, SFF offers tight control over 3D geometry of the scaffold, including porosity, fiber alignment and distribution, mechanical properties, and placement of different molecules relative to each other. While SSF technologies offer considerable advantages over other scaffold fabrication methods, they have not received widespread applicability across tissue engineering laboratories due to the high initial capital investment and the high degree of personnel training required for these methods.

SFF refers to a group of technologies used to fabricate 3D scaffolds including stereo lithography, selective laser sintering, and fusion deposition modeling (35,36). The principle is similar in all of these techniques; a polymer is transferred

to a surface using a nozzle, and the x-y position of the polymer is controlled by robotic arms using pre-programmed algorithms. Using this strategy, a complex 3D pattern is created by carefully controlling the position of the polymer. Once the first layer has been completed, a second layer of polymer is placed in the z-direction, and this process continues until a complex 3D scaffold has been fabricated. This strategy of fabricating a complex 3D scaffold by carefully positioning the polymer in the x, y, and z directions provides control over the scaffold's architecture and properties.

In the case of selective layer sintering, a laser beam is used to heat the polymer above its glass transition temperature, after it has been positioned on the platform (37,38). As the polymer cools, it transitions from a rubbery to a glassy state, resulting in bonding of neighboring particles. This process leads to formation of solid structures. In the case of stereolithography, a photocurable polymer is used. This means that polymerization takes place in the presence of ultraviolet light. As in the case for selective layer sintering, the polymer is positioned in the x-y plane, and UV light is used to photopolymerize the polymer. This results in binding of the neighboring molecules and formation of planar structures. As the process continues, a second layer of polymer is added in the z-direction and again treated with UV light. This process continues until a complex 3D scaffold has been fabricated. In the case of fusion deposition modeling, temperatures beyond the glass transition temperatures are used to melt the polymer, and the molten polymer is extruded through a nozzle onto a fabrication platform in the x-y plane. As the temperature is reduced, the melted polymer solidifies, resulting in the fabrication of a planar scaffold. This process is repeated, and second and additional layers are added in the z-plane; temperature is used to control binding of the layers resulting in 3D scaffolds.

4.7 SOFT LITHOGRAPHY AND MICROFLUIDICS

Soft lithography is a microfabrication technology used to engineer microfluidic devices, particularly microvascular networks (6,7). There are many applications of microfluidic devices. Microfluidic channels have been engineered to simulate the flow of red blood cells through capillary networks (39–41), or to study intercellular connectivity between cardiac myocytes (42,43). Microfluidics has also been used to study the effect of fluid shear stress on endothelial cells (44–46), and to regulate differentiation of stem cells in response to predefined flow regimes (47–52). Some other applications include separation of cells or analytical assessment of metabolic activity of cells (53–55); these concepts have even been extended to "lab-on-a-chip" models—in which complex analytical analysis can be performed on microfabricated devices (56–59). Another interesting application has been in the fabrication of micro-organs using microfabricated devices with the heart being one example (60,61). The microfabricated heart consists of a pumping chamber and microchannels to simulate capillaries, all within a closed loop configuration (61).

The strength of microfluidics technology lies in the fabrication of microchannels in complex configurations on a very small scale. These devices can be used

to perform complex operations on a very small scale, eliminating the need for the ancillary support apparatus required at larger scales. Microfabrication devices have not been used to support tissue fabrication, and it is difficult to envision a case where microfluidic devices will be used to bioengineer transplantable artificial tissue. Rather, microfluidics devices can be used as a tool to understand effects of the microenvironment on cellular interaction and function; this data can directly feed into the tissue fabrication process.

The process of microfabrication using soft lithography is shown in Figure 4.6.

This is a two-step process: the first step involves fabrication of a reusable stamp using replica molding, and the second step is microcontact printing in which a specific pattern is transferred to a surface using this stamp (62). The first step in the process is fabrication of a stamp using PDMS with a predefined pattern. A negative of the pattern is generated on a silicon wafer using photoresist (a chemical that responds to UV light) and exposure to ultraviolet light. The entire silicon wafer is coated with photoresist to form a uniform layer. The photoresist surface is then selectively exposed to UV light; photoresist regions exposed to UV light break and are washed away in a solvent. This process leaves a patterned surface of photoresist on the silicon wafer. PDMS is then poured on the silicon wafer, allowed to cure, and then removed to form a negative of the pattern created by the photoresist. At this stage, the PDMS surface is known as the "stamp". This stamp is

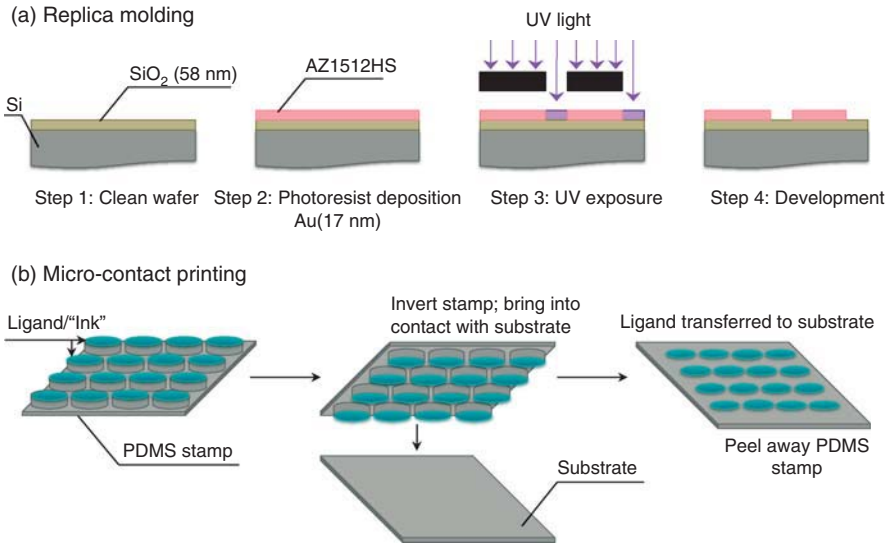

Figure 4.6 Soft Lithography—(a) Replica Molding—A PDMS surface is coated with photoresist and selectively exposed to UV light. Regions of photoresist that are not exposed to UV light are retained on the PDMS surface, resulting in formation of a specific pattern. This process leads to the formation of a stamp. (b) Microcontact Printing—The stamp that was fabricated in the first step of the process is coated with an ink and is transferred to a culture surface by direct contact with the surface.

used to create patterned surfaces over time and can be used over an extended time period.

The PDMS stamp fabricated using soft lithography is used in a process known as microsurface printing (62). The stamp is inked with a specific molecule and transferred to a substrate. This process is carried out by physical contact between the stamp and the substrate and requires a very short time—not more than seconds. Once the molecule has been transferred to the substrate, the void spaces can be filled by a second molecule of interest.

There are several interesting applications of microfabrication technologies in tissue engineering, particularly in the area of "organ-on-a-chip". The concept has been to recreate organ level functionality within a microfluidics device. These systems are not geared toward fabrication of transplantable tissue, but rather as model systems to understand organ-level function within an isolated microfluidics system. An example has been the fabrication of "organ-on-a-chip" models for lungs (63). During normal mammalian function, contraction of the diaphragm causes a reduction in intrapleural pressure and expansion of the alveoli, leading to influx of air in contact with the epithelium layer. Oxygen transfer takes place from the epithelium layer of the alveoli and to the endothelium layer of adjacent capillaries (63). This process has been recreated by fabricating microchannels, layering epithelium cells on one side and endothelial cells on the other, and fabricating side chambers maintained under a vacuum. As the air pressure increases in the microchamber from the side with epithelial cells (which represent alveoli function), the microchamber expands, resulting in flow of oxygen to the endothelium side, which represents capillary function (63).

Another interesting application of microfluidics technology has been in the fabrication of artificial heart muscle (60). In this case, a patterned surface was created by fabrication of microchannels on a substrate, and cardiac myocytes were cultured within these channels (60). The confined space of the microchannels supported intercellular connectivity between adjacent cardiac myocytes, while geometry of the channels resulted in alignment of newly formed heart muscle tissue. Microfluidic channels have also been used to fabricate microvascular networks by generating organized arrays of microchannels connected by fluid inlet and outlet channels, thereby creating a closed-loop circulatory system (64).

4.8 CELL PATTERNING

The process by which the spatial placement of cells is controlled to create an organized pattern of cell monolayers or 3D tissue is known as cell patterning (28–32). The rationale for development of cell patterning technologies is to control organization of different cell types relative to one and other and to the extracellular matrix, just as is found in mammalian tissue. Fabrication of 3D artificial tissue requires a high level of spatial resolution in order to bioengineer tissue that is similar in form and function to mammalian tissue. In an earlier section, we looked at some examples of technologies used for cell patterning, including cell/organ

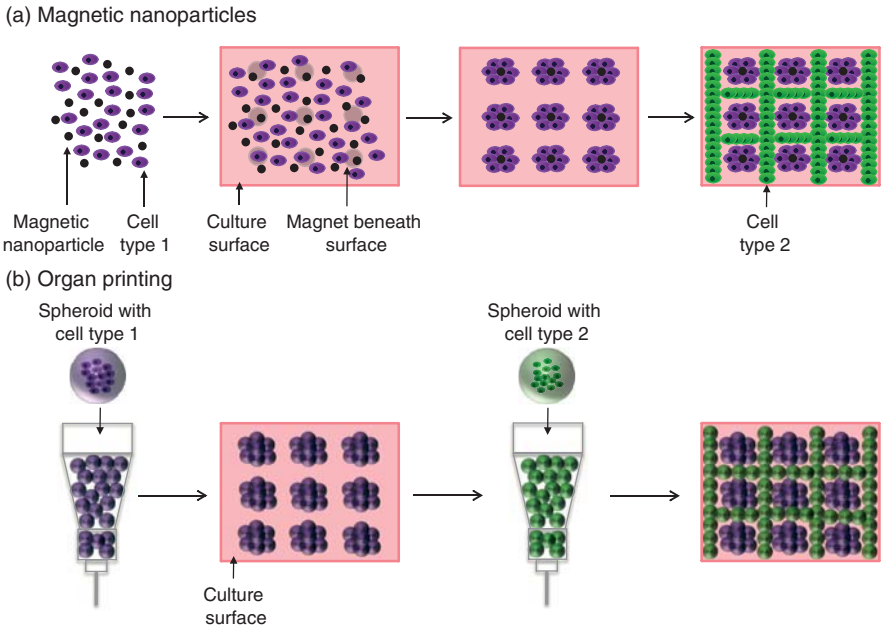

Figure 4.7 Cell Patterning. (a) Nanoparticles for Cell Patterning—Isolated cells are mixed with magnetically charged nanoparticles and plated on the culture substrate. A magnetic field is applied beneath the culture surface, and cells organize around this magnetic field. Cells form a pattern that correlates to the magnetic field. A second cell type is added directly to the culture surface without any nanoparticles, and it fills void spaces. (b) Organ Printing for Cell Patterning—Spheroids are formed using two different cell types and are loaded in cartridges. Spheroids are transferred on a culture surface, and their cell pattern is determined based on a predefined algorithm.

printing, soft lithography, and microfluidic channels. Here, we look at examples of these and other technologies as they are applied for cell patterning (Figures 4.7, 4.8 and 4.9).

One interesting approach to cell patterning has been the use of nanoparticles in conjunction with controlled magnetic fields (65). Nanoparticles are mixed with a cell suspension and plated on a culture surface. Application of a controlled magnetic field is used to guide placement of these cells at specific locations on the culture surface. Using this technology, isolated cells can be patterned into many different configurations, including small cell islands, during monolayer culture. In addition, nanoparticles can be used to preferentially regulate spatial placement of two different cell types. For example, the first cell type can be mixed with nanoparticles and patterned to any configuration using magnetic fields. The void spaces on the culture surface can be later filled by plating the second cell type without any nanoparticles. The second cell type will attach to the culture surface in the void spaces, leading to a controlled pattern of two different cell types.

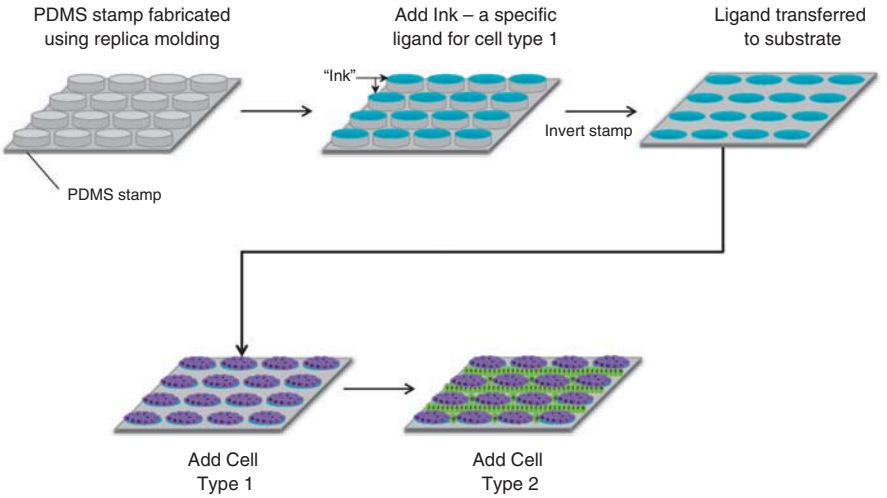

Figure 4.8 Soft Lithography for Cell Patterning—A PDMS stamp is fabricated using replica molding and coated with an adhesion protein . Adhesion protein is transferred to the culture surface by direct contact between the PDMS stamp and the culture surface. Cells are plated on the culture surface and attach to form a specific cell pattern that correlates with the pattern of the adhesion protein. A second cell type is plated on the culture surface and fills void spaces.

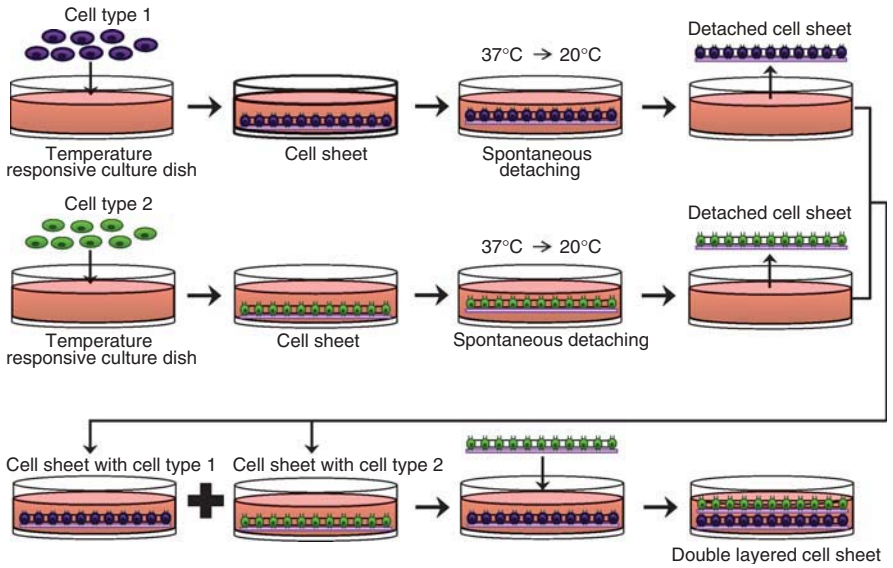

Figure 4.9 Cell Sheet Engineering for Cell Patterning—Cell sheets are fabricated using two different cell types by plating the cells on a culture surface that has been coated with temperature-responsive molecules. Individual sheets are fabricated using different cell types that can be layered on top of one another to form 3D tissue with a specific pattern.

Soft lithography has been extensively used for cell patterning (66). The first step in the process involves fabrication of a stamp using replica molding; this stamp can be designed in a specific configuration and can be used as a template to transfer cells or adhesion proteins on a culture surface. Once fabricated, cells or adhesion proteins can be coated on the stamp and directly transferred to the culture surface, thereby creating a pattern of cells/proteins that replicate the pattern of the stamp. Alternatively, an adhesion protein can be transferred to a culture surface and used as a binding site for cells. In both cases, the technology can be adapted to pattern two different cell types: the first cell type is coated on the culture surface using the stamp, and the second cell type is plated to fill the void spaces.

Using cell/organ printing, isolated cells are suspended within a spheroid and used as building blocks for cell/tissue patterning (67). Spheroids are transferred through a robotically controlled nozzle that regulates spatial placement. Using this technology, a single cell type can be suspended in a spheroid and patterned on the culture surface by controlling spatial placement of spheroids. This process can be adapted for two different cell types—a second nozzle can be used to deliver spheroids with a second cell type.

Cell sheet engineering can be used for cell patterning to fabricate multilayer tissue (68). As may be recalled, cell sheet engineering is based on detachment of a cohesive cell monolayer from an underlying substrate coated with a temperature-sensitive molecule. This process leads to formation of cell sheets that can be stacked together to form multilayer tissue. This process can be adapted to fabricate specific patterns of 3D tissue by fabricating a cell sheet using a single cell type and then layering it on the surface of the cell sheet fabricated with a second cell type. Using this method, 3D tissue can be fabricated using multiple cell sheets with different cell types.

4.9 IDEALIZED SYSTEM TO SUPPORT TISSUE FABRICATION

An idealized system to support fabrication of 3D artificial tissue is shown in Figure 4.10.

The system consists of modules for different cell types and for scaffolds that allow control over placement of scaffolds and cells in order to support 3D tissue formation. In this system, the scaffold provides temporary support during the initial stages of artificial tissue fabrication, degrades over time in a controlled manner, and is replaced by extracellular matrix produced by cells. This process leads to formation of scaffold-free artificial tissue, which has a physiological composition of proteins and other ECM compounds.

As shown in Figure 4.10, the idealized system is designed to support fabrication of artificial heart muscle. Isolated cardiac myocytes are suspended within spheroids and transferred to a nozzle; these spheroids are patterned on a culture substrate to support linear alignment of cells which supports cell-cell interaction. This process is carried out until a cohesive monolayer of cardiac myocytes has been formed, followed by layering of a sheet of polymeric molecules that serve to

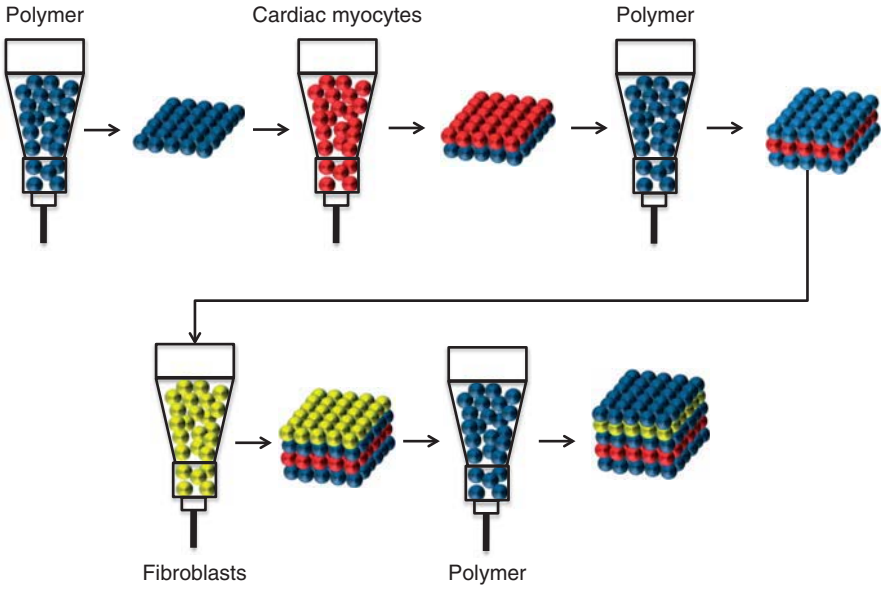

Figure 4.10 Idealized System for Heart Muscle Fabrication—Organ printing is used to fabricate a multilayer system of cardiac myocytes, fibroblasts and a polymer. Over time, the polymer degrades and is replaced by ECM produced by fibroblasts. This process leads to the fabrication of scaffold-free artificial heart muscle fabricated by organ printing.

provide structural support during tissue fabrication. After fabrication of these two layers (cardiac myocytes and polymeric molecules), the third layer is generated by layering fibroblast cells on top of the polymer sheet. The fourth layer is again fabricated using polymers to stabilize the multilayer heart muscle. The next step is formation of a capillary network by controlled patterning of endothelial cells on top of the polymer layer. This is followed by patterning another polymer layer, followed by fibroblast cells, polymers, and cardiac myocytes, leading to fabrication of complex multi-cellular 3D tissue. The polymer is designed to serve as a temporary scaffold and is replaced by ECM components produced by fibroblasts, thereby leading to 3D scaffold-free heart muscle tissue.

SUMMARY

Current State of the Art—Over the years, there have been several strategies that have been developed to support fabrication of 3D artificial tissue. In this chapter, we have looked at several such strategies, including scaffold-free methods, scaffold-based methods, cell and organ printing, solid freeform fabrication, soft lithography, and cell patterning. These techniques provide researchers with a vast tool kit to use when fabricating 3D artificial tissue. The objective for all of these strategies is the same: to replicate the complex 3D architecture of mammalian tissue.

Thoughts for the Future—The current generation of tissue fabrication technologies has been developed for the development of one aspect of artificial tissue. For example, cell and organ printing techniques are designed to support the fabrication of 3D tissue using cells, while solid freeform fabrication strategies are focused on the scaffold. In addition, cell patterning techniques are focused on the spatial alignment of cells, while soft lithography and microfluidics are geared toward the fabrication of microvasculature and more toward understanding cellular behavior. As tissue fabrication technologies are being developed, there is a growing need for a new generation of strategies that can incorporate multidimensional mammalian tissue. A new generation of tissue fabrication technologies needs to be developed that incorporates cellular and extracellular components, including vasculature and innervation. These tissue fabrication technologies will form a critical component of the tissue fabrication process, and these technologies are essential for the fabrication of 3D artificial tissue that mimics the complex architecture of mammalian tissue.

PRACTICE QUESTIONS

1. This chapter is about tissue fabrication technology. Provide a general discussion about why tissue fabrication is important for the development of artificial tissue and organs. What do you think is involved in the fabrication of artificial tissue and organs?
2. In this chapter we described the fabrication of artificial tissue using scaffold-free technology. We discussed self-organization strategies and cell sheet engineering. Describe what the term "scaffold-free technology" means. What are the relative advantages and disadvantages of using scaffold-free technology for the fabrication of artificial tissue? Describe self-organization strategies and cell sheet engineering.
3. Describe the process of self-organization for the fabrication of 3D artificial tissue. What variables affect tissue formation and function?
4. Describe the process of cell sheet engineering for the fabrication of 3D artificial tissue. What variables affect tissue formation and function?
5. We studied scaffold-based tissue engineering and looked at acellular scaffolds, polymers, and hydrogels. Discuss each of these as they relate to biomaterial synthesis and tissue fabrication. What are the relative advantages and disadvantages of acellular scaffolds, polymers, and hydrogels as applied to tissue engineering and tissue fabrication?
6. Scaffold-based and scaffold-free methods have been used extensively in tissue engineering. Describe the relative advantages and disadvantages of these two strategies.

7. Pick any tissue or organ fabrication application and develop a strategy for bioengineering your selected tissue or organ using self-organization strategies. What specific method will you choose and why?
8. Describe the process of cell and organ printing. Explain the process and describe how complex 3D structures can be formed using multiple cell types. What are some of the scientific and technological hurdles that need to be overcome in order to improve this technology?
9. Describe how you would use organ printing to fabricate functional 3D artificial heart muscle with embedded vasculature.
10. Explain how soft lithography is used to fabricate microfluidics channels.
11. Why are microfluidic channels important in tissue engineering? How can microfluidics channels be used to support tissue fabrication?
12. We looked at several models for tissue and "organ-on-a-chip": heart muscle, lungs, and blood vessels. Develop a model for a total bioartificial heart- on-a- chip.
13. What is cell patterning and why is it important for tissue engineering? During the course of this chapter, we looked at several strategies for cell patterning. Pick any one and describe how your chosen strategies can be used for cell patterning.
14. During the course of this chapter, we looked at several tissue fabrication technologies. What are some scientific and technological challenges associated with tissue fabrication technologies? What can be done to overcome these scientific and technological challenges?
15. We have studied several tissue fabrication technologies used in tissue engineering. However, there are many different fabrication technologies that are used in different fields, particularly in engineering. Based on your experiences with other fabrication technologies, develop a new method that can be used to support the fabrication of 3D artificial tissue.

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